Statin inhibits hypoxia-induced endothelin-1 via accelerated degradation of HIF-1α in vascular smooth muscle cells

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Aims
Endothelin-1 (ET-1) contributes to the pathogenesis of cardiovascular diseases with multiple properties such as vasoconstriction. Human ET-1 gene expression is up-regulated by the transcription factor hypoxia-inducible factor-1 (HIF-1) through hypoxia response element (HRE). Although previous studies suggested that 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins) alter HIF-1-related gene expression, it remained unclear whether statins modulate HIF-1-mediated ET-1 expression. Therefore, we investigated the effect of fluvastatin on hypoxia-induced human ET-1 expression in vascular smooth muscle cells (VSMC).

Methods and results
Hypoxia (1% O2), compared with the normoxic condition (21% O2), significantly induced the expression of pre-proET-1 mRNA, ET-1 protein, and ET-1 secretion in VSMC. Hypoxia induced a 2.3-fold increase in HRE-dependent ET-1 reporter gene activation. Under concentrations of 1 μmol/L or greater, fluvastatin attenuated the hypoxia-induced ET-1 gene expression through the accelerated ubiquitin/proteasome-dependent degradation of HIF-1α, thus consequently attenuating HIF-1α binding to the HRE of the ET-1 gene. These inhibitory effects of fluvastatin were cancelled by concomitant treatment with mevalonate, farnesyl pyrophosphate, or geranylgeranyl pyrophosphate, but not squalene.

Conclusion
The present study suggests that fluvastatin attenuates HIF-1-dependent ET-1 gene expression in conjunction with the stimulation of HIF-1α ubiquitin/proteasome-dependent degradation via isoprenoid-dependent mechanisms.

Keywords
Endothelin-1 • Statins • Hypoxia • Hypoxia-inducible factor-1 • Vascular smooth muscle cells

1. Introduction
Endothelin-1 (ET-1) plays an important role in various cardiovascular diseases such as coronary artery disease (CAD), pulmonary hypertension (PH), and high-altitude disease through its vasoconstrictive property and multiple cellular functions, including proliferation, hypertrophy, fibrosis, and activation of cardiac-specific genes. Not only endothelial cells but also vascular smooth muscle cells (VSMC) are considered as important sources of ET-1 secretion. ET-1 secretion by vascular cells is promoted by hypoxia which reportedly facilitates binding of the transcription factor hypoxia-inducible factor-1 (HIF-1) to the hypoxia response element (HRE) in the ET-1 promoter, thus resulting in the increased gene transcription. HIF-1 is composed of a heterodimer of HIF-1α and HIF-1β subunits, both of which belong to a basic helix-loop–helix Per-AhR/Amt- Sim transcription factor family. Under normoxic conditions, HIF-α is hydroxylated at two critical proline residues (proline-402 residue and proline-564) by the prolyl hydroxylases in
the presence of dioxygen, 2-oxoglutarate (2OG), and ferrous iron (Fe^{2+}). This hydroxylation allows the binding of von Hippel-Lindau (VHL)—E3 ubiquitin ligase complex to these proline residues, leading to ubiquitination of HIF-1α and the subsequent proteasomal degradation. Exposure to hypoxia or cobaltous ions inhibits this ubiquitination and proteasomal degradation of HIF-1α, resulting in increased expression of HIF-1α, which is translocated into the nucleus to transactivate its target genes. Factor-inhibiting HIF-1 (FIH-1), another iron- and oxoglutarate-dependent oxygenase, regulates the transcriptional activity of HIF-1 independently of HIF-1α degradation. FIH-1 binds to a critical asparagine residue of HIF-1α, asparagine-803, in normoxic conditions and prevents the interaction of the HIF-1 C-terminal transactivation domain (CTAD) with transcriptional coactivator CBP/p300.

The 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, also termed statins, reduce morbidity and mortality in patients with CAD mainly through a cholesterol-lowering effect. Statins also have lipid-independent effects, such as anti-inflammatory and anti-oxidative properties, which are attributable to inhibition of isoprenoid production.

Previous studies have demonstrated associations between HIF-1 and statins; simvastatin attenuated HIF-1α expression in the coronary artery wall especially the VSMC layers of the media isolated from hypercholesterolaemic pigs independent of its cholesterol-lowering effect. Statins also have lipid-independent effects, such as anti-inflammatory and anti-oxidative properties, which are attributable to inhibition of isoprenoid production.

