Statins Activate AMP-Activated Protein Kinase In Vitro and In Vivo

Wei Sun, MD*; Tzong-Shyuan Lee, DVM, PhD*; Minjia Zhu, BS; Chunang Gu, MS; Yinsheng Wang, PhD; Yi Zhu, MD; John Y-J. Shyy, PhD

Background—Statins exert pleiotropic effects on the cardiovascular system, in part through an increase in nitric oxide (NO) bioavailability. AMP-activated protein kinase (AMPK) plays a central role in controlling energy and metabolism homeostasis in various organs. We therefore studied whether statins can activate AMPK, and if so, whether the activated AMPK regulates nitric oxide (NO) production and angiogenesis mediated by endothelial NO synthase, a substrate of AMPK in vascular endothelial cells.

Methods and Results—Western blotting of protein extracts from human umbilical vein endothelial cells treated with atorvastatin revealed increased phosphorylation of AMPK at Thr-172 in a time- and dose-dependent manner. The AMPK activity, assessed by SAMS assay, was also increased accordingly. The phosphorylation of acetyl-CoA carboxylase at Ser-79 and of endothelial NO synthase at Ser-1177, 2 putative downstream targets of AMPK, was inhibited by an adenovirus that expressed a dominant-negative mutant of AMPK (Ad-AMPK-DN) and compound C, an AMPK antagonist. The positive effects of atorvastatin, including NO production, cGMP accumulation, and in vitro angiogenesis in Matrigel, were all blocked by Ad-AMPK-DN. Mice given atorvastatin through gastric gavage showed increased AMPK, acetyl-CoA carboxylase, and endothelial NO synthase phosphorylation in mouse aorta and myocardium.

Conclusions—Statins can rapidly activate AMPK via increased Thr-172 phosphorylation in vitro and in vivo. Such phosphorylation results in endothelial NO synthase activation, which provides a novel explanation for the pleiotropic effects of statins that benefit the cardiovascular system. (Circulation. 2006;114:2655-2662.)

Key Words: statins ■ angiogenesis ■ aorta ■ endothelium ■ nitric oxide ■ nitric oxide synthase ■ myocardium

The clinical efficacy of statin therapy in decreasing cardiovascular mortality and morbidity is clearly demonstrated by several cohort trials, such as the Heart Protection Study.1 Functioning as competitive inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A reductase, statins increase the hepatic expression of the low-density lipoprotein receptor, which results in enhanced low-density lipoprotein clearance in the circulation. In addition to their cholesterol-lowering effect, statins display other cardiovascular protective effects. Such pleiotropic effects on the vessel wall have been suggested to contribute to antioxidative, antiinflammatory, and improved endothelial functions through enhanced bioavailability of nitric oxide (NO; see Wolfrum et al2 for review). In the experimental myocardial infarction model in mice, atorvastatin treatment was shown to enhance neovascularization in myocardium.3 Simvastatin administration also promoted angiogenesis in ischemic limbs of normocholesterolemic rabbits.4 At the cellular and molecular levels, treatment of vascular endothelial cells (ECs) with statins activates endothelial NO synthase (eNOS), with increased NO production, which has been suggested to be mediated through the phosphatidylinositol-3-kinase–Akt/PKB pathway.5

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AMP-activated protein kinase (AMPK) is a trimeric enzyme comprising a catalytic α-subunit and regulatory β,γ-subunits.6 AMPK was first identified as an upstream kinase that phosphorylates and hence inactivates 3-hydroxy-3-methylglutaryl coenzyme A reductase and acetyl-CoA carboxylase (ACC), the key enzymes controlling cholesterol/isoprenoid and fatty acid biosynthesis, respectively. AMPK can function as a fuel gauge to regulate the homeostasis of energy in the form of glucose and fatty acids in skeletal muscles, liver, and adipocytes (see Kahn et al7 and Rutter et
al8 for review). Recent findings suggest that the fuel-sensing mechanism of AMPK is also present in the hypothalamus to regulate food intake, energy expenditure, and body weight.9,10 The involvement of AMPK in diabetes mellitus is demonstrated by insulin resistance, with associated high levels of plasma glucose and low levels of insulin in mice with ablated AMPK-α2.11

