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ORIGINAL ARTICLE

Pravastatin accelerates ischemia-induced angiogenesis through AMP-activated protein kinase

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Statins exert pleiotropic effects on the cardiovascular system, in part through an increase in nitric oxide (NO) bioavailability. In this study, we examined the role of pravastatin in ischemia-induced angiogenesis. Unilateral hindlimb ischemia was surgically induced in C57BL/6J mice. Phosphorylation of AMP-activated protein kinase (AMPK), acetyl-CoA carboxylase (ACC) and endothelial NO synthase (eNOS) was increased in ischemic tissues. Furthermore, mice treated with pravastatin showed higher increases in phosphorylation than did untreated mice. Laser Doppler analysis has shown that pravastatin treatment accelerates the development of collateral vessels and angiogenesis in response to hindlimb ischemia. Capillary density in the ischemic hindlimb was also increased by pravastatin treatment. An *in vitro* study on human umbilical vein endothelial cells (HUVECs) revealed that pravastatin increased the phosphorylation of AMPK. Pravastatin-induced phosphorylation of eNOS, one of the downstreams of AMPK, was inhibited by compound C, an AMPK antagonist. The increased migration and tube formation of HUVECs by pravastatin were significantly blocked by compound C treatment. The accelerated angiogenesis by pravastatin after hindlimb ischemia was significantly reduced after treatment with compound C. Thus, ischemia induced AMPK phosphorylation *in vivo*. Furthermore, pravastatin could also activate AMPK *in vivo* and *in vitro*. Such phosphorylation results in eNOS activation and angiogenesis, which provide a novel explanation for one of the pleiotropic effects of statins that is beneficial for angiogenesis.
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INTRODUCTION

The AMP-activated protein kinase (AMPK) is a trimeric enzyme comprising a catalytic α-subunit and regulatory-β, γ-subunits. AMPK was identified as an upstream kinase that phosphorylates and hence inactivates 3-hydroxy-3-methylglutaryl-coenzyme A reductase and acetyl-coenzyme A carboxylase (ACC), key enzymes that control choresterol/isoprenoid and fatty acid biosynthesis, respectively. AMPK is considered to be a cellular energy sensor that stimulates ATP-producing catabolic pathways and inhibits ATP-consuming anabolic pathways.[1] Thus, the AMPK pathway is thought to be a regulator of stress responses and cellular energy homeostasis. However, recent studies have shown that AMPK also plays an important role in maintaining endothelial functions.[2] AMPK activation has beneficial effects on endothelial functions and in antiatherogenesis. These effects include the induction of the endothelial nitric oxide synthase (eNOS) pathway to increase NO bioavailability; the suppression of endothelial reactive oxygen species production when stimulated by hyperglycemia or high free fatty acids to improve endothelial free fatty acid oxidation and limit lipid accumulation; the inhibition of apoptosis and inflammation; and the modulation of the vascular tone.[3,4]

3-Hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors (statins) belong to the class of lipid-lowering medications. Numerous studies, however, suggest that statin therapy has additional cardiovascular protective effects that may function independently of their ability to lower serum cholesterol levels.[5,6] In addition, the antithrombotic and anti-inflammatory effects of statins have been shown to contribute to the overall beneficial activity of these drugs.[7] Most importantly, statin therapy improves endothelial function by virtue of its antioxidant and anti-inflammatory effects,[8,9] as well as by its ability to upregulate eNOS.[10] The statin activation of Akt has been shown to inhibit apoptosis, acutely increase NO production, induce migration and promote angiogenesis.[11] These effects of statins were prevented by PI3K inhibitors or by the dominant-negative form of Akt.[11] Although many studies have reported that these effects depend on eNOS activation by Akt, their action cannot be explained by Akt activation alone.[12] Indeed, each physiological response downstream of Akt seems to be mediated by multiple targets.

Recent studies have reported that statins can activate AMPK in mouse aorta and myocardium.[13] Furthermore, AMPK signaling is required for angiogenic responses in endothelial cells (ECs)

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under hypoxic conditions *in vitro*.[14] In addition, adiponectin or 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) and AMPK activators promote angiogenesis in response to ischemic stress.[15,16] Furthermore, atrovastatin-induced eNOS phosphorylation was attenuated in the aorta of AMPKα2$^{-/-}$ mice.[17] Thus, eNOS phosphorylation may be at least partly mediated by AMPK activation. However, the molecular mechanism of angiogenesis by statins is not fully understood. Here, we investigated the role of AMPK in statin-induced eNOS phosphorylation *in vitro* and *in vivo*. Our results show that pravastatin can activate AMPK in an ischemic hindlimb, with the assistance of eNOS.

