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Atorvastatin promoted in vitro angiogenesis by reduction of geranylgeranyl pyrophosphate in a dose-dependent manner and protected against rho kinase-mediated endothelial cell damage caused by thromboxane A_2

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Abstract

Background: Atorvastatin can inactivate Rho/Rho kinase via a reduction in the synthesis of geranylgeranyl pyrophosphate (GGPP). Thromboxane A_2 (TxA_2) causes endothelial cell (EC) apoptosis via Rho/Rho kinase activation. We tested the hypothesis that atorvastatin protects against the Rho kinase-mediated anti-angiogenic effect of TxA_2.

Methods: We used human coronary artery ECs to form tubular structures on plates coated with a basement membrane matrix gel. The number of tubular structure was counted under a microscope. The caspase-3 activity was used as a determinant of apoptosis.

Results: Atorvastatin significantly increased the number of tubes in a dose-dependent manner, and this effect was blocked by mevalonate or geranylgeranyl pyrophosphate (GGPP). Similar to atorvastatin, a potent selective inhibitor of geranylgeranyl transferase type I enhanced tubular formation. A TxA_2 mimetic (IBOP) inhibited formation of EC tubular structures. The inhibitory effect was completely blocked by a TxA_2 antagonist (SQ29548), a Rho kinase inhibitor (Y27632), and by atorvastatin. The IBOP-induced increase in caspase-3 activity was attenuated by atorvastatin.

Conclusions: Atorvastatin promoted in vitro angiogenesis of ECs in a dose-dependent manner and reversed the TxA_2 receptor-mediated anti-angiogenic effect. We suggest that reduction of GGPP and inactivation of Rho kinase plays an important role in the proangiogenic effect of atorvastatin.

Key words：Angiogenesis, Endothelial cells, Statin, Geranylgeranyl pyrophosphate, Rho kinase, Thromboxane A_2, Caspase-3

Introduction

The pleiotropic anti-hyperlipidemic agent, atorvastatin, has effects other than its ability to block HMG-CoA reductase and lower serum low-density lipoprotein cholesterol. Statins primarily prevent cardiovascular diseases by inhibition of atherosclerosis [1,2] and can reduce myocardial damage [3].

Another effect of statins is the ability to modulate angiogenesis, which is a complex process involving the various components of vessels, such as endothelial cells, smooth muscle cells or extracellular matrix. Angiogenesis plays a critical role in a variety of physiologic and pathological conditions. Statins promote angiogenesis during myocardial ischemia [4], stroke [5], and skin wound healing [6], although one animal study suggested that atorvastatin impairs the angiogenic response to chronic ischemia [7]. On the other hand, statins can induce an anti-angiogenic response in cancer cells [8] or immortalized endothelial cells [9]. Thus,

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the effect of statins on angiogenesis are complex and controversial. Differences in results might be attributed to the use of different statin doses in experimental models; low-dose statin therapy (nano-molar range) might be proangiogenic, while high-dose statin therapy (micro-molar range) might be anti-angiogenic [10-13]. Further, the differential effect of statins on angiogenic gene expression might also determine whether it has proangiogenic or anti-angiogenic effects [14].

The HMG CoA pathway yields farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP) through mevalonate. GGPP modifies small GTP-binding proteins, such as Rho, by prenylation in the cytosol, so that the prenylated Rho translocates to the plasma membrane where it becomes activated. Activated Rho then reacts with effectors, triggering downstream reactions involved in apoptosis [15]. Thus, statins are suggested to inhibit Rho kinase and apoptosis by reducing the level of GGPP.

The eicosanoid thromboxane A_2 (TxA_2) is a well-known mediator released from activated platelets during acute coronary syndrome. It causes platelet aggregation, vasoconstriction, and inhibition of endothelial migration, proliferation, and tube formation. The inhibition of such endothelial cell responses results from endothelial cell apoptosis via its specific receptor during angiogenesis [16]. Mechanisms involved in apoptosis induced by TxA_2 in endothelial cells include inhibition of the PI3K/Akt pathway [17] and/or activation of Rho kinase [18].

Thus, we hypothesized that atorvastatin protects against the anti-angiogenic effect of TxA_2 by suppressing Rho kinase activation that otherwise leads to apoptosis. To investigate the protective effect of atorvastatin on the TxA_2-induced anti-angiogenic effect in vitro, we used human coronary artery endothelial cells that formed tubular structures on plates coated with a basement membrane matrix gel as an in vitro model of angiogenesis.