In the present study, we demonstrate that fluvastatin attenuated hypoxia-induced ET-1 expression in VSMC by attenuating HIF-1α protein levels. This inhibitory effect may be attributable to accelerated HIF-1α degradation in association with the down-regulation of isoprenoids by fluvastatin.

2. Methods

2.1 Materials

Fluvastatin, pravastatin, and simvastatin were donated by Mitsubishi Tanabe (Osaka, Japan), Daiichi Sankyo (Tokyo, Japan), and Banyu (Tokyo, Japan), respectively. Mevalonate, squalene, farnesyl pyrophosphate (FPP), geranylgeranyl pyrophosphate (GGPP), and camptothecin were purchased from Wako Pure Chemical (Osaka, Japan). Mevalonate, squalene, farnesyl pyrophosphate (FPP), geranylgeranyl pyrophosphate (GGPP), and camptothecin were purchased from Wako Pure Chemical (Tokyo, Japan).

2.2 Cell culture and hypoxic treatment

Human coronary smooth muscle cells were purchased from Kurabo (Osaka, Japan). Cells were grown at 37°C in Hamedia-SG2 medium (Kurabo) containing 5% foetal bovine serum (FBS), 5.0 μg/mL insulin, 2.0 ng/mL human basic fibroblast growth factor-B, and 0.5 ng/mL human epidermal growth factor. All assays were carried out in human SB2 basic medium (Kurabo) with 1% FBS. The cells were exposed to hypoxia by culturing at 1% O2, 5% CO2, and 94% N2 at 37°C using a Napco 7000 incubator (Thermo Scientific, Waltham, MA, USA). Fluvastatin and pravastatin, water-soluble drugs, were dissolved in distilled water. A lipid-soluble simvastatin was dissolved in DMSO before use. DMSO was added at a concentration equal (final 0.1%) to all samples in the evaluation of simvastatin efficacy.

2.3 Total RNA isolation and real-time quantitative RT–PCR analysis

At the indicated hours after treatment with the compounds, total RNA was extracted using the SV Total RNA Isolation System (Promega, Madison, WI, USA) as reported previously. Transcript levels of target genes were identified as described in the Supplementary material online.

2.4 Preparation of whole-cell extract and western blot assay

Cells were harvested and protein extracts were prepared as previously described, and western blot analyses were conducted as described in the Supplementary material online.

2.5 ET-1 and BigET-1 immunoassay

ET-1 in whole-cell extracts and cell culture media was measured using the Human Endothelin-1 Immunoassay (R&D Systems, Minneapolis, MN, USA) according to the manufacturer’s instructions, and the Big-ET1 level in whole-cell extracts was also detected by Big Endothelin-1 (human) EIA kit (Enzo, PA, USA).

2.6 Plasmids

The human ET-1 promoter region spanning –176 to +173 bp or –98 to +173 bp relative to the transcription start site was PCR-amplified using human genomic DNA (Roche, Indianapolis, IN, USA) as a template and ET-1-specific primers (forward primer with KpnI site, 5′-TAAGGTACCGTTCTCTGAAAGTTACGTG-3′ for –176 and 5′-TAAGGTACCCACAATAACATTGTCTGGGGG-3′ for –98; reverse primer with BglII sites, 5′-TATAGATCTAAAGCCGATCCTTCGAGCC-3′). The PCR products were ligated into a pGL3-basic vector (Promega) to create ET-1–176/+173-Luc and ET-1 –98/+173-Luc. 3xHRE-kt-Luc was kindly gifted from Dr Steven L. McKnight of the University of Texas Southwestern Medical Center. HIF-1α expression vectors HA-HIF1α-pcDNA3 and HA-HIF1αP402A/P564A-pcDNA3 were purchased from Addgene (Cambridge, MA, USA). pGL3-Control vector was purchased from Promega.

