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Increased expression and catalytic activity of prostacyclin synthase after simvastatin application to human umbilical vein endothelial cells

Sun-Ah Cho and Su-Jun Lee*

Department of Pharmacology and Pharmacogenomics Research Center, Inje University College of Medicine, Inje University, Busan, South Korea

***Corresponding author**: 2sujun@inje.ac.kr

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Abstract: In addition to lowering blood cholesterol levels, statins are known to exert antiplatelet effects. One of the key factors contributing to the antiplatelet effects of statins includes the upregulation of prostacyclin (PGI_2) level. The present study was undertaken to determine the effects of statins on prostacyclin synthase (PGIS, CYP8A1) and PGI_2 synthesis at the molecular level. Human umbilical vein endothelial cells (HUVEC) were exposed to five structurally different statins (atorvastatin, simvastatin, pravastatin, lovastatin, and rosuvastatin) and changes in CYP8A1 expression levels and the metabolic activities of CYP8A1 were investigated. Among the tested statins, simvastatin induced significant PGIS expression at both transcriptional (2.9-fold, $P<0.05$) and translational (1.8-fold, $P<0.05$) levels. Treatment with a constitutive androstane receptor (CAR) agonist, phenobarbital, significantly increased CYP8A1 mRNA expression (3-fold, $P<0.01$). A metabolite of prostacyclin, 6-keto prostaglandin F1α, was significantly increased by treatment with simvastatin ($P<0.01$) and markedly repressed by the CYP8A1 inhibitor tranylcypromine ($P<0.01$) and the CAR antagonist clotrimazole ($P<0.01$) in HUVEC. The results of this study improve our understanding of the inter-individual variations in PGI_2 levels. Clinical studies in humans are necessary to confirm the present *in vitro* results.

Keywords: prostacyclin; prostacyclin synthase; CYP8A1; simvastatin; CAR; cardiovascular disease

INTRODUCTION

Statins, inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, are the most common cholesterol-lowering drugs [1]. Prescriptions of statins have increased for the management of high blood cholesterol levels and prevention of cardiovascular diseases [2,3], although high statin concentrations have been associated with the increased risk of myopathy [4,5]. In addition to the inhibition of HMG-CoA reductase, multiple studies have reported that statins exhibit other pharmacologically important roles, such as antiinflammatory action [6], repression of vascular hyporeactivity [6], thromboxane A_2 (TXA_2) reduction [7], prostacyclin increase [8] and upregulation of endothelial nitric oxide synthase (eNOS) [9]. Statins have been reported to influence platelet eicosanoid synthesis, resulting in reduced platelet aggregation without blocking TXA_2 synthesis [10]. These data imply that the expression of prostacyclin synthase (CYP8A1, PGIS) is upregulated by statins, resulting in increased levels of prostacyclin (prostaglandin I_2 (PGI_2)). CYP8A1, is an enzyme responsible for the conversion of prostaglandin H_2 (PGH_2) to PGI_2, and PGI_2 is known to be a potent mediator of vasodilation and inhibitor of platelet aggregation [11,12]. Statins were found to activate human constitutive androstane receptor (CAR) at the transcriptional level through a phenobarbital-responsive enhancer element [13]. Major expression sites of CYP8A1 in humans are vascular endothelial and smooth muscle cells [14]. Multiple studies have reported that variations in CYP8A1 activities or expression levels can affect PGI_2 levels in the blood, and these altered PGI_2 levels have been associated with increased risk of cardiovascular diseases, such as cerebral infarction [15], hypertension [16,17] and myocardial infarction [18,19].

Although statin-induced variations in PGI_2 levels have been shown [20], there is a lack of studies on

statin-mediated transcriptional regulation of CYP8A1. It was reported that statins induced CYP2C9 mRNA through the CAR-mediated mechanism in endothelial cells of human blood vessels [21]. Therefore, the aim of the present study was to determine whether CYP8A1 is transcriptionally induced by statins in human endothelial cells. Five statins, selected on the basis of their chemical structures, were screened to determine the transcriptional regulation of CYP8A1. The results obtained in the present study will be helpful for understanding statin-induced variations of PGI_2 levels and will be useful in PGI_2-related fields, such as research on cardiovascular diseases.