Methods

Materials, Cell Culture

Human coronary artery endothelial cells (HCAECs), culture medium (EGM-2MV®), and basal medium (EBM®-2) were purchased from Lonza (MD, USA). A TxA_2 mimetic [1S-[1α, 2α(Z), 3β (1E, 3S*), 4α]]-7-[3-[3-hydroxy-4-(4-iodophenoxy)-1-butenyl]-7-oxabicyclo [2. 2. 1] hept-2-yl]-5-heptenoic acid (IBOP) and an inhibitor of Rho kinase (Y27632) were purchased from Cayman Chemicals (MI, USA). A selective TxA_2 receptor antagonist (SQ29548) was purchased from Biomol International (PA, USA). Atorvastatin was a generous gift from Pfizer, Inc. (NY, USA). Mevalonic acid 5-phosphate trilithium salt hydrate (mevalonate) and geranylgeranyl pyrophosphate (GGPP) were obtained from Sigma-Aldrich (MO, USA). A selective inhibitor of geranylgeranyl transferase I (GGTI-286) was obtained from Calbiochem (CA, USA).

In Vitro Tube Formation Assay

The tube formation assay was performed on the surface of a BD Matrigel® Basement membrane matrix obtained from BD Biosciences (MA, USA). One hundred and ten microliters of Matrigel was dispensed into a four-well plate and allowed to polymerize at 37 °C for 1 hour. Cultured HCAECs were trypsinized, resuspended in EGM-2MV, and seeded at a density of 7×10^4 cells per well on four-well plates coated with Matrigel. After the cells had been cultured for 17 hours, tube formation was observed using a microscope (Nikon ECLIPSE TS100), and images were captured with a cooled charge-coupled device (CCD) camera (VB-7000, KEYENCE co.). The tube formation was determined by counting the number of enclosed networks of tubes in eight random fields from each well, as previously described [16].

Caspase-3 Assay

The activity of caspase-3 was determined using the Caspase-3/CPP32 Colorimetric Assay Kit (BioVision, CA, USA). HCAECs were pre-incubated in basal medium for 8 hours and then incubated for 17 hours with vehicle, atorvastatin, and/or IBOP. The caspase-3 activity was measured following the manufacturer's protocol. Briefly, cells were harvested, resuspended in 50 µl of chilled cell lysis buffer, and centrifuged for 1 min in a microcentrifuge (10,000 x g). Reaction buffer was added to cytosolic extract of each sample. Then four mM of the mammalian caspase-3/7-specific colorimetric substrate (DEVD-pNA) was added to each sample for reaction at 37 °C for 1-2 hours. Finally samples were read at 405 nm in a microtiter plate reader.

Data Analysis

Multiple comparisons were performed by one-way ANOVA. A post hoc test was performed by

Bonferroni's method. Differences were considered significant at probability values less than 0.05. Graphs were expressed as mean and standard deviation.

Results

Atorvastatin Promotes Tubular Structure of HCAECs

HCAECs were incubated with atorvastatin on the gel as described in Materials and Methods. Atorvastatin increased the number of tubes when compared with control in a dose-dependent manner in the range of 0.1 through 10 μM (Fig. 1A and 1B). One μM of atorvastatin approximately doubled the number of tubes. This increase was mimicked by a potent selective inhibitor of geranylgeranyl transferase type I (GGTI-286) (Fig. 1C), although its effect was not as strong as that of atorvastatin. Ten μM of GGPP (Fig. 2A) or 10 mM of mevalonate (Fig. 2B) completely abolished the increase by atorvastatin, although farnesylpyrophosphate (FPP) did not (data not shown). GGPP, but not mevalonate decreased the number of tubes when compared with control.