2.7 DNA transfection and luciferase assays

VSMC were cultured in six-well plates and transfection was performed with Lipofectamine LTX and Plus Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. Transfection was performed with 300 ng of each reporter plasmid and 20 ng of phRL-TK (Promega). After 6 h of transfection, media were replaced with human SB2 medium (Kurabo) with 1% FBS, and the cells were further cultured in the presence or absence of fluvastatin or other compounds for 24 h. The cells were then lysed with 100 μL of passive lysis buffer (Promega). Luciferase activity assays were performed using a Dual-Luciferase Reporter Assay System (Promega) and luciferase activity was measured using a Luminometer GeneLight 55 (Microtec, Tokyo, Japan).

2.8 Immunoprecipitation: HIF-1α polyubiquitination

After treatment with the compounds, whole-cell extracts (500 μg) of VSMC were prepared with lysis buffer. The immunoprecipitation assay was performed using Immunoprecipitation Kit Dynabeads Protein G (Invitrogen) with 4 μg anti-HIF-1α antibody (Santa Cruz Biotechnology, CA, USA) according to the manufacturer’s instructions with the following modification: an immunoprecipitation reaction was conducted with cell lysis buffer for 1 h at 4°C. Subsequently, polyubiquitination of HIF-1α was assessed by western blotting with anti-ubiquitin antibody (sc-8017, Santa Cruz Biotechnology) at 1:10,000 dilution.
2.9 Chromatin immunoprecipitation assay
We performed the chromatin immunoprecipitation (ChIP) assay using the method reported by Setoguchi et al. as described in the Supplementary material online.

2.10 Statistical analysis
Data presented are mean ± SD of three separate experiments. Statistical significances between experimental groups were calculated using Student's t-test and one-way or two-way ANOVA followed by the Bonferroni post hoc test. A value of \( P < 0.05 \) was considered significant.

3. Results
3.1 Fluvastatin attenuates hypoxia-induced ET-1 gene expression in VSMC
Initially, we analysed the effect of fluvastatin on hypoxia-induced ET-1 gene expression in human coronary artery smooth muscle cells. As expected, preproET-1 mRNA increased significantly during hypoxia (1% \( O_2 \) condition) for 12 h, by 2.4-fold compared with the normoxic condition (21% \( O_2 \)), and treatment of fluvastatin with concentrations of \( 1 \mu \text{mol/L} \) or greater inhibited this induction (Figure 1A). As shown in Figure 1B, preproET-1 mRNA levels evidently increased 8 h after exposure to hypoxia and 1 \( \mu \text{mol/L} \) fluvastatin completely attenuated this hypoxia-induced preproET-1 mRNA expression. Mirroring the mRNA levels, ET-1 immunoassays revealed that fluvastatin reduced hypoxia-induced ET-1 protein levels in whole-cell extracts (Figure 1C) and media (Figure 1D). Further, we confirmed that the inhibitions of preproET-1 mRNA expression, ET-1 protein level, and ET-1 secretion by fluvastatin were also evident under the normoxic condition (see Supplementary material online, Figure S1). The inhibitory effects of fluvastatin on BigET-1 protein levels in hypoxia induction were similar to both those of preproET-1 mRNA and ET-1 expressions as shown in Supplementary material online, Figure S2.

3.2 Fluvastatin inhibits HIF-1-dependent ET-1 transcripational activation during hypoxia in VSMC
To elucidate the molecular mechanisms underlying the attenuation of hypoxia-induced ET-1 gene expression by 1 \( \mu \text{mol/L} \) fluvastatin, we investigated the effect of fluvastatin and hypoxia on ET-1 transactivation using luciferase reporter plasmids containing 5' flanking regions of the ET-1 gene with or without HRE (Figure 2A). As shown in Figure 2B and C, hypoxia activated the ET-1 promoter by 2.3-fold in an HRE-dependent fashion. Concomitant treatment with fluvastatin diminished this hypoxia-induced ET-1 transactivation, supporting the observations shown in Figure 1.