METHODS

Materials

Pravastatin was provided as a gift from Daiichi Sankyo Co. Ltd. (Tokyo, Japan). The antibodies used in this study and their commercial sources were as follows: anti-phospho-AMPK (p-Thr172), anti-AMPK, anti-phospho-Akt (p-Ser473), anti-Akt, anti-phospho-ACC (p-Ser79), anti-ACC and anti-phospho-eNOS (p-Ser1177) (Cell Signaling Technology, Beverly, MA, USA); anti-eNOS (BD Biosciences, San Jose, CA, USA); anti-β-actin (SIGMA, St Louis, MO, USA); and the horseradish-peroxidase-coupled secondary antibodies (GE Healthcare, Chalfont St Giles, UK). Compound C, an AMPK inhibitor, was purchased from Calbiochem (San Diego, CA, USA). Matrigel was obtained from BD Biosciences.

Animal experiments

All procedures were in accordance with the institutional guidelines for animal research. All experiments were performed using male C57BL/6J mice aged 8–9 weeks (Japan CREA, Tokyo, Japan). Unilateral hindlimb ischemia was induced by resecting the left femoral arteries and veins of mice under anesthesia with sodium pentobarbital (50 mg kg^{-1} intraperitoneally), as described previously.[18]

In the first experiment, we examined the time course of ischemia-induced AMPK, eNOS and Akt activities. Next, the mice were fed with pravastatin at 20 mg kg^{-1} body weight by gastric gavage. Saline was fed to control animals as control. After 4, 8, 16 or 24 h, the mice were killed and their hindlimbs were removed and stored in −80 °C for western blotting. Furthermore, hindlimb ischemia was induced after 7 h of pravastatin administration by gastric gavage, and the mice were killed after 60 min of ischemia induction.

In the second experiment, to investigate the effect of pravastatin on hindlimb blood flow, the mice were administered pravastatin (20 mg kg^{-1} day^{-1}) in drinking water from the day of ischemia induction.

Laser Doppler blood flow analysis

Hindlimb blood flow was measured using a laser Doppler blood flow (LDBF) analyzer (Moor LDI; Moor Instruments, Axminster, UK) as described previously.[18] Before the operation and on postoperative days 4, 7, 14, 21 and 28, we performed LDBF analysis on the legs and feet. After scanning the blood flow twice, the stored images were subjected to computer-assisted quantification, and the average flows of the ischemic and non-ischemic limbs were calculated. To avoid data variations due to ambient light and temperature, hindlimb blood flow was expressed as the ratio of left (ischemic) to right (non-ischemic) LDBF.

Capillary density

Capillary density within the ischemic thigh adductor skeletal muscles was analyzed to obtain a specific evidence of vascularity at the microcirculation level, similar to our previous method.[18] Three pieces of ischemic muscles were harvested from each animal, sliced and fixed in methanol. The tissues were embedded in paraffin, and multiple tissue slices of 5 μm thickness were prepared. Capillary ECs were identified by immunohistochemical staining with a rat anti-mouse CD31 antibody (Ab) (BD Biosciences). Fifteen random microscopic fields from three different sections in each tissue block were examined for the presence of capillary ECs, and capillary density was expressed as the number of capillaries per high-power field (×400).

Preparation of extracts and western blot analysis

Our detailed method has been previously described.[18,19] Protein extracts were obtained from homogenized ischemic or non-ischemic skeletal muscles. After an electrophoretic transfer to polyvinylidine difluoride membranes (Immobilon-P, Millipore, Billerica, MA, USA), the membranes were probed with each primary antibody.

Effects of AMPK inhibitor on ischemia-induced angiogenesis

Mice were injected intraperitoneally with 10 mg kg^{-1} of compound C dissolved in DMSO (Wako Chemicals, Osaka, Japan) or with DMSO alone before inducing ischemia thrice weekly. We measured hindlimb blood flow until day 28, as mentioned above.

Cell culture, migration and tube formation

EGM2 and EBM2, culture media and human umbilical vein ECs (HUVECs, passage 3–5) were purchased from Lonza (Basel, Switzerland). The cells were cultured at 37 °C, in 5% CO_2 in EGM2 medium. Three μmol l^{-1} of compound C was treated before 30 min of pravastatin stimulation. All experiments were performed at least four times repeatedly.