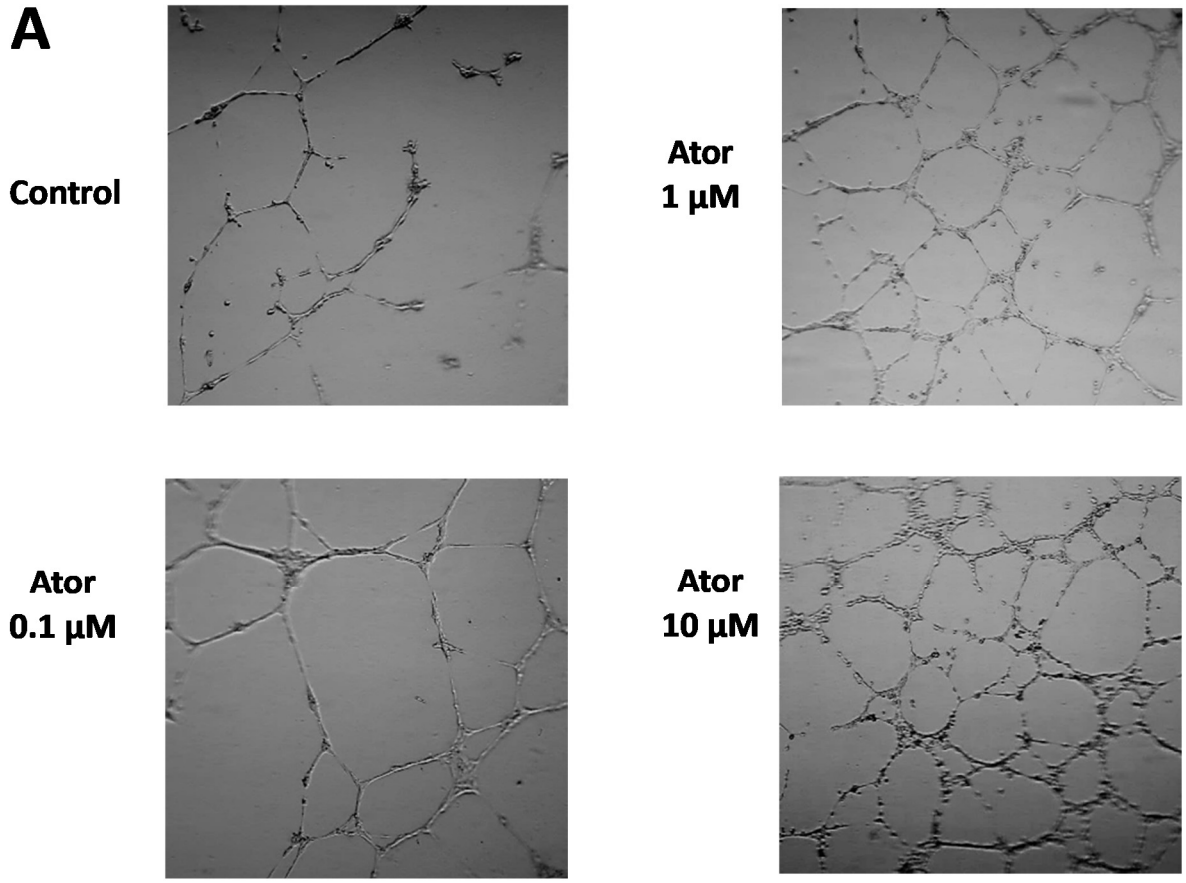

Fig. 1A In vitro tubular formation of HCAECs on the Matrigel observed at 17 hours after seeding under microscopy at a magnification of 40X

Atorvastatin (Ator) increases the number of tubes when compared with control in a dose-dependent manner in the range of 0.1 through 10 μM. Representative pictures for each concentration are shown.

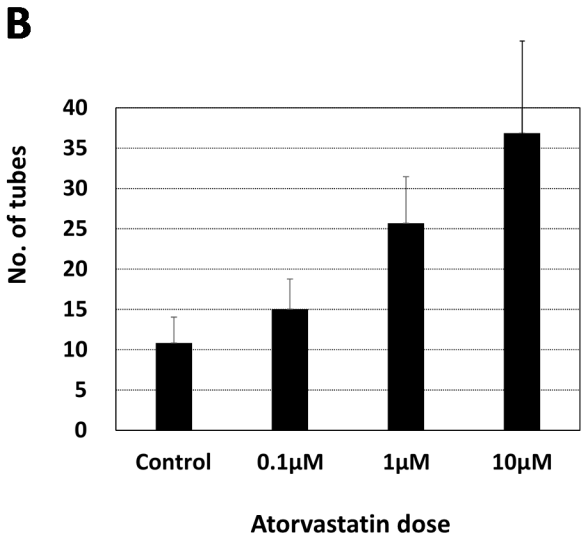

Fig. 1B Promotion of in vitro tube formation by Atorvastatin

Atorvastatin increases the number of tubes on the Matrigel when compared with control in a dose-dependent manner in the range of 0.1 through 10 μM. Results are shown as mean ± SD of four independent experiments. $p < 0.01$ vs. control or between different doses.