Although Akt has been demonstrated to be a major kinase phosphorylating human eNOS at Ser-1177 (Ser-1179 in bovine eNOS), with ensuing increased activity of eNOS,12 AMPK can also phosphorylate eNOS at Ser-1177/1179, particularly in ECs.13 AMPK may have a beneficial effect on the vessel wall, because several recent studies demonstrated that adiponectin, high-density lipoprotein, apolipoprotein AI, estradiol, and shear stress all activate AMPK in ECs, with augmented NO production.13–18 Given the pleiotropic effects of statins on endothelium-mediated vascular functions, we investigated the role of AMPK in statin-induced eNOS phosphorylation and NO bioavailability in vitro and in vivo. Our results demonstrate for the first time that 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors activate AMPK in ECs. At a clinical dosage, atorvastatin activates AMPK in the vessel wall and the myocardium in mice, with attendant activation of eNOS.

Methods

Materials

Antibodies used in the present study and their commercial sources were as follows: anti-phospho-AMPK Thr-172, anti-pan-α-AMPK, anti-phospho-Akt Ser-473, and anti-phospho-ACC Ser-79 (Cell Signaling Technology, Beverly, Mass); anti-phospho-eNOS Ser-1177/1179, polyclonal anti-eNOS (BD Biosciences Pharmingen, San Diego, Calif); and anti-α-tubulin (Santa Cruz Biotechnology, Santa Cruz, Calif). Griess reagent, 5-aminooimidazole-4-carboxamide 1-ribofuranoside (AICAR), and 2,4-dinitrophenol were purchased from Sigma (St. Louis, Mo), whereas compound C was from Calbiochem (San Diego, Calif). Matrigel was obtained from BD Biosciences (San Diego, Calif) and bovine aortic ECs isolated from bovine aorta were cultured in Dulbecco's modified Eagle's medium (In- cision, Carlsbad, Calif) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. Human capillary ECs obtained from Clonetics (San Diego, Calif) and bovine aortic ECs isolated from bovine aorta were cultured in Dulbecco's modified Eagle's medium (In-vitrogen, Carlsbad, Calif) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. ECs were maintained in a humidified 95% air-5% CO2 incubator at 37°C. Cells within passages 2 to 5 were used in all experiments. We used a recombinant adenovirus expressing a dominant-negative mutant of AMPKα1, henceforth referred to as Ad-AMPK-DN, described previously.18,19 The parental adenoviral vector was referred to as null when used as a control. Confluent ECs were infected with recombinant adenoviruses at the indicated multiplicity of infection and incubated for 24 hours before experiments.

Detection of NO and cGMP Assay

We determined accumulated nitrate (NO−3), a stable breakdown product of NO, in culture media by mixing an aliquot of cell culture media with an equal volume of Griess reagent and then incubating it at room temperature for 15 minutes. The azo dye production was analyzed by use of a spectrophotometer with absorbance set at 540 nm. Sodium nitrite was used as a standard. Intracellular levels of cGMP in ECs were assessed over 4 hours. After removal of culture media, ECs were lysed, and the extracts were collected and centrifuged for 5 minutes at 5000g. cGMP level was determined by use of a cGMP enzyme immunoassay kit (R&D Systems, Minneapolis, Minn), then normalized to protein content as determined by Bradford assay.