MATERIALS AND METHODS

Chemicals and reagents

Atorvastatin was purchased from Sigma-Aldrich (St. Louis, MO, USA); simvastatin, pravastatin, lovastatin, and rosuvastatin were purchased from Toronto Research Chemicals (Toronto, ON, Canada). Actinomycin D, dimethyl sulfoxide (DMSO), ethylenediaminetetraacetic acid (EDTA), sodium phosphate dibasic, potassium phosphate monobasic, ammonium persulfate and N,N,N',N'-tetramethylethylenediamine were purchased from Sigma-Aldrich, USA. Skimmed milk was purchased from BD Difco Laboratories (Sparks, MD, USA). All other chemicals and solvents used in the experiments were of the highest purity grade available from the supplier.

Cell culture

Primary human umbilical vein endothelial cells (HUVEC) were purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were seeded in flasks precoated with 0.2% gelatin in culture medium (Sigma-Aldrich, USA) containing 20% heat-inactivated fetal bovine serum (Invitrogen, Burlington, ON, Canada) and 2.5% penicillin-streptomycin (Invitrogen) and incubated at 37°C with 5% CO_2 [22]. After two cycles of cell culture (72 h), adherent cells were detached using 0.25% trypsin-EDTA (Invitrogen) and seeded in a 100-mm dish at 3×10^5 cells. All experimental data were obtained between 3 and 6 passage cycles. All statins were dissolved in DMSO. Cells were exposed to 0.1% (v/v) DMSO (vehicle) or 10 μM statins for 36 h according to a previous study [23].

RNA extraction and quantitative reverse transcription PCR (qRT-PCR)

Total RNA extraction was performed using TRIzol reagent according to the manufacturer's instructions (Invitrogen). Each RNA sample was quantified and qualified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). cDNA synthesis reactions were prepared by adding 3 μg total RNA to a reaction mixture of 50 μM random hexamers, 10 mM NTPs and RNase-free water to a volume of 27 μL. After heating the reaction mixtures, 0.1 M dithiothreitol and 200 U of M-MLV reverse-transcriptase were added and the reaction mixtures were incubated at 42°C for 1 h. The reaction was terminated at 72°C for 5 min. The TaqMan Universal Master Mix (Applied Biosystems, Foster City, CA, USA) and TaqMan Gene Expression Assay were used for qRT-PCR in a 0.2-mL RNase-free tube. The cDNA prepared by reverse transcription was added to a final concentration of 30 ng per assay. qRT-PCR was performed using the following primer and probe sets from Applied Biosystems: CYP8A1 (Hs00168766_m1) and GAPDH (Cat. No. 4326317E). The PCR conditions used were as follows: denaturation at 95°C for 10 min, followed by 35 cycles of denaturation at 95°C for 15 s, and annealing and extension at 60°C for 1 min. The reactions were placed in an ABI Prism 7900 Sequence Detector (Applied Biosystems). Relative CYP8A1 mRNA expression was calculated using the ΔCt method as described in the literature [24]. The results are presented as the ratio of the target mRNA to *GAPDH* mRNA ($2^{-(\Delta\Delta CT)}$). The assays were performed twice in triplicate for each experiment.