In addition, hypoxia markedly induced the HIF-1-dependent reporter gene expression using 3xHRE-tk-Luc that was attenuated by fluvastatin (Figure 2D), an observation similar to the result in the endogenous ET-1 promoter. To confirm whether this inhibitory property of fluvastatin was a class effect of statins, we performed the same experiments using other statins. Supplementary material online, Figure S3, demonstrates that both pravastatin and simvastatin had similar inhibitory effects on HIF-1-dependent ET-1 transactivation. Fluvastatin did not affect transcripational activities using pGL3-Control.
vector (positive control) and pGL3-Basic vector (negative control) in both normoxic and hypoxic conditions (data not shown). To eliminate the possibility of cell damage and changing cell cycle in these inhibitory effects by this concentration (1 μmol/L) of fluvastatin, we assessed the effects of fluvastatin on cell death including apoptosis as shown in Supplementary material online, Figure S4. There was no effect of fluvastatin on cell death and apoptosis in our experimental conditions: during normoxia (21% O2) or hypoxia (1% O2) in the presence or absence of 1 μmol/L fluvastatin for both 12 and 24 h.

Although there was no effect during normoxia and hypoxia in the presence or absence of 1 μmol/L fluvastatin for 12 h on cell growth, hypoxia exposure slightly increased cell growth compared with normoxia for 24 h and fluvastatin inhibited this increase.

3.3 Fluvastatin inhibits hypoxia-induced, but not CoCl2-induced, ET-1 transactivation via reduced HIF-1α expression in VSMC

To further investigate the underlying mechanisms, we assessed the effects of 1 μmol/L fluvastatin on HIF-1α expression in VSMC. As expected, fluvastatin significantly increased LDL-R mRNA levels under both the normoxic (21% O2) and hypoxic (1% O2) conditions (Figure 3B); in contrast, it did not alter HIF-1α mRNA levels in either condition (Figure 3A). Figure 3C demonstrates that HIF-1α protein expression evidently decreased by hypoxia together with fluvastatin treatment, whereas protein expressions of HIF-1β, VHL, and FIH-1 did not.

Treatment with cobaltous ions is known to inhibit the interaction between HIF-1α and VHL by replacing the iron atom of Fe2+, which in turn stabilizes HIF-1α protein, thereby resulting in transactivation of the target genes. Therefore, we investigated whether fluvastatin attenuated ET-1 transactivation induced by CoCl2 treatment. In contrast to hypoxia, fluvastatin did not induce any changes in CoCl2-induced activation of the ET-1 promoter (Figure 4A). Supporting the observations in the promoter assay, fluvastatin did not affect CoCl2-mediated HIF-1α protein accumulation (Figure 4B), suggesting that the mechanisms of hypoxia- and CoCl2-induced HIF-1α expression and ET-1 transactivation in relation to statin treatment may be distinct.

3.4 Fluvastatin stimulates HIF-1α ubiquitination and proteasomal degradation during hypoxia exposure in VSMC

Next, to explore the precise mechanisms of inhibitory effects on hypoxia-induced ET-1 transactivation in fluvastatin treatment, we investigated the role of the two critical prolines in HIF-1α, P402 and P564, which are reportedly essential for degradation by VHL. When wild-type HIF-1α was overexpressed in VSMC, exposure to hypoxia stimulated ET-1 transactivation, which was markedly inhibited by 1 μmol/L fluvastatin through attenuation of exogenous expression of HIF-1α (Figure 4C and D), similar to endogenous expression (Figure 3). In contrast, overexpression of P402A/P564A HIF-1α, which resulted in enhanced expression due to escape from degradation by VHL, increased HIF-1-dependent ET-1 transactivation compared with wild-type HIF-1α even under normoxia (Figure 4C and D). This enhanced activity was affected by neither hypoxia nor fluvastatin treatment. In
parallel with ET-1 reporter gene expression, hypoxia-induced accu-

mulation of ectopic expression of wild-type HA-HIF-1α was inhib-

ited by fluvastatin; in contrast, neither hypoxia nor fluvastatin

affected protein levels of HA-HIF1αP402A/P564A (Figure 4C

and D). These results indicate that VHL-mediated degradation of

HIF1α may be involved in both oxygen- and statin-mediated reduc-

tion in HIF-1α protein.