The cells were washed with phosphate-buffered saline and lysed in RIPA buffer containing 50 mmol l^{-1} HEPES (pH 8.0), 150 mmol l^{-1} NaCl, 5 mmol l^{-1} EDTA, 1% CHAPS, 10% glycerol, 100 mmol l^{-1} NaF, 0.2 mmol l^{-1} DTT, 1 mmol l^{-1} phenylmethylsulfonyl fluoride, protease inhibitor cocktail (Nacalai Tesque, Osaka, Japan) for western blotting.

Transwell inserts (Nalgen Nunc, Rochester, NY, USA) were coated with 0.1 mg ml^{-1} collagen. Pravastatin (1 μmol l^{-1}) dissolved in EBM-2 medium containing 0.25% bovine serum albumin was added to the bottom chamber of the Boyden apparatus. HUVECs (1×10^5 cells) suspended in a 100 μl aliquot of EBM-2 containing 0.25% bovine serum albumin were added to the upper chamber. After 5 h incubation, the non-migrating cells in the upper part of the chamber were removed mechanically, and the remaining cells on the lower part were fixed with methanol. The migrated cells were counted in six random microscopic fields after staining with Diff-Quik solutions (Sysmex International Reagents Co. Ltd, Kobe, Japan).

The formation of vascular-like structures by HUVECs was assessed on growth factor-reduced Matrigel (BD Biosciences). Cells were plated at 1×10^4 well^{-1} in a 96-well-multidish, precoated with 30 ml of Matrigel in the absence or presence of pravastatin (1 μmol l^{-1}). After 5 h, the length of the completed tube-like structure in the center field (magnification, ×10) was quantified. Images were captured with a CCD color camera attached to the microscope and the tube length was measured using a Micro Analyzer (JPD, Tokyo, Japan).

Statistical analysis

All data are presented as mean ± s.e.m. Comparisons among groups were made by one-way analysis of variance. For differences between two groups, Student's *t*-test was used when appropriate. Differences were considered statistically significant at a value of $P<0.05$.

RESULTS

Time course of AMPK activation after ischemia and pravastatin treatment

All mice survived after the surgical induction of unilateral hindlimb ischemia. We investigated AMPK, Akt and eNOS activities in ischemic hindlimb. As shown in Figure 1a, AMPK was remarkably activated at 15 min after hindlimb ischemia and peaked at 60 min. Akt and eNOS activities were also increased in response to ischemia. The level of phosphorylated AMPK in the hindlimb increased 4 h after pravastatin administration and lasted for at least 24 h (Figure 1b). The phosphorylation of ACC, Akt and eNOS was elevated. Therefore, we next examined the effect of pravastatin on the phosphorylation of AMPK, ACC, Akt and eNOS in the ischemic tissue. AMPK phosphorylation after 60 min of ischemia induction was additionally increased with pravastatin administration (Figure 1c). The level of AMPK and β-actin was not changed.

Unilateral hindlimb ischemia and time course of LDBF analysis

Immediately after the left femoral artery and vein were resected, the ratio of ischemic (left) to non-ischemic (right) hindlimb LDBF (the LDBF ratio) decreased from 1.06 ± 0.05 to 0.08 ± 0.01 without pravastatin and from 1.00 ± 0.01 to 0.10 ± 0.01 with pravastatin. Thus, the severity of induced ischemia was comparable in the two groups.

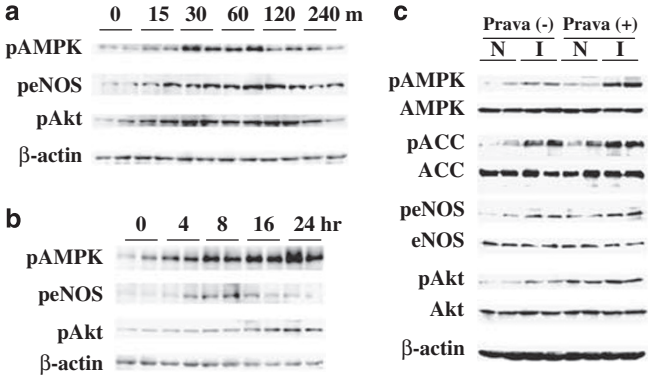

Figure 1 Time course of ischemia-induced AMPK, Akt, ACC and eNOS activation in mice. (**a**) Representative western blot analysis of these activations at 0, 15, 30, 60, 120 and 240 min after ischemia. (**b**) Representative western blot analysis of AMPK, Akt and eNOS phosphorylation at 0, 4, 8, 16 and 24 h after pravastatin treatment. (**c**) Representative western blot analysis of AMPK, ACC, eNOS and Akt. AMPK phosphorylation after 60 min of ischemia induction was further increased after pravastatin administration (after 8 h). pAMPK, phospho-AMPK; pAkt, phospho-Akt; peNOS, phospho-eNOS; pACC, phospho-ACC; Prava, pravastatin; N, non-ischemic muscle; I, ischemic muscle.