Membrane protein extraction and Western blot assay

Western blotting was performed to determine the protein level of PGIS in HUVEC after simvastatin treatment. The control group was treated with 0.1% DMSO (v/v) and the experimental groups were treated with 0.1 or 10 μM simvastatin for 36 h. To determine whether the result of the experiment was due to transcriptional regulation by statin, the cells were treated with the RNA polymerase inhibitor actinomycin D for 2 h before simvastatin treatment. Following treatment, the cells were harvested, washed with phosphate-

buffered saline (PBS), and lysed in RIPA buffer (1.5 M NaCl, 0.1% NP-40, 0.5 M Tris-HCl, 0.1% sodium dodecyl sulfate (SDS), and 0.05 M NaF) containing a protease inhibitor mix (104 mM 4-(2-aminoethyl) benzenesulfonyl fluoride, 80 μM aprotinin, 4 mM bestatin, 1.4 mM E-64, 2 mM leupeptin and 1.5 mM pepstatin). The sample mixture was sonicated on ice two times for 30 s. The cell lysates were centrifuged at 10000 ×g for 10 min to separate the membrane fraction from the soluble fraction. The membrane fraction was resuspended in 500 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% sodium deoxycholate and 0.1% SDS buffer containing the protease inhibitor mix. The mixture was solubilized by incubation with shaking at 4°C for 2 h and sonicated for 10 s three times on ice. The prepared samples were centrifuged again at 10000 ×g for 10 min and the supernatant was collected for Western blotting and enzyme assays. The protein (25 μg/lane) was separated on a NuPAGE 4-12% bis-Tris gel (Invitrogen) and transferred to a polyvinylidene difluoride membrane (GE Healthcare Bio-Sciences, Little Chalfont, Buckinghamshire, UK). The membrane was blocked with 5% skimmed milk in (Tris-buffered saline (TBS): 10 mM Tris-HCl (pH 7.4), 0.9% NaCl). Specific anti-PGIS antiserum (Cayman Chemical, Ann Arbor, MI, USA) was used as the primary antibody at 1:2000 dilution for 2 h at room temperature. The membranes were washed thoroughly with TBS containing 0.1% Tween-20. The immunoreactive proteins were detected with an enhanced chemiluminescence system (GE Healthcare Bio-Sciences, UK). Protein bands were visualized by autoradiography in a LAS 3000 imaging system (Fujifilm Life Science, Stamford, CT, USA). The relative CYP8A1 protein levels were normalized to β-actin, which was immunoblotted as an internal control. The band densities were quantified using Multi Gauge software (Fuji Photo Film, Tokyo, Japan).

Enzyme assays

CYP8A1 enzyme activity was determined using a 6-keto prostaglandin F1α (PGF1α) enzyme immunoassay (EIA) kit (Cayman Chemical) according to the manufacturer's instructions and our previous report [25,26]. Briefly, the enzyme reaction contained the substrate U46619, a stable analog of PGH_2 (ranging from 1 to 200 μM), and the membrane fraction (50 pg/mL) in an EIA buffer containing 100 mM potassium phosphate (pH 7.4), 0.1% bovine serum albumin (BSA), 0.4 M sodium chloride, 1 mM EDTA, and 0.01% sodium azide at 23°C in 50 μL total reaction volume, and incubated for 1 min [26]. After the addition of the extracted membrane protein and standards, 6-keto-PGF1α (tracer) and antiserum specific to 6-keto-PGF1α were added to the reaction. The nonenzymatic metabolite of PGI_2, 6-keto-PGF1α, was measured. The amount of metabolite was determined by standard curves and the detection limit of 6-keto-PGF1α was 6 pg/mL in the assay system.

Statistical analyses

At least two independently prepared RNA samples were assayed in triplicate to confirm the CYP8A1 mRNA levels in the qRT-PCR analysis. Analysis of the CAR response element (CAR-RE) in the CYP8A1 promoter region was performed using TRANSFAC software (BIOBASE, Wolfenbüttel, Germany) as described [27]. Statistical analysis of differences between treatment and control groups was performed using the Student's *t*-test. All data are presented as means ± standard error of three independent experiments. A P-value <0.05 was considered to indicate statistical significance. All analyses were performed using SAS version 9.1.3 (SAS Institute, Cary, NC, USA).