Since HIF-1α protein is known to undergo polyubiquitination and

subsequent degradation by the ubiquitin–proteasome system, we

further investigated whether proteasomal inhibition affected the

statin-mediated effect on the HIF-1α level and whether statin treat-

ment affected the polyubiquitination of HIF-1α. As expected,

HIF-1α accumulations were observed in the presence of MG132, a

proteasome inhibitor, under both normoxic and hypoxic conditions

(Figure 5A, lanes 2, 5, and 6). Notably, 1 μmol/L fluvastatin-mediated

reduction of HIF-1α accumulation (Figure 5A, lane 4) diminished in

the presence of MG132 under hypoxia (Figure 5A, lane 6). Figure 5B

demonstrates that polyubiquitinated HIF-1α accumulated in the presence of MG132 under hypoxia (Figure 5B, lane 2) and exposure to hypoxia attenuated the accumulation of HIF-1α polyubiquitination (Figure 5B, lane 5). In the presence of fluvastatin, this hypoxia-induced attenuation of HIF-1α polyubiquitination was cancelled (Figure 5B, lane 6). These findings indicate that fluvastatin accelerates the polyubiquitination of HIF-1α under hypoxic condition, thus resulting in enhanced degradation by proteasomes.

3.5 Mevalonate and isoprenoids restore the inhibitory effects of fluvastatin on protein levels of HIF-1α and HIF-1α binding to the ET-1 promoter

We investigated whether the products catalysed by HMG-CoA

reductase or the downstream enzymes were involved in the

statin-inducible effects demonstrated above. Figure 6A demonstrates

that the inhibitory effect of 1 μmol/L fluvastatin on hypoxia-induced

ET-1 promoter activity was cancelled by concomitant treatments

with mevalonate and the isoprenoids FPP and GGPP, but not squa-

lene. In parallel with promoter activity, diminished HIF-1α expression

as a result of fluvastatin treatment was restored in the presence of

mevalonate, FPP, or GGPP, but not squalene (Figure 6B).

Next, we performed ChIP assays to confirm that HIF-1α bound to

the HRE of the ET-1 promoter in accordance with its expression

levels as altered by hypoxia, fluvastatin, or other compounds. Figure 6C demonstrates that hypoxia enhanced HIF-1α binding to the HRE of the ET-1 promoter (lanes 1 and 2), and this enhancement was attenuated in the presence of 1 μmol/L fluvastatin (lane 3). Concomitant treatment with mevalonate, FPP, and GGPP, but not squalene, restored the attenuated HIF-1α binding to the HRE induced by fluvastatin, in parallel with the HIF-1α expression levels shown in Figure 6C (lanes 4–7). Taken together, these observations suggest that reduced amounts of the downstream products of
statins, mevalonate, FPP, and GGPP, caused the inhibitory effect of fluvastatin on HIF-1α accumulation followed by transrepression of ET-1 gene in VSMC.

4. Discussion

In the present study, we demonstrated, for the first time to the best of our knowledge, that statins inhibit hypoxia-induced ET-1 gene transactivation by accelerating HIF-1α polyubiquitination and proteasomal degradation in VSMC. The present study also suggests that this inhibitory effect of fluvastatin on HIF-1-dependent ET-1 gene expression was due to the down-regulation of isoprenoid intermediates.

Our new findings revealed the details of the mechanism whereby fluvastatin inhibits hypoxia-induced ET-1 transactivation through the stimulation of HIF-1α protein polyubiquitination and the inhibition of HIF-1α binding to the HRE of the ET-1 promoter, using a ChIP assay in VSMC (Figures 5 and 6). Using an ELISA-based transactivation assay in endothelial cells, Loboda et al. demonstrated that atorvastatin inhibited hypoxia-induced ET-1 activity. In addition, using an electromobility shift assay in endothelial cells and VSMC, Dichtl et al. reported the diminishment of HIF-1 binding to the HRE of the PAI-1 gene by simvastatin, atorvastatin, andLovastatin. We also demonstrated that pravastatin and simvastatin inhibited HIF-1-dependent ET-1 promoter activity (see Supplementary material online, Figure S3), thus suggesting that the inhibitory effect on HIF-1-dependent ET-1 gene expression is a common effect of statins in VSMC.

Regarding the therapeutic value of 1 μmol/L fluvastatin, Holdaas et al. previously reported that the C_{max} per day of oral fluvastatin (40 mg twice a day) in healthy subjects was \sim 1 μmol/L fluvastatin.