Figure 2a shows the representative LDBF images of hindlimb blood flow. A serial LDBF examination disclosed a progressive recovery of hindlimb blood flow in the pravastatin-untreated mice after ischemia was induced. On the other hand, the blood flow of mice treated with pravastatin was accelerated during the follow-up period. Compared with control mice, the LDBF ratio in the pravastatin-treated mice was persistently high after 4 days following the induction of ischemia, and significantly increased except on day 14 ($P<0.05$) (Figure 2b).

Tissue capillary density

To investigate the extent of angiogenesis at the microcirculation level, we measured capillary density in histological sections harvested from ischemic tissues. Quantitative analysis revealed that the capillary density was significantly augmented by pravastatin on postoperative day 28, compared with that in control mice (Figure 2c).

Effects of compound C on pravastatin-induced eNOS phosphorylation, migration and tube formation in HUVECs

To determine whether AMPK activation is directly concerned with endothelial functions, we studied the effects of compound C, an AMPK inhibitor, on angiogenesis. Angiogenesis incorporates migration, intercellular connections with other ECs and lumen formation *in vitro*. Angiogenesis was assessed by tube formation assay using Matrigel. Compound C ($3\,\mu mol\,l^{-1}$) significantly inhibited pravastatin-induced tube formation (Figure 3a). As angiogenesis involves cell migration, we investigated the role of compound C in the pravastatin-induced migration of HUVECs, using a modified Boyden chamber method. Compound C treatment inhibited pravastatin-induced cell migration (Figure 3b).

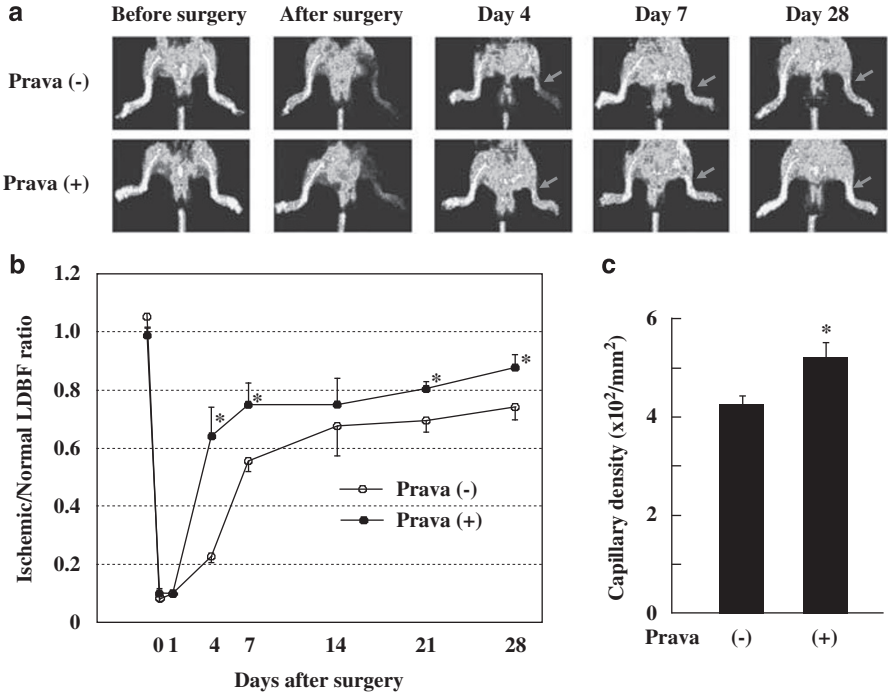

Figure 2 Laser Doppler blood flow (LDBF) analysis and capillary density. (**a**) Representative LDBF image. A low-perfusion signal was observed in the ischemic hindlimb, whereas a high-perfusion pattern (white to red) was detected in pravastatin-treated mice. (**b**) Computer-assisted quantitative analysis of hindlimb blood perfusion showed a significant improvement in the ischemic/normal hindlimb blood flow ratio in pravastatin-treated mice ($n=6$) compared with that in the untreated animals ($n=6$). Each bar represents mean ± s.e.m. $^*P<0.01$ *vs.* Prava (−) at each time point. (**c**) Capillary density assessed by staining with anti-CD31 Ab on postoperative day 28 ($n=6$ in each group). pAMPK, phospho-AMPK; pAkt, phospho-Akt; peNOS, phospho-eNOS; pACC, phospho-ACC; Prava, pravastatin; N, non-ischemic muscle; I, ischemic muscle. Each bar represents mean ± s.e.m. $^*P<0.01$ *vs.* Prava (−). A full color version of this figure is available at the *Hypertension Research* journal online.