RESULTS

The effects of statin on CYP8A1 expression in HUVEC were investigated by qRT-PCR, Western blot analysis and the enzyme activity assay. The five statin drugs, atorvastatin, simvastatin, pravastatin, lovastatin and rosuvastatin, showed different influences on CYP8A1 mRNA expression in qRT-PCR (Fig. 1). Among the five statins, simvastatin induced 2.9-fold higher CYP8A1 expression compared to the control group (DMSO; P<0.05). However, the other statins exhibited weak effects on CYP8A1 mRNA expression that were not statistically significant. Therefore, simvastatin was selected for immunoblot analysis.

A significant increase in CYP8A1 protein expression was observed with simvastatin treatment compared to the control (Fig. 2). Western blot band density was quantified and expressed as an arbitrary unit. The ex-

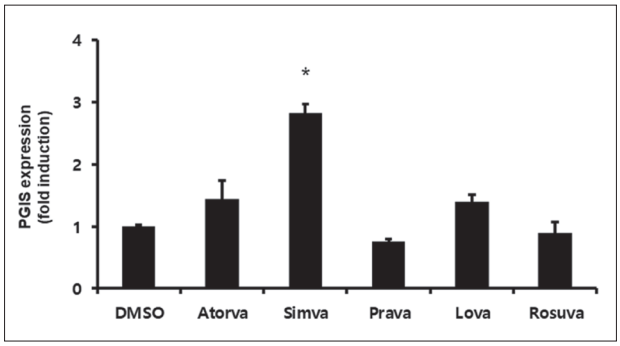

Fig. 1. Effects of statins on CYP8A1 mRNA expression in HUVEC treated with 10 μM of statins for 36 h. Values are means ± SE of three independent experiments. The fold-change in CYP8A1 expression was calculated using the $2^{-\Delta\Delta CT}$ equation. Statistically significant changes compared to the DMSO treatment group are indicated with an asterisk; *P<0.05. Atorva – atorvastatin; Simva – simvastatin; Prava – pravastatin; Lova – lovastatin; Rosuva – rosuvastatin.

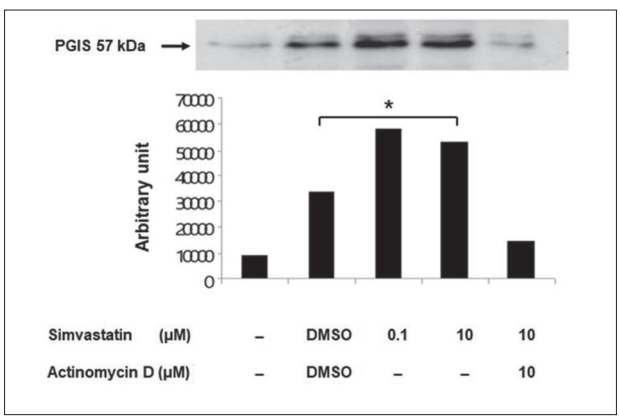

Fig. 2. Differential expression of CYP8A1 protein with simvastatin after actinomycin D treatment of HUVEC exposed to 0.1% DMSO and simvastatin (0.1 μM or 10 μM) for 36 h. Actinomycin D (10 μM) was applied 2 h before simvastatin treatment. The band densities of the immunoreactive bands were quantified and expressed in arbitrary units. Statistically significant changes compared to the DMSO-treatment group are indicated with an asterisk; *P<0.05.