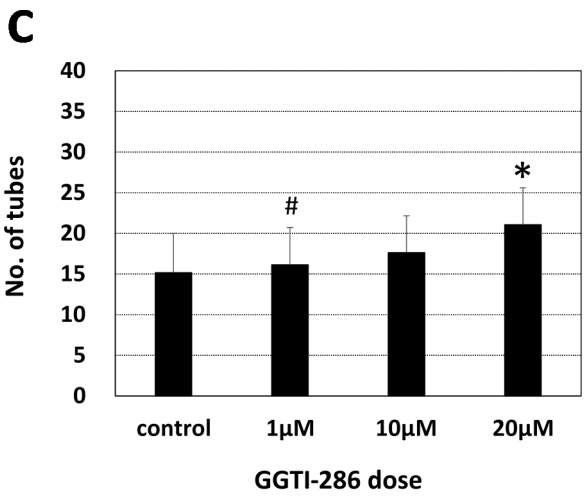

Fig. 1C Promotion of in vitro tube formation by GGTI-286

A selective inhibitor of GGT-I (GGTI-286) mimics the effect of atorvastatin. Results are shown as mean ± SD of four independent experiments. *$p < 0.001$ vs. control. $^{\#}p < 0.01$ vs. cells treated with 20 μM of GGTI-286.

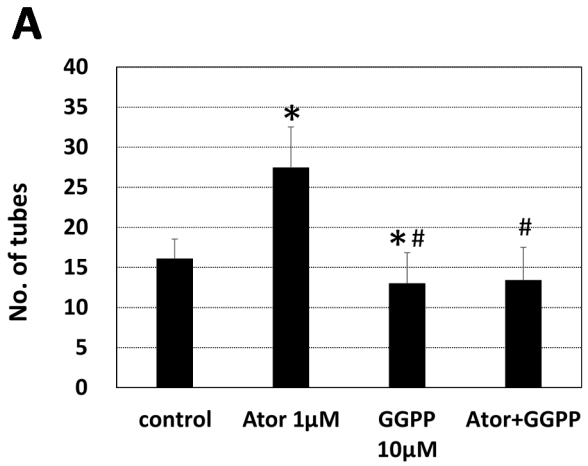

Fig. 2A Blockade of promotive effect of Atorvastatin by GGPP

GGPP completely blocks the effect of 1 μM of atorvastatin (Ator). GGPP per se significantly decreases the number of tubes when compared with control. Results are shown as mean ± SD of four independent experiments. *$p < 0.05$ vs. control. $^{\#}p < 0.001$ vs. cells treated with 1 μM of Ator.

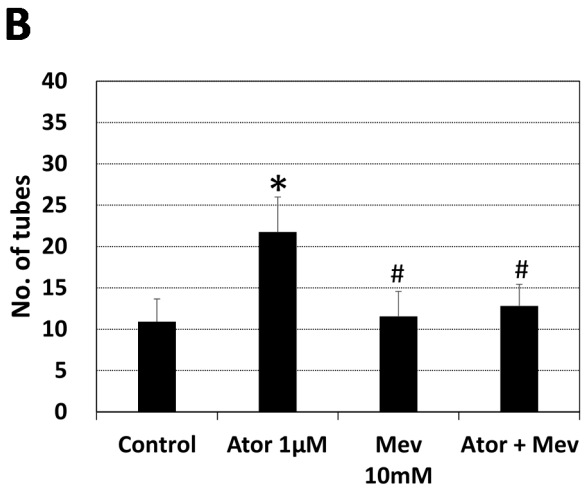

Fig. 2B Blockade of promotive effect of Atorvastatin by Mevalonate

Mevalonate (Mev) blocks the effect of 1 μM of atorvastatin (Ator), in a manner similar to that of GGPP. Results are shown as mean ± SD of four independent experiments. *$p < 0.001$ vs. control. $^{\#}p < 0.001$ vs. cells treated with 1 μM of Ator.