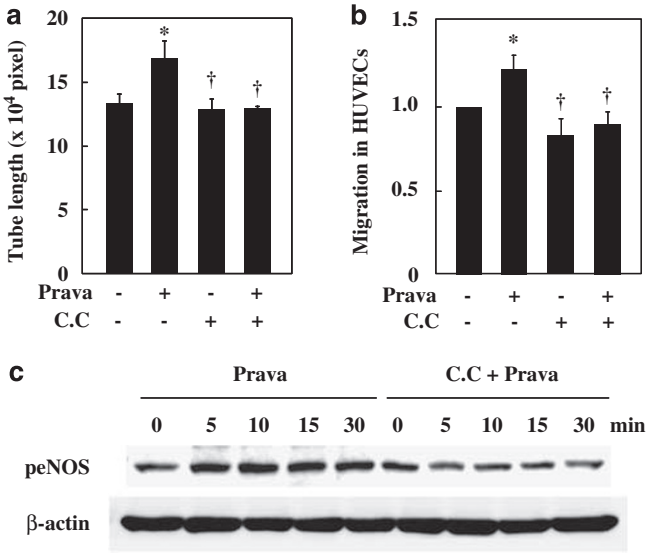

Figure 3 Effects of compound C on pravastatin-induced tube formation (**a**), migration (**b**), and eNOS phoshorylation (**c**) in HUVECs. C.C, compound C; pAMPK, phospho-AMPK; pAkt, phospho-Akt; peNOS, phospho-eNOS; pACC, phospho-ACC; Prava, pravastatin; N, non-ischemic muscle; I, ischemic muscle. Each bar represents mean ± s.e.m. (n=4). $^{*}P<0.01$ *vs.* Prava (−)/C.C (−), $^{\dagger}P<0.01$ *vs.* Prava (+)/C.C (−). Experiments were repeated three times and similar results were obtained.

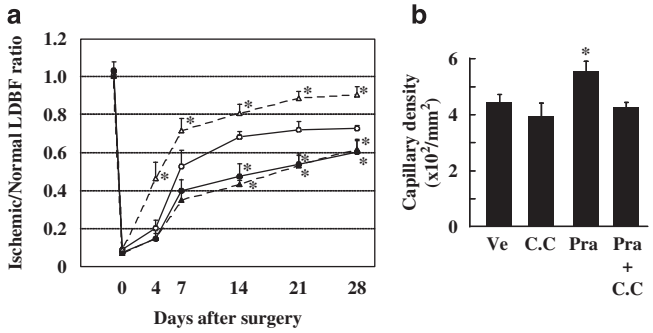

Figure 4 Effects of compound C on angiogenesis. (**a**) Computer-assisted quantitative analysis of hindlimb blood perfusion by LDBF analyzer. (**b**) Capillary density on postoperative day 28. ○, vehicle; ●, compound C; △, pravastatin; ▲, pravastatin and compound C; C.C, compound C; pAMPK, phospho-AMPK; pAkt, phospho-Akt; peNOS, phospho-eNOS; pACC, phospho-ACC; Prava, pravastatin; N, non-ischemic muscle; I, ischemic muscle. Each bar represents mean ± s.e.m. (n=5–6). $^{*}P<0.05$ *vs.* vehicle.

As eNOS activation is important in endothelial function, we next measured the phosphorylation of eNOS. Pravastatin ($1\,\mu mol\,l^{-1}$) induced the phosphorylation of eNOS at Ser1177, which could be blocked by compound C pretreatment for 30 min (Figure 3c). Taken together, these results suggest that blockade of AMPK suppressed angiogenesis, which indicates that endogenous AMPK is important for the angiogenic activity of ECs.

In vivo blockade of AMPK weakens angiogenesis

We finally examined the effect of compound C on pravastatin-induced angiogenesis *in vivo*. The recovery of hindlimb blood flow in mice treated with compound C was weaker than that in control (DMSO)-treated mice after 14 days following the induction of ischemia. The increased LDBF ratio by pravastatin administration was also reduced with compound C (Figure 4a). On postoperative day 28, the increase in capillary density by pravastatin was also reduced with the treatment of compound C (Figure 4b).