Western Blotting

ECs were lysed with a buffer that contained 10 mmol/L Tris, pH 7.4, 100 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L NaF, 20 mmol/L Na3PO4, 2 mmol/L Na2VO4, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton-X 100, 100 μg/mL leupeptin, 60 μg/mL aprotinin, and 1 mmol/L phenylmethylsulfonyl fluoride. Frozen mouse aortas and hearts were thawed and homogenized in the same buffer as above. EC lysates and mouse aortic and myocardial extracts were resolved on SDS-PAGE according to standard protocols. After being transferred to membranes, the samples were revealed with primary antibodies, followed by secondary antibodies conjugated with horseradish peroxidase. Bands were revealed by use of an enzyme-linked chemiluminescence detection kit (Amersham Biosciences, Piscataway, NJ), and density was quantified by use of Scion Image software (Scion Corp, Frederick, Md).

In Vitro Angiogenesis (Tube Formation) Assays

The angiogenic effect of the statin-activated AMPK was examined with the use of human capillary ECs. Cells were infected with recombinant adenovirus for 24 hours, then seeded on Matrigel (3×104 cells/cm2) in 24-well culture plates for another 24 hours, in the presence or absence of atorvastatin. Tube formation was assessed by microscopic imaging and quantified by counting the number of branch points.

AMPK Activity Assay and High-Performance Liquid Chromatography Determination of Cellular AMP and ATP

EC lysates were incubated with SAMS peptide and (γ-32P)ATP, and the catalytic activity of AMPK was determined by the incorporation of 32P into SAMS peptide. HUVECs were treated with atorvastatin or 2,4-dinitrophenol, and nucleotides were extracted according to published procedures.20 The high-performance liquid chromatography used was composed of a Surveyor MS pump, a Surveyor PDA (Thermo, Waltham, Mass), and a YMC ODS-AQ S-5 column (4.6×250 mm, 5 μm in particle size, and 120 Å in pore size; YMC Co, Ltd, Kyoto, Japan). The flow rate was 500 μL/min, and a UV detector was set at 260 nm to monitor the fractions. The mobile phases were 50 mmol/L triethylammonium acetate, pH 6.5 (buffer A) and 30% acetonitrile in A (buffer B), and the gradient program was composed of 40-minute 0% to 90% buffer B, 5-minute 90% to 100% buffer B, and 1-minute 100% to 0% buffer B.

Animal Experiments

The animal experimental protocols were approved by the UCR institutional Animal Care and Use Committee. All experiments were performed in 8-week-old male C57BL/6j mice (The Jackson Laboratory, Bar Harbor, Me). Mice were fed with atorvastatin at 50 mg/kg body weight by gastric gavage. Saline was fed to control animals as a vehicle control. After 2, 4, 8, 12, or 24 hours, mice were killed. Abdominal aortas and hearts were removed and stored in −80°C.
Statistical Analysis
The significance of variability was determined by unpaired Student t test or ANOVA. Each experiment included triplicate measurements for each condition tested, unless otherwise indicated. All results are expressed as mean±SD from at least 3 independent experiments. In all cases, P<0.05 was considered to be statistically significant.

The authors had full access to the data and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results
Statins Increase AMPK, ACC, and eNOS Phosphorylation in Cultured ECs
To test whether statins can phosphorylate AMPK in ECs, HUVECs were treated with atorvastatin for up to 60 minutes. As shown in Figure 1A, the phosphorylation of Thr-172 of AMPK in HUVECs increased transiently, with a peak level at 15 minutes. Treatment with atorvastatin did not change the levels of AMPK in ECs. The transient increase in AMPK phosphorylation was accompanied by transient augmentation of phosphorylation of ACC at Ser-79, a downstream target of AMPK. As a putative effector of AMPK, eNOS was also phosphorylated at Ser-1177 in HUVECs in response to atorvastatin. The increased AMPK phosphorylation coincided with an augmented AMPK activity, as assessed by SAMS peptide assay (Figure 1B). In control cells, the addition of MeOH (atorvastatin vehicle) with a dilution of 1:104 (vol/vol) did not increase any of these phosphorylation events (data not shown). As shown in Figure 1C and 1D, AMPK, ACC, and eNOS phosphorylation and AMPK activity increased in an atorvastatin dose-dependent manner. The activation of AMPK by the statin was also observed in bovine aortic ECs stimulated with lovastatin (online-only Data Supplement, Figure I).