Inhibition of Tube Formation of HCAECs by TxA_2 Receptor Stimulation

HCAECs were incubated with 100 nM of IBOP on the Matrigel for 17 hours. Inhibition of tubular structure formation by IBOP was observed and this inhibition was completely reversed by 10 μM of the TxA_2 antagonist, SQ-29548 (Fig. 3), indicating that this inhibition was specifically mediated by the TxA_2 receptor. We demonstrated TxA_2 receptor expression on HCAECs by reverse-transcriptase polymerase chain reaction (RT-PCR) as previously described [19].

Rho Kinase-Mediated Inhibition of Tube Formation of HCAECs by TxA_2 Receptor Stimulation

Inhibition of tubular structure formation by IBOP was completely counteracted by adding 10 μM of the Rho kinase inhibitor, Y27632 (Fig. 4A). Y27632 per se produced a strong proangiogenic effect, doubling the number of tubes, in an effect similar to that of atorvastatin.

Reversal of the TxA_2 Effect by Atorvastatin

One μM of atorvastatin protected against the decrease in the number of tubular structures caused by 100 nM of the TxA_2 mimetic, IBOP (Fig. 4B).

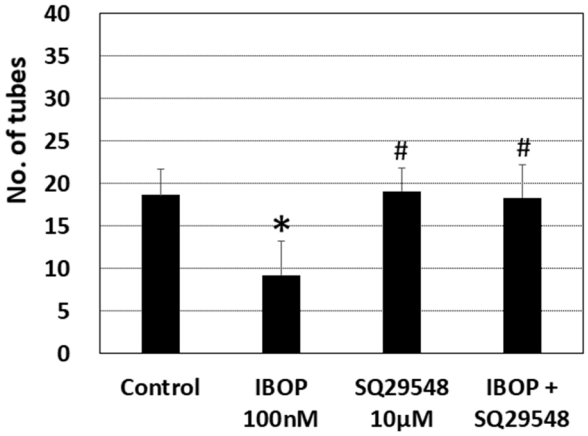

Fig. 3 Inhibition of tubular structure formation specifically mediated by TxA2 receptor

HCAECs incubated with 100 nM of IBOP on the Matrigel show reduced tubular structure when compared with control. This effect is completely blocked by 10 μM of the TxA_2 antagonist, SQ29548. Results are shown as mean ± SD of three independent experiments. *p < 0.001 vs. control. #p<0.001 vs. cells treated with 100nM IBOP.

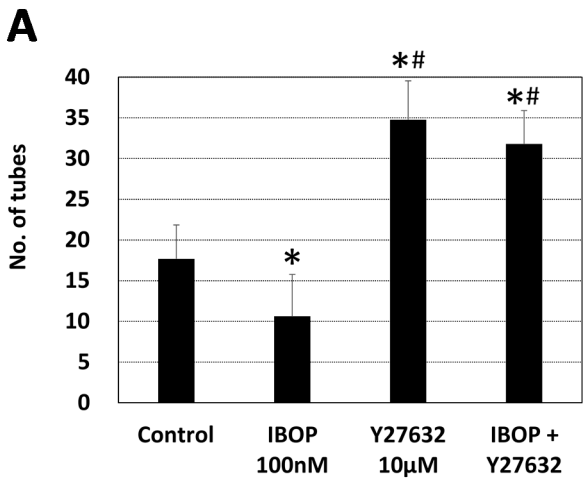

Fig. 4A Proangiogenic effect of Rho kinase inhibitor

Inhibition of tubular structure by IBOP is completely counteracted by adding 10 μM of the Rho kinase inhibitor, Y27632, which produces a strong proangiogenic effect. Results are shown as mean ± SD of seven independent experiments. *p < 0.001 vs. control. #p<0.001 vs. cells treated with 100nM IBOP

