Reactive Nitrogen Species Is Required for the Activation of the AMP-activated Protein Kinase by Statin in Vivo*

Received for publication, April 21, 2008, and in revised form, May 9, 2008. Published, JBC Papers in Press, May 12, 2008, DOI 10.1074/jbc.M803020200

Hyoung Chul Choi, Ping Song, Zhonglin Xie, Yong Wu, Jian Xu, Miao Zhang, Yunzhou Dong, Shuangxi Wang, Kai Lau, and Ming-Hui Zou

From the Sections of Endocrinology and Nephrology, Department of Medicine, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma 73104 and the Department of Pharmacology, College of Medicine, Yeungnam University, Daegu 705-717, Korea

The AMP-activated protein kinase (AMPK) is reported to mediate the beneficial effects of statin on the vascular functions, but the mechanisms underlying the benefit are unknown. The aim of the study was to determine how statin activates AMPK. Owing to the anti-atherogenic effects observed in vivo and in vitro, statins are widely prescribed for the prevention and treatment of cardiovascular diseases. Treatment of AMPK-Thr172, acetyl-CoA carboxylase (ACC)-Ser 79, and LKB1 nuclear export. Furthermore, statin treatment of AMPK at Thr172 and activities of AMPK, which was in its dephosphorylated form, increased. Similarly, further activation of AMPK. The undeclared splicing in Fig. 5 (A and B) were between the same immunoblots. The authors offered to published amended figures based upon those data and, alternatively, offered to repeat the experiments. However, the Journal declined both offers, a decision with which the authors respectfully disagree.

This article has been withdrawn by the authors. The LKB1 immunoblot from Fig. 1D was reused in Fig. 7D. The AMPK immunoblot from Fig. 2A was reused in Fig. 3A. Figs. 3C and 5 (A and B) contained splices. The authors state that the undeclared splicings in Fig. 5 (A and B) were between the same immunoblots. The authors state that any errors in the construction of figures in the paper do not alter the scientific conclusions of the work, and they stand by the conclusions of the paper.

© 2008 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in the U.S.A.

2 The abbreviations used are: eNOS, endothelial nitric-oxide synthase; NO, nitric oxide; BAEC, bovine aortic endothelial cell(s); HUVEC, human umbilical vein endothelial cell(s); ACC, acetyl-CoA carboxylase; ONOO⁻, peroxynitrite; PKC, protein kinase C; siRNA, small interference RNA; WT, wild type; ROS, reactive oxygen species; AMPK, AMP-activated protein kinase; BAPTA, 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid; BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid acetoxymethyl ester; HPLC, high pressure liquid chromatography; CaMKK, Ca²⁺/calmodulin-dependent kinase kinase; Ad, adenovirus; DN, dominant negative; 3-NT, 3-nitrotyrosine; HG, high glucose; Ang-II, angiotensin II; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling; Con, control; DHE, dihydroethidium.

WITHDRAWN
June 10, 2019
catabolic pathways and inhibits ATP-consuming anabolic pathways (17). Although the AMPK pathway is traditionally thought of as a regulator of metabolism, recent studies have demonstrated that AMPK may also act to maintain normal endothelial functions (18). AMPK exerts pleiotropic effects believed to be beneficial to endothelial functions and antiatherogenesis. These effects include, among others, induction of the eNOS/nitric oxide (NO) pathway to increase NO bioavailability; suppression of endothelial ROS production when stimulated by hyperglycemia or high FFA to improve endothelial FFA oxidation and limit lipid accumulation; inhibition of apoptosis and inflammation; and modulation of vascular tone (19–21).

Since many of the metabolic benefits and the endothelial protection conferred by statin are similar to those elicited by up-regulation of AMPK, we hypothesized that AMPK activation may mediate many of the pleiotropic and salutary activities exerted by statins on the cardiovascular system. Consistent with our hypothesis, several recent studies (22, 23) demonstrated that statin can rapidly activate AMPK via increased Thr\(^{172}\) phosphorylation \textit{in vitro}, resulting in eNOS activation in cultured cells. Although statin is known to activate AMPK, the mechanism(s) by which statins activate AMPK was not yet defined. To this end, we examined the effects of statin on the kinases upstream of AMPK, specifically evaluating the activity on LKB1 and PKC-\(\zeta\). Here we report that statin treatment results in LKB1-dependent AMPK activation through a nitrogen species, including peroxynitrite (ONOO\(^-\)), hydroxyl radical (OH\(^-\)), and nitric oxide (NO), and that PKC-\(\zeta\)-dependent mechanisms may mediate the pleiotropic and salutary activities of statin.