Statin-Activated AMPK Is Involved in eNOS Phosphorylation and NO Production
We have previously demonstrated that ECs infected with a recombinant adenovirus expressing the constitutively active form of AMPK (Ad-AMPK-CA) showed activated eNOS and increased NO production. Given that statins activate both AMPK and eNOS in ECs in a time- and dose-dependent manner, we then investigated whether the statin-activated eNOS and the consequent NO production involves AMPK. HUVECs infected with Ad-AMPK-DN were incubated with atorvastatin 1 μmol/L for 10 minutes. As shown in Figure 2A, cells infected with the Ad-null control showed an increased phosphorylation of AMPK at Thr-172, ACC at Ser-79, and eNOS at Ser-1177. However, Ad-AMPK-DN expression, as indicated by positive antihemagglutinin Western blotting, attenuated these phosphorylation events. Functional assays shown in Figure 3A and 3B demonstrated that treatment with atorvastatin enhanced the NO production and
intracellular cGMP accumulation, which was attenuated in cells expressing AMPK-DN. Interestingly, atorvastatin and AICAR, an AMPK agonist, had similar effects in augmenting the phosphorylation of ACC and eNOS in HUVECs (Figure 2B). Such atorvastatin-increased phosphorylation of ACC and eNOS was also abolished by compound C, an AMPK antagonist (Figure 2B). The AMPK activity in both the Ad-AMPK-DN–infected and compound C–treated cells was reduced to a level lower than that of control cells (Figure 2C). However, the lack of AMPK activity by either Ad-AMPK-DN or compound C increased Akt Ser-473 phosphorylation regardless of the presence or absence of atorvastatin, as shown in Figure 2A and 2B.

**AMPK Mediates Statin-Induced Tube Formation**

Statins have been shown to facilitate EC-derived angiogenesis in vitro and in vivo, which is mediated through the eNOS-produced NO. To investigate further the role of AMPK in statin-enhanced NO bioavailability, we performed tube formation assays. Human capillary ECs infected with null adenovirus or Ad-AMPK-DN were seeded on Matrigel in the presence or absence of atorvastatin. As shown in Figure 4, atorvastatin enhanced EC tube formation in Matrigel regardless of the presence of the Ad-null. However, the atorvastatin-induced tube formation was significantly attenuated with Ad-AMPK-DN infection or compound C treatment. These results agree with the notion that AMPK is involved in
statin-enhanced angiogenesis because of increased NO bioavailability.

**Atorvastatin Activates AMPK Phosphorylation In Vivo**

To explore whether AMPK in the vessel wall and heart can be activated by statin in vivo, C57BL/6J mice were given atorvastatin at 50 mg/kg body weight, then aorta and apex myocardium were removed at different time points (2 to 24 hours) to detect the level of AMPK, ACC, and eNOS phosphorylation. As shown in Figure 5A and 5B, the level of phosphorylated AMPK Thr-172 in aorta and myocardium increased 2 to 4 hours after atorvastatin administration and lasted for at least 24 hours. The phosphorylation of ACC and eNOS was elevated, with a similar pattern to that of phosphorylated AMPK. The activity of AMPK increased in these tissues as well, as revealed by SAMS assay (Figure 5C and 5D).

**Discussion**

Results from the Scandinavian Simvastatin Survival Study (4S) and the Heart Protection Study demonstrate clearly that statin therapy achieves a greater reduction of both myocardial infarction and death rate. Although statins reduce cardiovascular incidents, anti-type II diabetic drugs such as metformin and rosiglitazone, a peroxisome proliferator–activated receptor-γ agonist, can activate AMPK. Although AMPK has emerged as a new target for treating metabolic syndromes in various tissue types, the principal finding of the present study is that statins also activate AMPK in the cardiovascular system.