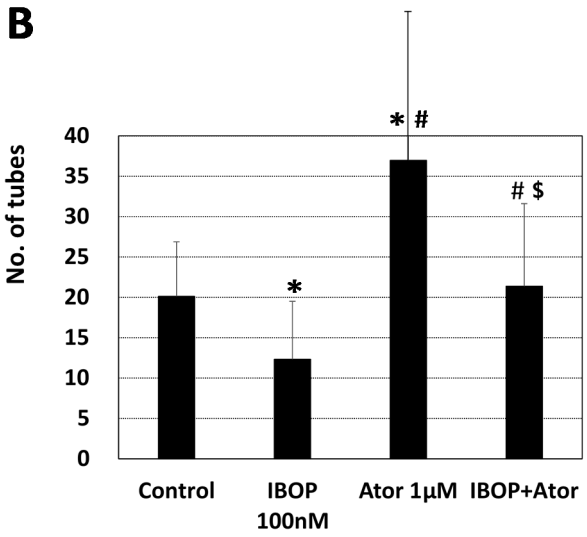

Fig. 4B Reversal of the TxA_2 effect by atorvastatin (Ator)

One μM of Ator abolishes the effect of 100 nM of IBOP. Results are shown as mean ± SD of six independent experiments. *p < 0.001 vs. control. #p < 0.001 vs. cells treated with IBOP. $ p<0.001 vs. cells treated with Ator.

Atorvastatin Lowered Caspase-3 Activity Induced by the TxA_2 Mimetic, IBOP

To determine whether IBOP caused an apoptotic response, capase-3 activity of HCAECs incubated on the plates was measured. The increase in caspase-3 activity induced by 100 nM of IBOP was attenuated by 1 μM of atorvastatin. Atorvastatin per se did not significantly decrease caspase-3 activity as compared with control (Fig. 5).

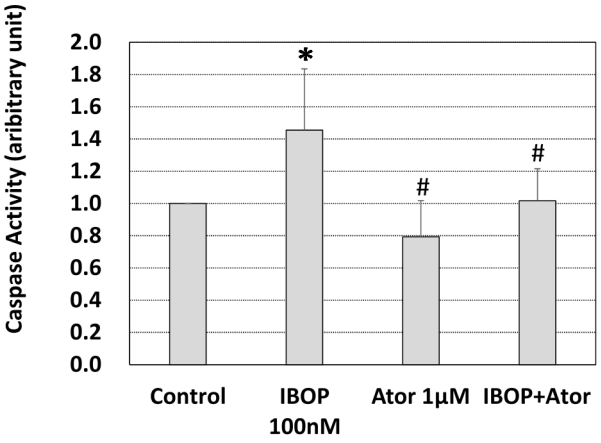

Fig. 5 Attenuation of IBOP-induced apoptotic effect by atorvastatin

Atorvastatin (Ator) lowers caspase-3 activity induced by the TxA_2 mimetic, IBOP (100 nM). HCAECs are pre-incubated in basal medium for 8 hours and then incubated for 17 hours with vehicle, Ator, and/or IBOP. Then, they are harvested and treated for colorimetric assay, as described in the Methods. Results are shown as mean ± SD of six independent experiments performed in duplicate. *$p < 0.05$ vs. control. $^{\#}p < 0.05$ vs. cells treated with IBOP.

Discussion

We demonstrated that atorvastatin apparently promoted in vitro tube formation of HCAECs on a gel in a dose-dependent manner, suggesting a proangiogenic effect on human coronary endothelial cells. Furthermore, this angiogenic effect reversed the TxA_2-induced anti-angiogenic effect caused by the Rho kinase-dependent pathway. These observations are new and support clinical observations in which statins protect against cardiovascular diseases. Underlying mechanisms could be explained by promotion of angiogenesis during myocardial ischemia and/or reperfusion, leading to a reduction of necrosis in the tissue at risk.

An interesting result in the present study was the dose-dependency of the proangiogenic effect of atorvastatin. We did not observe a biphasic effect of statins on angiogenesis, but rather a dose-dependent one (0.1 through 10 μM). This might be due to a difference in the cell types used. Weis et al. reported biphasic angiogenesis effects in human dermal microvascular ECs in a similar Matrigel system[13]. A low dose (0.005 μM) of atorvastatin enhanced tube formation, while a high dose (0.5 μM) conversely inhibited tube formation. We used primary cultured human coronary artery ECs, while they used an immortalized human dermal microvascular EC line. Thus, the angiogenic response to atorvastatin might differ in normal tissue vs. malignant tissue. However, biphasic results in human umbilical vein ECs was also reported[12]; angiogenesis was enhanced at low statin doses (0.01 to 0.1 μM) and was inhibited at high statin doses (1 μM). It is not clear whether this difference is dependent on cell type. Clinically it is unknown how much dose of statin we should increase to prevent coronary events, that is, whether the more dose the better results. Therefore, it is important that human coronary endothelial cells showed not a biphasic but dose-dependent effect of statin.