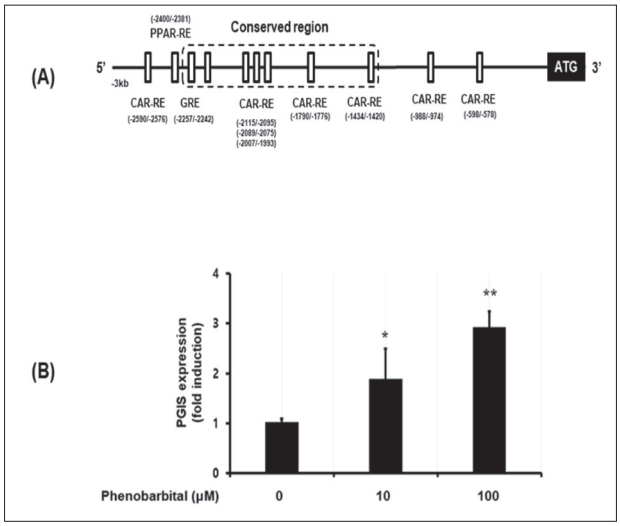

Fig. 3. *In silico* promoter analysis of the CYP8A1 gene and the influence of phenobarbital on CYP8A1 mRNA expression in HUVEC treated with phenobarbital for 36 h. **A** – Prediction of CAR-REs in the CYP8A1 promoter region. CAR-RE – constitutive androstane receptor-response element; GRE – glucocorticoid response element; PPAR-RE – peroxisome proliferator-activated receptor-response element. **B** – Induction of CYP8A1 mRNA expression by phenobarbital. Values are means ± SE from triplicate experiments. The fold-change in CYP8A1 expression was calculated using the $2^{-\Delta\Delta CT}$ equation. Statistically significant changes compared to the DMSO treatment group are indicated with asterisks; *P<0.05; **P<0.01.

pression of CYP8A1 protein increased about 1.8-fold in cells treated with simvastatin compared to cells treated only with DMSO (P<0.05). To determine whether the effect of simvastatin on CYP8A1 induction was due to increased transcription, actinomycin D, a chemical that blocks transcription, was added to the HUVEC culture 2 h before simvastatin treatment. As a result, the cells treated with simvastatin after actinomycin D exhibited repressed CYP8A1 protein expression that was similar to the control group (Fig. 2). This implied that simvastatin increased CYP8A1 expression by acting on transcription of the CYP8A1 gene. Since statins are reported as CAR agonists [13], the promoter region of the CYP8A1 gene was analyzed and multiple regions of putative CAREs were identified (Fig. 3A) using TRANSFAC (27). Interestingly, the region containing multiple CAREs (between -1415 and -2257 bp from the first methionine codon (+1)) was highly conserved among multiple mammalian species (data not shown). Therefore, the effects of phenobarbital, a prototype CAR agonist, on the expression of CYP8A1 in HUVEC were examined. Treatment of phenobarbital induced a large increase in CYP8A1 mRNA in a dose-dependent manner when compared to the control group (3-fold, P<0.01) (Fig. 3B).

To determine the activity of the CYP8A1 identified by both qRT-PCR and Western blotting analyses, an enzyme immunoassay was performed as described previously [26]. The assay indicated that CYP8A1 activity was present in HUVEC and that enzyme activity was significantly increased with simvastatin treatment in a dose-dependent manner (P<0.01) (Fig. 4). In ad-

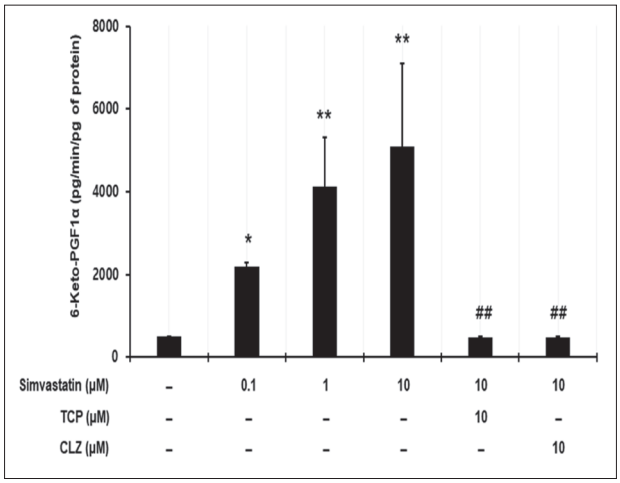

Fig. 4. Assays of 6-keto-PGF1α levels in HUVEC. Simvastatin (0.1, 1 and 10 μM) was applied for 36 h at 37°C to HUVEC. Values are means ± SE of triplicate assays. *P<0.05 and **P<0.01 – compared to the control group (DMSO); ##P<0.01 – compared to the simvastatin-only (10 μM) treatment group.