We used atorvastatin to demonstrate the positive effect of statins on the phosphorylation of AMPK Thr-172 in ECs. Such phosphorylation is essential for AMPK activation, which is also revealed by increased AMPK activity and increased phosphorylation of ACC Ser-79, a direct target of AMPK. The positive effect of statins should not be limited to atorvastatin, because lovastatin can also cause AMPK and ACC phosphorylation in bovine aortic ECs (online Data Supplement, Figure I). A previous study by Xenos et al showed an increase in the level of AMPK protein in human ECs treated with fluvastatin for 48 hours. The present data indicate that AMPK phosphorylation/activity in ECs was increased by statins in a rapid and transient manner. This temporal response is similar to that stimulated by shear stress, peroxisome proliferator–activated receptor-γ agonists, adiponectin, metformin, estradiol, and high-density lipoprotein.
Rosiglitazone activates AMPK by increasing the cellular AMP/ATP ratio.20 With examination by high-performance liquid chromatography, atorvastatin treatment did not alter the AMP/ATP ratio (online Data Supplement, Figure II), which is similar to the effect of metformin treatment.20 The phosphorylation/activation of AMPK in mouse aorta and myocardium was observed as early as 2 hours and peaked at 4 to 8 hours after atorvastatin administration (Figure 5). The delay of atorvastatin delivery to circulation due to gastric administration in these mice may account for the discrepant temporal responses of AMPK in vitro in cultured cells and in vivo in mice. Notably, cardiomyocytes constitute the major cell types in the heart, and atorvastatin also caused increased AMPK and ACC phosphorylation in cardiomyocytes (W.S. and J.Y.-J.S., unpublished data). Thus, the tissue sources of the detected AMPK activation in the myocardium in vivo would be both endothelium and cardiomyocytes.