![Figure 4](https://example.com/figure4.jpg)

**Figure 4** Fluvastatin affects neither CoCl₂-induced ET-1 transcriptional activity nor ectopic expression of HIF-1α P402A/P564A, including two critical proline mutations. (A) Reporter activity of ET-1 -176/+173-Luc with or without 100 μmol/L CoCl₂ in the presence or absence of 1 μmol/L fluvastatin for 24 h. *\P < 0.05 compared with normoxic control using two-way ANOVA followed by the Bonferroni post hoc analysis. n = 6/group. (B) Protein levels of HIF-1α and β-actin (loading control). n = 3/group. (C) Reporter activity of ET-1 -176/+173-Luc with transfection of 0.5 μg of the ectopic expression plasmid of HA-HIF-1α or HA-HIF-1αP402A/P564A in six-well plates. *\P < 0.05 compared with normoxic control using one-way ANOVA followed by the Bonferroni post hoc analysis. n = 6/group. (D) Protein levels of HA-HIF-1α and β-actin (loading control) with transfection of 3 μg of the ectopic expression plasmid of HA-HIF-1α or HA-HIF-1αP402A/P564A in 100 mm cell culture dishes. n = 3/group.

![Figure 5](https://example.com/figure5.jpg)

**Figure 5** Fluvastatin stimulates polyubiquitination of HIF-1α under hypoxia. (A) Protein levels of HIF-1α and β-actin (loading control) in input samples. (B) HIF-1α polyubiquitination. VSMC were cultured with (lanes 2, 5, and 6) or without (lanes 1, 3, and 4) 10 μmol/L MG132 and in the presence (lanes 4 and 6) or absence (lanes 1, 2, 3, and 5) of 1 μmol/L fluvastatin and exposed to normoxia (21% O₂) (lanes 1 and 2) or hypoxia (1% O₂) (lanes 3–6) for 8 h. n = 3/group.
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Therefore, we consider this concentration to have a clinical value in higher dose intakes of fluvastatin. We confirmed that at this higher concentration, fluvastatin had no effect on cell death in exposure to hypoxia or normoxia for 12 and 24 h as shown in Supplementary material online, Figure S4. Neither was an inhibitory effect on cell growth observed during normoxia and hypoxia for 12 h in the presence or absence of 1 μmol/L fluvastatin, although fluvastatin inhibited hypoxia-induced ET-1 expression.

There was a significant increase in cell growth under hypoxic conditions for 24 h compared with that under normoxic conditions for 24 h, and 1 μmol/L fluvastatin inhibited this increase (see Supplementary material online, Figure S4). This increased cell growth may be because of hypoxia-induced ET-1 secretion (Figure 1D) since previous studies have demonstrated that ET-1 stimulates VSMC growth. 28– 30 On the basis of this, the inhibitory effect of fluvastatin on VSMC proliferation after exposure to hypoxia for 24 h may be associated with that of fluvastatin on ET-1 induction by HIF-1.

There are some discrepancies in the effects on HIF-1-mediated gene expressions by statins, which are probably due to cell specificities and/or gene specificities. Our findings about the inhibitory effect of fluvastatin in VSMC are consistent with several previous studies demonstrating that statins attenuated HIF-1-related vascular endothelial growth factor (VEGF) expression in an animal model and PAI-1 expression in human endothelial cells and VSMC. 19,20 In contrast, several other studies have demonstrated that statins up-regulated HIF-1 expression 11,32 and stimulated HIF-1-dependent VEGF expression through the down-regulation of the HIF-1α inhibitor prolyl hydroxylase domain 2 protein in vascular endothelial cells. 33 These differences may be related to specific subsets of transcriptional factors or intracellular signalling, as Yamashita et al. 34 previously indicated that GATA-2, activator protein-1, and CAAT-binding factor nuclear factor 1 are important for the stabilization of the binding of HIF-1 to the HRE of the ET-1 promoter in human umbilical vein endothelial cells (HUVEC), and other investigations demonstrated that the PI3K/Akt pathway and the mitogen-activated protein kinase pathway are engaged in HIF-1-dependent gene expression in lung vascular endothelial cells and retinal epithelial cells. 35,36

In accelerating HIF-1α polyubiquitination and proteasomal degradation by statin, the hydroxylation of two proline residues of HIF-1α by proline hydroxylases in an O2-, ZGO-, and Fe2+-dependent manner allows interaction between HIF-1α and the VHL–E3 ubiquitin ligase complex (elongin-C, elongin-B, CUL2, and RBX1). 37 This interaction is followed by ubiquitination mediated by E1 of Uba1 and E2 of Ubc5a and, thereafter, degradation by the 26S proteasome. 37 The effects of statin on these factors, which are involved in O2-dependent HIF-1α ubiquitin/proteasome-dependent degradation, have been less studied. Although no significant change in the VHL protein level was observed in our study, as shown in Figure 3C, additional analyses in the effect of statin on the expression of these factors and in complex formation may be needed.