dition, the CYP8A1 inhibitor tranylcypromine (TCP; 10 μM) and the CAR inhibitor clotrimazole (CLZ; 10 μM) were added to the cell culture. As can be seen in Fig. 4, CYP8A1 activity decreased significantly with the addition of TCP (90.5% decrease, P<0.01) when compared to the simvastatin-only treatment group. CYP8A1 activity was significantly reduced with treatment with the CAR antagonist (90.7% decrease, P<0.01) compared to the control group. Collectively, induction of CYP8A1 expression by simvastatin significantly increased the level of the PGI_2 metabolite 6-keto-PGF1α, and the increased metabolite was substantially repressed with the CAR antagonist CLZ and the CYP8A1 inhibitor TCP.

DISCUSSION

The major pharmacological effect of statins is the lowering of cholesterol synthesis by inhibition of HMG-CoA reductase. However, several lines of evidence suggest additional roles of statins, such as reduced synthesis of TXA_2 [10], suppression of platelet activation [9] and stimulation of PGI_2 synthesis [8]. Therefore, the present study was undertaken to determine the effects of statins on CYP8A1 expression and PGI_2 synthesis at the molecular level using human endothelial cells (HUVEC). PGI_2 can inhibit platelet aggregation in addition to vasodilation [11]. Although statins have been reported to have antiplatelet effects through increased expression of eNOS [9], the molecular mechanism of antiplatelet action by statins remains unknown. In the present study, among five different statins, simvastatin significantly induced CYP8A1 at both transcriptional and translational levels, resulting in increased synthesis of PGI_2.

When the five different statins were applied to HUVEC, the mRNA of CYP8A1 was significantly increased 3-fold in simvastatin-treated cells compared to the vehicle-treated group. However, atorvastatin, pravastatin, lovastatin and rosuvastatin exhibited insignificant differences in CYP8A1 mRNA expression compared to the control group. To confirm whether the increased expression of CYP8A1 with simvastatin was the result of transcriptional regulation, CYP8A1 protein levels were determined with and in the absence of pretreatment with actinomycin D, a transcription inhibitor. Consistent with transcriptional regulation, the CYP8A1 protein level was increased with simvastatin treatment alone, but not after actinomycin D pretreatment. This suggests that different statins have different influences on CYP8A1 expression, in addition to the common mechanism of HMG-CoA reductase inhibition. Many studies have reported cholesterol-independent effects of statins, including reduction of C-reactive protein [28], modulation of vascular inflammation [29, 30], upregulation of scavenging enzymes (catalase, superoxide dismutase (SOD) and thioredoxin reductase) [31], inhibition of NF-κB activation [32], and inhibition of inflammation-mediated proliferation and migration of vascular smooth cells [33]. Since our data pointed to the induction of CYP8A1 after simvastatin treatment, we investigated the molecular mechanism of CYP8A1 expression by simvastatin. It was reported that hemodynamic changes are observed with simvastatin treatment in rats, including the finding of an increased level of 6-keto-PGF1α, a nonenzymatic hydrolysis product of PGI_2 [34]. Although this finding was from an animal study, it suggested the possibility of CYP8A1 induction by simvastatin in human cells. In the course of a screening of human drug activators of CAR (NR1I3), which is a member of the nuclear receptor family and a major regulator of CYP2B and CYP2C genes via phenobarbital, statins causing 3-fold human CAR-mediated transcriptional activation of the phenobarbital-responsive enhancer module (PBREM)