The effect of statins on eNOS activation and the resultant NO release in ECs have been documented to be dependent on the phosphatidylinositol-3 kinase–Akt pathway.4,12 The present results indicate that AMPK is also engaged in the upregulation of eNOS-NO by statins. The experimental evidence supporting such an argument is the inhibition of eNOS Ser-1177 phosphorylation, NO/cGMP production, and tube formation by Ad-AMPK-DN and compound C in vitro. The association between Akt and AMPK in phosphorylating eNOS Ser-1177/1179 is elusive (see Sessa27 for review). Wortmannin, a phosphatidylinositol-3 kinase inhibitor, could block the phosphorylation of eNOS Ser-1177/1179 in ECs in response to shear stress, which indicates that eNOS is phosphorylated by phosphatidylinositol-3 kinase–Akt.12 However, later studies showed that dominant-negative mutants of Akt were unable to inhibit eNOS phosphorylation, although these mutants could inhibit shear-dependent NO release.28,29 Results of the present immunoprecipitation kinase activity assays revealed that AMPK immunoprecipitated from sheared ECs phosphorylated glutathione S-transferase–eNOS, which indicates that AMPK can phosphorylate eNOS directly.18 This result agrees with those by Chen et al13 showing that a dominant-negative mutant of AMPK but not of Akt significantly inhibited eNOS phosphorylation and NO production in ECs in response to adiponectin. Although eNOS Ser-1177 phosphorylation was largely inhibited in the Ad-AMPK-DN–infected or compound C–treated HUVECs, Akt Ser-473 phosphorylation was drastically increased in these cells that lacked AMPK activity (Figure 2). The increased Akt phosphorylation may be due to the loss of feedback inhibition of insulin receptor signaling that has been observed with ablated AMPK.30,31 Data presented in Figure 2 support the hypothesis that eNOS activation does not depend on Akt, because inhibition of AMPK mitigated the statin-activated eNOS despite increased Akt phosphorylation. However, others have shown that the dominant-negative mutant of Figure 5. Atorvastatin activates AMPK in mouse models in vivo. C57BL/6 mice were killed after atorvastatin treatment (50 mg/kg) for the indicated times. In the control group, mice received the same amount (0.5 mL) of saline 2 hours before they were killed. Tissue extracts from aorta (A) and heart (B) were analyzed by Western blotting with various antibodies as indicated. The data represent results of 6 independent sets of experiments. The bottom panels of A and B are densitometry quantification revealing the ratio between levels of phosphorylated AMPK normalized to those of total AMPK. AMPK kinase activity assays with the use of SAMS peptide as a substrate were also performed for extracts from aorta (C) and myocardium (D). Results shown are mean±SD averaged from 4 animals for each time point. *P<0.05 between saline-treated controls and atorvastatin-treated groups.
Akt blocked adiponectin-stimulated Akt and eNOS phosphorylation without altering AMPK phosphorylation, but dominant-negative AMPK inhibited adiponectin-induced Akt phosphorylation.26 These results suggest that AMPK is upstream of Akt. Regardless of the hierarchy or parallel role of AMPK in activating eNOS-NO in relation to Akt, data presented in Figure 4 demonstrate that statin-induced angiogenesis, a crucial indication of endothelial NO bioavailability, is mediated at least in part through AMPK. A dose of 50 mg of atorvastatin per kilogram of body weight in mice corresponds to \( \sim 80 \) mg/d in humans.3,32 The rapid activation of AMPK-eNOS/ACC in the aorta and myocardium by this therapeutic dose may have clinical implications. Endothelium has been considered an organ system that is not meant for fatty acid biosynthesis and storage. Fatty acids have been suggested to be a major energy source of ECs,33 however, and thus the effect of statin-activated ACC in ECs remains to be investigated. In the ischemic heart, the oxidation of both free fatty acids and glucose is inhibited, but glucose transport and ATP production resulting from glycolysis are increased (see Young et al34 for review). Apparently, AMPK in cardiomyocytes plays a central role in these metabolic regulations. Transgenic mice expressing a kinase-dead mutant of AMPK showed increased apoptosis and cardiac dysfunction after ischemia-reperfusion injury ex vivo.35 Furthermore, adiponectin has been shown to protect the heart from ischemia-reperfusion injury, which is AMPK dependent.36 Thus, during ischemic heart disease, statin-activated AMPK may be beneficial not only through improved coronary endothelial function and myocardial neovascularization but also through exertion of a cardioprotective effect. Given that metformin, thiazolidinediones, and adiponectin can activate AMPK in various metabolically related organs, including skeletal muscle, liver, and pancreatic islets,23,37–40 statin-activated AMPK is also likely to be present in tissues other than cardiovascular cells. If so, the advantages of AMPK activation in response to statins may extend beyond the protection of vascular endothelium and myocardium.

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**Disclosures**

None.

**References**

CLINICAL PERSPECTIVE

The efficacy of 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors in preventing and treating cardiovascular diseases is supported by the results of many evidence-based clinical studies. In addition to cholesterol lowering, the benefits of statins include pleiotropic effects on the cardiovascular system. Because multiple mechanisms are involved in the efficacious effects of statins, we explored the connection between statins and AMP-activated protein kinase (AMPK), a protein kinase that modulates metabolic homeostasis and energy balance in individual cell and in multiple organs. We demonstrate for the first time that atorvastatin, one of the most widely prescribed statins, can rapidly activate AMPK in cultured endothelial cells. At a clinical dosage, atorvastatin also activates AMPK in the mouse aorta and myocardium, with attendant endothelial nitric oxide synthase activation. The activated endothelial nitric oxide synthase in turn increases nitric oxide production and enhances endothelial cell–mediated angiogenesis. Such an augmentation of nitric oxide bioavailability may enhance the functional modulation of blood vessels, increase blood and oxygen supply, and promote revascularization after the onset of ischemic myocardial injury. Our finding provides a novel explanation for the pleiotropic effects of statins on the cardiovascular system. Because AMPK has emerged as a new target for treating metabolic syndromes in various tissue types, our results suggest that statins, as a modulator of AMPK, may help maintain metabolic homeostasis and energy balance in metabolic syndromes.
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