The present study characterized the involvement of intermediate products of cholesterol synthesis during angiogenesis. The proangiogenic effects of atorvastatin were reversed in the presence of GGPP or mevalonate. Furthermore, an inhibitor of geranylgeranyl transferase type I (GGTI) mimicked the effect of atorvastatin. This indicated that metabolites of the cholesterol synthesis pathway regulated angiogenesis. We did not know whether atorvastatin really suppressed GGPP or mevalonate in this cell culture model. However, as shown in this study, many papers has reported that GGPP or mevalonate is able to modify various statin-induced endothelial cell functions or intracellular signaling pathways [12,13,20]. Thus we believe reduction of mevalonate or GGPP occurs in human coronary artery endothelial cells in this study. GGPP modifies small GTP-binding proteins Rho via prenylation in the cytosol. Prenylated Rho proteins translocate to and get activated at the plasma membrane [15]. Activated Rho/Rho kinase is mostly reported to have a role in EC dysfunction and EC apoptosis in various tissue and organs [21-25]. However, the role of GGPP and Rho/Rho kinase activation are controversial. Lovastatin (3 μM-30 μM) or Y27632 (3 μM-30 μM) dose-dependently increased cell death of human umbilical vein ECs and was associated with an increase in caspase-3 activity. This

increased cell death was restored by geranylgeranylation with geranylgeraniol, although the investigators did not assess angiogenesis [26]. Ikeda et al. investigated the role of Rho/Rho kinase pathway in cell survival. C3 or Y27632, an inhibitor of Rho/Rho kinase, caused apoptosis of rat hepatic stellate cells [27]. Furthermore, the essential role of Rho kinase in angiogenesis of pluripotent embryogenic stem cells was reported [28]. Yanae et al. reported that simvastatin and other statins induced apoptosis of glioblastoma [8]. They discussed the role of Ras rather than Rho; reduction of GGPP inhibited Ras but not Rho and suppressed ERK1/2 and Akt activation that otherwise inhibits caspase-3. Thus the role of GGPP or Rho/Rho kinase appears different according to the specific type of cells studied.

In this study, atorvastatin and the Rho kinase inhibitor, Y27632, reversed TxA_2-induced inhibition of angiogenesis. TxA_2 causes endothelial cell apoptosis during angiogenesis [16]. Mechanisms involved in apoptosis induced by TxA_2 in endothelial cells include inhibition of the PI3K/Akt pathway [17] and activation of Rho kinase [18]. Thus, we suggest that the mechanisms involved in proangiogenic effect of atorvastatin might be inhibition of Rho kinase with suppression of GGPP, although we did not directly prove inactivation of Rho kinase by atorvastatin. Furthermore, we demonstrated that TxA_2-induced caspase-3 activation was inhibited by atorvastatin in cultured HCAECs, while the inhibitory effect of atorvastatin on caspase-3 against cells without IBOP stimulation was weak and not statistically significant. This suggests that atorvastatin might inhibit EC apoptotic signaling caused by Rho kinase activation via TxA_2 receptor stimulation during angiogenesis.

Clinically, statins have a prophylactic effect on coronary artery disease via suppression of LDL-cholesterol deposition on vessel walls. This study might support another beneficial effect of statins on acute coronary syndrome by promoting angiogenesis during ischemia and reperfusion against TxA_2 secreted by platelets.

In conclusion, we described the proangiogenic effect of atorvastatin on human coronary artery endothelial cells in vitro, and reversed TxA2 induced anti-angiogenic effect. Notably, atorvastatin worked on human coronary endothelial cell in a dose-dependent manner. This might be another mechanism by which statins protect against acute coronary syndrome. Suppression of Rho kinase by reducing GGPP, a substrate in the cholesterol synthesis, and inhibition of apoptosis might be involved in the proangiogenic effect.

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Conflicts of Interest

All authors declare that they have no conflicts of interest regarding the authorship or publication of this contribution. None of the authors has received or will receive benefits in any form from a commercial party related to this article.

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