were identified [13]. Therefore, the CARE was analyzed in the PGIS promoter region using TRANSFAC. The CYP8A1 promoter up to -3 kb was found to have eight putative CAREs and the region between -2257 bp and -1420 bp was particularly highly conserved among species (data not shown). Treatment with phenobarbital led to the induction of CYP8A1 mRNA, which was in good agreement with the increased CYP8A1 expression after simvastatin treatment. There are many xenobiotics and drugs that are agonists of CAR, and CAR activation can influence the upregulation of many genes through CAREs [35,36]. The induction of CYP8A1 through CAR activation suggests that the expression of CYP8A1 can be affected by foods, different chemicals and drugs. This variable expression may result in inter-individual variations in blood PGI_2 levels, providing for increased complexity in blood homeostasis. The balance between TXA_2 and PGI_2 levels is important in the regulation of platelet activation and blood coagulation [37]. To determine the effects of statins, CYP8A1 inhibitors and CAR inhibitors on the level of PGI_2, the HUVEC were treated with simvastatin and TCP, and CLZ and PGI_2 levels were measured. In agreement with the results of CYP8A1 expression, PGI_2 levels were significantly increased with simvastatin treatment and the increased level of PGI_2 was repressed by treatment with the CYP8A1 inhibitor TCP, indicating that PGI_2 in the experimental system was solely synthesized from CYP8A1 enzyme activity. In addition to the effect of TCP, the CAR antagonist CLZ also significantly repressed PGI_2 levels, suggesting that PGI_2 levels in HUVEC can be regulated by CAR activation.

Although these *in vitro* studies showed the induction of CYP8A1 by simvastatin through the CAR-mediated mechanism, the involvement of CAR in transcriptional regulation may be different, depending on the expression locations or sources of the endothelial cells. For example, CYP2C9 expression was induced by statins and the transcriptional mechanism of the induction was attributed to the strong expression of CAR in the endothelial cells [21, 38]. However, it was reported that there are different expressions of the pregnane X receptor (PXR) and CAR between HUVEC and human cardiac microvascular endothelial cells (hCMEC) [39] as it was found that strong expression of the Ah receptor occurred in both cell lines, but very low expression of CAR and PXR was detected in HUVEC when compared to the hCMEC [39]. Since CAR and PXR have a broad overlapping set of ligands that recognize similar DNA elements [40,41], the transcriptional regulation of PGIS could be influenced by both CAR and PXR.

In human blood vessels, PGI_2 synthesized from PGH_2 in endothelial cells is released into the blood and binds to prostacyclin receptors on platelets, resulting in the inhibition of platelet activation [42]. PGI_2 also binds to endothelial prostacyclin receptors and leads to the sequential cascade of cAMP signaling, resulting in the activation of protein kinase A, promotion of phosphorylation of myosin light chain kinase, and finally, smooth muscle relaxation and vasodilation [37,42]. The regulation of prostacyclin levels is important in the maintenance of normal blood homeostasis together with other signaling molecules such as TXA_2. Most data concerning inter-individual variations in PGI_2 levels in humans have been reported from genetic polymorphisms in the CYP8A1 gene [26,43]. However, the present results suggest that PGI_2 levels can be affected by xenobiotic factors, and our study indicates that simvastatin can upregulate CYP8A1 expression through a CAR-mediated mechanism. Further research is needed to determine the effects of simvastatin on PGI_2 levels *in vivo* in clinical settings.

CONCLUSION

Among five different statin treatments, simvastatin treatment resulted in upregulation of CYP8A1 and an increase in the PGI_2 metabolite, indicating that different statins exert different influences on PGI_2 levels. The present study suggests that PGI_2 levels can be affected by exogenous induction mechanisms in addition to endogenous genetic factors. Therefore, this result can serve to investigate further the inter-individual variations in PGI_2 levels, since there are many CAR and PXR activators in foods and drugs. This study will be helpful for researchers in studies of cardiovascular diseases with respect to PGI_2.

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