DISCUSSION

In this study, angiogenesis and blood flow recovery in response to hindlimb ischemia were significantly advanced by pravastatin, and increased angiogenesis was blocked by compound C. Thus, pravastatin displays proangiogenic effects through AMPK-eNOS activation on ECs.

Recent studies have suggested that AMPK is necessary for hypoxia-induced VEGF mRNA stabilization, and regulates VEGF expression and capillarization.[20,21] Furthermore, recent reports state that statins reduce cardiovascular events and enhance ischemia-induced angiogenesis.[11,12,22] In this study, we investigated whether the statin activation of AMPK involved angiogenesis.

We used pravastatin to demonstrate the positive effect of statins on the phosphorylation of AMPK (Thr172) in ECs. Such phosphorylation is essential for AMPK activation, which is also demonstrated by increased AMPK activity and increased phosphorylation of ACC (Ser79), a direct target of AMPK.[23] Other statins such as atrovastatin and lovastatin can also cause AMPK and ACC phosphorylation in ECs.[13] Furthermore, Xenos *et al.*[24] showed that the level of AMPK protein in human ECs increased following 48 h of fluvastatin treatment. On the other hand, AMPK phosphorylation in ECs was increased by statins in a rapid and transient manner. This temporal response is similar to that stimulated by shear stress, by adiponectin and by metformin.[25–28] Sun *et al.*[13] showed that the phosphorylation/activation of AMPK in mouse aorta and myocardium was observed as early as 2 h and peaked at 4–8 h after atrovastatin administration. However, the role of AMPK in angiogenesis *in vivo* has not been fully elucidated. The present data indicate that AMPK was transiently phosphorylated by ischemia (Figure 1a). Furthermore, the phosphorylation of AMPK in the mouse hindlimb was observed as early as 4 h and peaked at 16–24 h after pravastatin administration (Figure 1b). From this finding and from the report stating that activated AMPK in muscle induces VEGF production and promotes angiogenesis,[16] the tissues in which AMPK activation was observed in the hindlimb *in vivo* were the endothelium and mycocytes.

The effect of statins on eNOS activation in ECs is dependent on the phosphatidylinositol-3 kinase (PI3K)-Akt pathway.[11,29] Our data indicate that AMPK is also engaged in the upregulation of eNOS by pravastatin. The association between Akt and AMPK in eNOS phosphorylation (Ser 1177) is elusive.[30] Dimmeler *et al.*[29] have shown that shear stress-induced eNOS phosphorylation was blocked by a PI3K inhibitor, wortmannin, in ECs. On the other hand, Chen *et al.*[25] have shown that a dominant-negative mutant of AMPK (DN-AMPK), but not of Akt, significantly inhibited eNOS phosphorylation and NO production in ECs in response to adiponectin. Very recently, Sun *et al.*[13] have shown that eNOS phosphorylation in HUVECs was inhibited by DN-AMPK or by compound C, whereas Akt phosphorylation was drastically increased by the downregulation of AMPK. Our present data support the hypothesis that eNOS activation depends not only on Akt but also on AMPK, because inhibition of AMPK attenuated statin-activated eNOS. A recent study[17] that atrovastatin-induced eNOS phosphorylation was attenuated in AMPK$\alpha2^{-/-}$ mice supports our results. On the other hand, Ouchi *et al.*[26] have shown that DN-Akt blocked adiponectin-stimulated Akt and eNOS phosphorylation without altering AMPK phosphorylation, whereas DN-AMPK inhibited adiponectin-induced Akt phosphorylation. These results suggest that AMPK may be upstream of Akt.

The AMP-activated protein kinase seems to play a central role in metabolic regulations as its activation improves metabolic abnormalities that are found in diabetic mice and rats.[31,32] Transgenic mice expressing a kinase-dead mutant of AMPK showed increased apoptosis and cardiac dysfunction after an ischemia–reperfusion injury.[33] Furthermore, adiponectin has been shown to protect the heart from ischemia–reperfusion injury through the AMPK pathway.[34] We found that compound C significantly decreased pravastatin-induced eNOS phosphorylation in HUVECs, thereby confirming that pravastatin regulates eNOS activity through AMPK. Thus, the pharmacological activators of AMPK signaling could produce beneficial effects. In addition, the effects of statin-activated AMPK could extend beyond the protection of the vascular endothelium.