**EXPERIMENTAL PROCEDURES**

**Animals—**Male eNOS knock-out (eNOS \textit{H9256}/H9251) and wild-type mice, C57BL6 mice, 10 weeks of age, were obtained from the Jackson Laboratory (Bar Harbor, ME). Mice were housed in temperature-controlled cages with a 12-h light-dark cycle and given free access to water and non-fiber chow. These mice were randomly divided into control and statin-treated groups. Mice were abdominally injected with statin (5 mg/kg) for 4 h, and the control mice received a 0.9% physiological saline injection. The mice were euthanized with inhaled isoflurane. Mouse hearts, kidneys, livers, and aorta were removed and immediately frozen in liquid nitrogen.

**Materials—**Bovine aortic endothelial cells (BAEC), human umbilical vein endothelial cells (HUVEC), and cell culture media were obtained from Cambrex Bio Science Walkersville, Inc. (Walkersville, MD). A549 and HeLa S3 cells were grown in F-12K medium supplemented with 10% serum. BAEC and HUVEC were maintained in endothelial basal medium with 2% serum and growth factors prior to use. BAEC were serum-deprived overnight prior to experiments. For adenoviral infection experiments, infected BAEC were treated with statin in the presence of normal or high glucose concentrations and for the indicated length of time.

**Cell Culture—**All culture media were supplemented with penicillin (100 units/ml) and streptomycin (100 \(\mu\)g/ml). A549 and HeLa S3 cells were grown in F-12K medium supplemented with 10% serum. BAEC and HUVEC were maintained in endothelial basal medium with 2% serum and growth factors prior to use. BAEC were serum-deprived overnight prior to experiments. For adenoviral infection experiments, infected BAEC were treated with statin in the presence of normal or high glucose concentrations and for the indicated length of time.

**Western Blot and Preparation of Subcellular Fractions—**Western blots and preparation of nuclear, cytosol, and membrane fractions were performed as described previously (24). Western blot bands were visualized using the National Institutes of Health ImageJ program (version 1.37v).

**Immunocytochemical Staining of LKB1 (25)—**The HUVEC were cultured on coverslips and then fixed with 4% paraformaldehyde. The slides were incubated with a fluorescein isothiocyanate-conjugated donkey anti-rabbit and a rhodamine-conjugated donkey anti-mouse secondary antibody. The slides were then exposed to a 1:150 dilution of goat anti-LKB1 antibody (Santa Cruz Biotechnology) overnight at 4 °C. The slides were rinsed, counterstained with 4′,6-diamidino-2-phenylindole, mounted in VectashieldTM mounting medium (Vector Laboratories, Burlingame, CA), and viewed on an SLM 510 laser-scanning confocal microscope (Carl Zeiss Meditec, Inc., Jena, Germany).

**Assay of Protein Kinase C-\(\zeta\) Activity—**PKC-\(\zeta\) was immunoprecipitated from untreated (control) or treated cells with an antibody against PKC-\(\zeta\) overnight at 4 °C in the presence of protein A/G-agarose. PKC-\(\zeta\) activity present in the immunoprecipitates was determined by its ability to activate pseudosubstrate derivative (50 \(\mu\)M; ERMRPRKRQGSVRRR) as described previously (24, 25).

**AMPK Activity Assay—**Total AMPK was immunoprecipitated from 500 \(\mu\)g of protein using an antibody against AMPK\(\alpha\), and AMPK activity was assessed by determining the incorporation of \(^{32}\)P into the synthetic SAMS peptide as described (24, 25). The difference between the presence and absence of AMPK is calculated as the AMPK activity.

**siRNA Gene Silencing of PKC-\(\zeta\) (25)—**Small interfering RNA (siRNA) duplex oligonucleotides used in this study are based on the human cDNAs encoding PKC\(\zeta\). PKC\(\zeta\) siRNA as well as a nonsilencing control siRNA were obtained from Santa Cruz Biotechnology. The working concentration of siRNA duplexes applied was 100 nM. HUVEC were transfected with PKC\(\zeta\) siRNA or nonspecific control siRNA by using LipofectamineTM 2000 (Invitrogen) according to the manufacturer’s instructions. Transfected cells were starved in serum-free medium for 6 h and then exposed to the indicated concentrations of statin.
Statin via PKC-ζ Activates AMPK

**RESULTS**

Statin Increases Phosphorylation of AMPK at Thr\(^{172}\) and ACC at Ser\(^{79}\) in BAEC—Statin has been shown to activate AMPK in cultured endothelial cells (22, 23). To define the mechanism, we first examined whether the Ser\(^{79}\) phosphorylation of LKB1, a well characterized AMPK kinase in endothelial cells (24), was altered by statin. Consistent with previous studies, statin (1–50 \(\mu\)M) caused a dose-dependent increase in AMPK activity in cultured BEAC, as determined by examining AMPK-Thr\(^{172}\) phosphorylation (Fig. 1A). AMPK activation by statin was further confirmed by enhanced phosphorylation of ACC at Ser\(^{79}\). No change in the expression of AMPK or ACC was observed by immunoblot analysis (Fig. 1B). Moreover, incubation of BAEC with statin (50 \(\mu\)M) for 30 min before cell harvest, and then the cells were methanol-extracted. HPLC was used to separate and quantify oxyethidium (product of DHE and O\(_2^•\)) were assessed by the dihydroethidine (DHE) fluorescence/HPLC assay with minor modifications (26).