An O2-independent pathway of HIF-1α ubiquitin/proteasome-dependent degradation has also been reported: receptor for activated C-kinase 1 (RACK1) and heat-shock protein (HSP90) compete for binding to the PAS-A domain of HIF-1α. RACK1, in addition to VHL, recruits the elongin-C and -B subunits of E3 ubiquitin ligase and increases HIF-1α ubiquitination and proteasomal degradation. 38 The effect of statin on RACK1 and HSP90 remains unknown. Because the decrease in HIF-1α accumulation was cancelled by CoCl2 or mutation of two critical prolines (P402 and P564), as shown in Figure 4A and B, it appears unlikely that this O2-independent pathway involves the accelerated degradation of HIF-1α by statin.

The function of the HIF-1α CTAD is also important for the transactivation of HIF-1 target genes such as ET-1. O2-dependent FIH-1 binding and MAP kinase (p42/44 and p38 MAP kinase)-dependent HIF-1α phosphorylation regulate the recruitment of coactivator CBP/p300 to HIF-1α CTAD. 15 Previous studies reported the influence of statin on these MAP kinase pathways. 39,40 Although our study found no change of the FIH-1 protein level, as shown in Figure 3C, additional investigation of the extent to which these regulatory mechanisms affect the inhibitory effect of statin on hypoxia-induced ET-1 may be needed.

Fluvastatin regulates HIF-1α-dependent gene expression via isoprenoids in VSMC (Figure 6). Isoprenylation is attributed to the post-
translational modification and function of various proteins, including small GTP-binding proteins. The pleiotropic effects of statins have been associated with the inhibition of isoprenylation of Ras, RhoA, Rac1, and Cdc42 downstream of FPP and GGPP. Previous studies reported that inhibition of the Rho/Rho-associated kinase pathway induced HIF-1α degradation in HUVEC and Ras inhibition down-regulated HIF-1α in glioblastoma cells. The inhibitory effect of statins on HIF-1α-regulated gene expression may be mediated by the inhibition of these small GTP-binding proteins through the down-regulation of important isoprenoid intermediates in VSMC.

The relation between increased plasma levels of ET-1 and cardiovascular diseases such as vasospastic angina has been described in previous investigations, and the therapeutic potential of ET-1 antagonism in cardiovascular diseases. Furthermore, the addition of fluvastatin to calcium channel blockers reportedly reduced acetylcholine-induced coronary spasm compared with calcium channel blocker therapy alone in vasospastic angina patients, presumably independent of lipid lowering. Therefore, the antagonistic regulation of hypoxia-induced ET-1 expression by statins in VSMC may be a potential therapeutic application for these atherosclerotic diseases. ET-1 has been found to have strong associations with other hypoxia-related diseases such as PH and high-altitude disease, demonstrated that pravastatin significantly improved PH and exercise tolerance in chronic obstructive pulmonary disease patients, presumably by decreasing ET-1 synthesis. On the other hand, a previous investigation showed the beneficial effect of ET-1 antagonism (using an ET receptor A and B antagonist) on PH induced by high-altitude climbing. Hence, the inhibitory effects of statin on hypoxia-induced ET-1 expression observed in this study may be a promising therapeutic strategy for these diseases. Although the change of O2 concentration from 21 to 1% in our experimental setting was designed to emphasize those effects on HIF-1 transactivation, the evaluation of these effects by statins in various changes of O2 concentrations and the time course involved should be investigated further in order to determine more clearly how that applies to these diseases.

In conclusion, this study demonstrated that fluvastatin inhibited HIF-1-induced ET-1 gene expression by the acceleration of HIF-1α protein ubiquitination through an isoprenoid-dependent mechanism. Therefore, statin, in particular fluvastatin, may provide a novel therapeutic strategy in patients with PH and high-altitude disease.

Supplementary material
Supplementary material is available at Cardiovascular Research online.

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