In conclusion, our present work has provided the first *in vivo* evidence that angiogenesis and blood flow recovery in response to hindlimb ischemia are accelerated by pravastatin and that the increased angiogenesis is blocked by compound C. Thus, pravastatin will have proangiogenic effects through AMPK-eNOS activation on ECs. These findings suggest that AMPK could be a new therapeutic target for vascular diseases.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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1 Kahn BB, Alquier T, Carling D, Hardie DG. AMP-activated protein kinase: ancient energy gauge provides clues to modern understanding of metabolism. *Cell Metab* 2005; **1**: 15–25.
2 Zou MH, Wu Y. AMP-activated protein kinase activation as a strategy for protecting vascular endothelial function. *Clin Exp Pharmacol Physiol* 2008; **35**: 535–545.
3 Hattori Y, Suzuki K, Hattori S, Kasai K. Metformin inhibits cytokine-induced nuclear factor kappaB activation via AMP-activated protein kinase activation in vascular endothelial cells. *Hypertension* 2006; **47**: 1183–1188.
4 Kobayashi H, Ouchi N, Kihara S, Walsh K, Kumada M, Abe Y, Funahashi T, Matsuzawa Y. Selective suppression of endothelial cell apoptosis by the high molecular weight form of adiponectin. *Circ Res* 2004; **94**: e27–e31.
5 West of Scotland Coronary Prevention Study Group. Influence of pravastatin and plasma lipids on clinical events in the West of Scotland Coronary Prevention Study (WOSCOPS). *Circulation* 1998; **97**: 1440–1445.
6 Liao JK. Clinical implications for statin pleiotropy. *Curr Opin Lipidol* 2005; **16**: 624–629.
7 Vaughan CJ, Delanty N. Neuroprotective properties of statins in cerebral ischemia and stroke. *Stroke* 1999; **30**: 1969–1973.
8 Cahoon Jr WD, Crouch MA. Preprocedural statin therapy in percutaneous coronary intervention. *Ann Pharmacother* 2007; **41**: 1687–1693.
9 Dilaveris P, Giannopoulos G, Riga M, Synetos A, Stefanadis C. Beneficial effects of statins on endothelial dysfunction and vascular stiffness. *Curr Vasc Pharmacol* 2007; **5**: 227–237.
10 Laufs U, Endres M, Stagliano N, Amin-Hanjani S, Chui DS, Yang SX, Simoncini T, Yamada M, Rabkin E, Allen PG, Huang PL, Bohm M, Schoen FJ, Moskowitz MA, Liao JK. Neuroprotection mediated by changes in the endothelial actin cytoskeleton. *J Clin Invest* 2000; **106**: 15–24.
11 Kureishi Y, Luo Z, Shiojima I, Bialik A, Fulton D, Lefer DJ, Sessa WC, Walsh K. The HMG-CoA reductase inhibitor simvastatin activates the protein kinase Akt and promotes angiogenesis in normocholesterolemic animals. *Nat Med* 2000; **6**: 1004–1010.
12 Sata M, Nishimatsu H, Suzuki E, Sugiura S, Yoshizumi M, Ouchi Y, Hirata Y, Nagai R. Endothelial nitric oxide synthase is essential for the HMG-CoA reductase inhibitor cerivastatin to promote collateral growth in response to ischemia. *FASEB J* 2001; **15**: 2530–2532.
13 Sun W, Lee TS, Zhu M, Gu C, Wang Y, Zhu Y, Shyy JY. Statins activate AMP-activated protein kinase *in vitro* and *in vivo*. *Circulation* 2006; **114**: 2655–2662.
14 Nagata D, Mogi M, Walsh K. AMP-activated protein kinase (AMPK) signaling in endothelial cells is essential for angiogenesis in response to hypoxic stress. *J Biol Chem* 2003; **278**: 31000–31006.
15 Shibata R, Ouchi N, Kihara S, Sato K, Funahashi T, Walsh K. Adiponectin stimulates angiogenesis in response to tissue ischemia through stimulation of AMP-activated protein kinase signaling. *J Biol Chem* 2004; **279**: 28670–28674.
16 Ouchi N, Shibata R, Walsh K. AMP-activated protein kinase signaling stimulates VEGF expression and angiogenesis in skeletal muscle. *Circ Res* 2005; **96**: 838–846.
17 Chen Z, Peng IC, Sun W, Su MI, Hsu PH, Fu Y, Zhu Y, DeFea K, Pan S, Tsai MD, Shyy JY. AMP-activated protein kinase functionally phosphorylates endothelial nitric oxide synthase Ser633. *Circ Res* 2009; **104**: 496–505.