**Statistical Analysis**—Results are expressed as mean ± S.E. of at least three independent experiments. Comparisons of the means were performed by the one-way or two-way analysis of variance. A value of \(p < 0.05\) was considered statistically significant.
**Statin via PKC-ζ Activates AMPK**

**Statin Increases LKB1 Phosphorylation at Ser\(^{428}\) in a Dose-dependent Manner**—Previous studies have identified LKB1 as a kinase upstream of AMPK and determined that LKB1 phosphorylation at Ser\(^{428}\) is important for peroxynitrite (ONOO\(^-\))-enhanced (24) or metformin-enhanced (25) AMPK activation in cultured endothelial cells. Since statin treatment activates AMPK, we next examined whether statin would affect LKB1 phosphorylation at Ser\(^{428}\). Statin (10–50 \(\mu M\)) for 2 h of treatment did not alter overall LKB1 levels but did result in significantly increased phosphorylation of LKB1-Ser\(^{428}\) compared with untreated cells (Fig. 1C). Combined with statin-mediated AMPK phosphorylation, there was a dose-dependent increase in LKB1 phosphorylation at Ser\(^{428}\) in response to statin (28, 29).

**Inhibition of Ca\(^2+\)/Calmodulin-dependent Kinase Kinase (CaMKK) Does Not Alter Statin-induced AMPK Activation**—Recent studies (28, 29) have suggested that CaMKKK-β functions as AMPK kinase under conditions in which intracellular Ca\(^{2+}\) increases. We first determined if STO-609, a selective CaMKKK-β inhibitor, or BAPTA-AM (20 \(\mu M\)), an intracellular Ca\(^{2+}\) chelator, altered calcium-dependent AMPK activation in BAEC. As expected, exposure of BAEC to A23187 (1 \(\mu M\)) significantly enhanced the phosphorylation of AMPK-Thr\(^{172}\) in BAEC. Preincubation of BAEC with either STO-609 (1 \(\mu M\)) or BAPTA-AM (20 \(\mu M\)), significantly suppressed calcium ionophore A23187-enhanced phosphorylation in BAEC (Fig. 2A).

To determine if Ca\(^{2+}\) or CaMKKK-β is required for statin-enhanced AMPK activation, STO-609 (1 \(\mu M\)) or BAPTA were preincubated with BAEC prior to statin treatment. Neither STO-609 nor BAPTA-AM at the stated concentrations altered the basal or statin-enhanced phosphorylation levels of AMPK at Thr\(^{172}\) or ACC at Ser\(^{79}\) in BAEC (Fig. 2, B–E). These results suggest that CaMKKK-β was not required for statin-induced AMPK activation in BAEC.

**Transfection of LKB1-expressing Plasmid Is Required for Statin-induced AMPK Activation in A549 Cells, Which Are Otherwise Deficient in LKB1**—Since statin results in a parallel increase in both AMPK and LKB1 (Ser\(^{428}\)) phosphorylation, we next evaluated whether LKB1 was required for statin-induced AMPK activation. We first examined if statin activated AMPK in A549 or HeLa-S3, two tumor cell lines that have been

**Increased AMPK activity (6.5 ± 0.7 versus 4.1 ± 0.4 pmol/min/mg protein, statin versus control, \(n = 6, p < 0.05\)).** AMPK activation by statin was also concentration-dependent. Although the maximal phosphorylation of AMPK by statin occurred at 50 \(\mu M\) (Fig. 1A), the level of AMPK activation by statin at 50 \(\mu M\) was comparable with that of 5-aminoimidazole-4-carboxamide-1-d-ribofuranoside at 1 \(\mu M\) (data not shown).

Our initial studies suggest that AMPK activation by statin is biphasic. Acute exposure for 45 min was required for statin (50 \(\mu M\)) to activate AMPK. Increased phosphorylation of AMPK and ACC occurred 45 min following the initiation of statin treatment, with peak levels of activated protein occurring at 120 min (Fig. 1B). Chronic exposure of BAEC (>24 h) lowered the effective concentrations of statin to 5–10 \(\mu M\) (data not shown). Compared with control, statin (5 \(\mu M, 72 \text{ h}\)) caused a drastic increase in phosphorylation of both AMPK (Thr\(^{172}\)) and ACC (Ser\(^{79}\)) (data not shown). Collectively, these data confirm that AMPK is activated by statin treatment.