18 Izumi Y, Kim-Mitsuyama S, Yoshiyama M, Omura T, Shiota M, Matsuzawa A, Yukimura T, Murohara T, Takeya M, Ichijo H, Yoshikawa J, Iwao H. Important role of apoptosis signal-regulating kinase 1 in ischemia-induced angiogenesis. *Arterioscler Thromb Vasc Biol* 2005; **25**: 1877–1883.
19 Izumi Y, Kim S, Yoshiyama M, Izumiya Y, Yoshida K, Matsuzawa A, Koyama H, Nishizawa Y, Ichijo H, Yoshikawa J, Iwao H. Activation of apoptosis signal-regulating kinase 1 in injured artery and its critical role in neointimal hyperplasia. *Circulation* 2003; **108**: 2812–2818.
20 Lee M, Hwang JT, Yun H, Kim EJ, Kim MJ, Kim SS, Ha J. Critical roles of AMP-activated protein kinase in the carcinogenic metal-induced expression of VEGF and HIF-1 proteins in DU145 prostate carcinoma. *Biochem Pharmacol* 2006; **72**: 91–103.
21 Zwetsloot KA, Westerkamp LM, Holmes BF, Gavin TP. AMPK regulates basal skeletal muscle capillarization and VEGF expression, but is not necessary for the angiogenic response to exercise. *J Physiol* 2008; **586**: 6021–6035.
22 Llevadot J, Murasawa S, Kureishi Y, Uchida S, Masuda H, Kawamoto A, Walsh K, Isner JM, Asahara T. HMG-CoA reductase inhibitor mobilizes bone marrow—derived endothelial progenitor cells. *J Clin Invest* 2001; **108**: 399–405.
23 Stein SC, Woods A, Jones NA, Davison MD, Carling D. The regulation of AMP-activated protein kinase by phosphorylation. *Biochem J* 2000; **345** (Part 3): 437–443.
24 Xenos ES, Stevens SL, Freeman MB, Cassada DC, Goldman MH. Nitric oxide mediates the effect of fluvastatin on intercellular adhesion molecule-1 and platelet endothelial cell adhesion molecule-1 expression on human endothelial cells. *Ann Vasc Surg* 2005; **19**: 386–392.
25 Chen H, Montagnani M, Funahashi T, Shimomura I, Quon MJ. Adiponectin stimulates production of nitric oxide in vascular endothelial cells. *J Biol Chem* 2003; **278**: 45021–45026.
26 Ouchi N, Kobayashi H, Kihara S, Kumada M, Sato K, Inoue T, Funahashi T, Walsh K. Adiponectin stimulates angiogenesis by promoting cross-talk between AMP-activated protein kinase and Akt signaling in endothelial cells. *J Biol Chem* 2004; **279**: 1304–1309.
27 Schulz E, Anter E, Zou MH, Keaney Jr JF. Estradiol-mediated endothelial nitric oxide synthase association with heat shock protein 90 requires adenosine monophosphate-dependent protein kinase. *Circulation* 2005; **111**: 3473–3480.
28 Zhang Y, Lee TS, Kolb EM, Sun K, Lu X, Sladek FM, Kassab GS, Garland Jr T, Shyy JY. AMP-activated protein kinase is involved in endothelial NO synthase activation in response to shear stress. *Arterioscler Thromb Vasc Biol* 2006; **26**: 1281–1287.
29 Dimmeler S, Fleming I, Fisslthaler B, Hermann C, Busse R, Zeiher AM. Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. *Nature* 1999; **399**: 601–605.
30 Sessa WC. eNOS at a glance. *J Cell Sci* 2004; **117**: 2427–2429.
31 Fiedler M, Zierath JR, Selen G, Wallberg-Henriksson H, Liang Y, Sakariassen KS. 5-aminoimidazole-4-carboxy-amide-1-beta-D-ribofuranoside treatment ameliorates hyperglycaemia and hyperinsulinaemia but not dyslipidaemia in KKAy-CETP mice. *Diabetologia* 2001; **44**: 2180–2186.
32 Hardie DG. Minireview: the AMP-activated protein kinase cascade: the key sensor of cellular energy status. *Endocrinology* 2003; **144**: 5179–5183.
33 Russell III RR, Li J, Coven DL, Pypaert M, Zechner C, Palmeri M, Giordano FJ, Mu J, Birnbaum MJ, Young LH. AMP-activated protein kinase mediates ischemic glucose uptake and prevents postischemic cardiac dysfunction, apoptosis, and injury. *J Clin Invest* 2004; **114**: 495–503.
34 Shibata R, Sato K, Pimentel DR, Takemura Y, Kihara S, Ohashi K, Funahashi T, Ouchi N, Walsh K. Adiponectin protects against myocardial ischemia-reperfusion injury through AMPK- and COX-2-dependent mechanisms. *Nat Med* 2005; **11**: 1096–1103.