**FIGURE 2.** Statin-induced AMPK activation in BAEC is independent of intracellular Ca\(^{2+}\). A, BAEC were treated with A23187 (1 \(\mu M\)) for 30 min with or without STO-609 (1 \(\mu M\)) or BAPTA-AM (20 \(\mu M\)) for 2 h in the presence or absence of statin (50 \(\mu M\)) or STO-609 (1 \(\mu M\)) or BAPTA-AM (20 \(\mu M\)) for 2 h in the presence or absence of intracellular Ca\(^{2+}\) chelator BAPTA-AM (20 \(\mu M\), given 30 min prior to statin). The blot is representative of five blots from five independent experiments. +, \(p < 0.05\) (STO-609 versus control); †, \(p < 0.05\) (BAPTA-AM versus control); ††, \(p < 0.05\) (STO-609 versus STO-609 control). B, the phosphorylation at Thr\(^{172}\) of AMPK by statin was also concentration-dependent. Although the maximal phosphorylation of AMPK by statin occurred at 50 \(\mu M\) (Fig. 1C), the level of AMPK activation by statin at 50 \(\mu M\) was comparable with that of 5-aminoimidazole-4-carboxamide-1-d-ribofuranoside at 1 \(\mu M\) (data not shown).

Our initial studies suggest that AMPK activation by statin is biphasic. Acute exposure for 45 min was required for statin (50 \(\mu M\)) to activate AMPK. Increased phosphorylation of AMPK and ACC occurred 45 min following the initiation of statin treatment, with peak levels of activated protein occurring at 120 min (Fig. 1B). Chronic exposure of BAEC (>24 h) lowered the effective concentrations of statin to 5–10 \(\mu M\) (data not shown). Compared with control, statin (5 \(\mu M, 72 \text{ h}\)) caused a drastic increase in phosphorylation of both AMPK (Thr\(^{172}\)) and ACC (Ser\(^{79}\)) (data not shown). Collectively, these data confirm that AMPK is activated by statin treatment.
Statin via PKC-ζ Activates AMPK

A.

![Graph A](image)

B.

![Graph B](image)

C.

![Graph C](image)

FIGURE 3. LKB1 is required for statin-induced AMPK activation in BAEC. A, LKB1-deficient A549 cells infected with adenovirus expressing the wild type LKB1 (Ad-LKB1-WT) for 48 h, were then treated with statin (50 μM) for 2 h. Adenoviral overexpression of wild type LKB1 resulted in increased phosphorylation of AMPK-Thr172 in A549 cells. The blot is a representative of five independent experiments. B, BAEC was transfected with control siRNA or LKB1 siRNA. Cells were then treated with statin (50 μM) for 1 h. C, BAEC was infected with adenovirus encoding either wild type LKB1 (Ad-D149A, Ad-S428A) for 48 h. The infected cells were then treated with statin (50 μM) for 1 h. The blot is a representative of five independent experiments.

Phosphorylation of LKB1 at Serine 428 Is Required for Statin-induced AMPK Activation in Endothelial Cells—Since statin increased levels of LKB1 phosphorylation at Ser428 (Fig. 1, C and D), we next evaluated whether LKB1 phosphorylation at Ser428 was required for statin-induced AMPK activation. Using site-directed mutagenesis, we developed two LKB1 mutants in which an amino acid essential for LKB1 activation, aspartic acid 194 or serine 428, was mutated to alanine (LKB1-D194A or LKB1-S428A) (24, 25). Adenoviral overexpression of the LKB1 mutant (Ad-PKC-ζ) would prevent statin-induced AMPK activation. Although overexpression of the phosphorylation-defective LKB1 mutant (LKB1-S428A) did not significantly increase basal level of AMPK-Thr172 in BAEC, it did abolish statin-enhanced phosphorylation of both AMPK-Thr172 and ACC-Ser79 (Fig. 3C). These data suggest that Ser428 phosphorylation of LKB1 was essential for statin-induced AMPK activation in BAEC.

Statin Increases PKC-ζ Phosphorylation at Thr410/403 in Both a Time- and Dose-dependent Manner—Previously we had determined that PKC-ζ regulates AMPK activity by increasing AMPK-Thr172 phosphorylation (24). Therefore, we next determined whether PKC-ζ activation was involved in statin-enhanced LKB1-dependent AMPK activation. Incubation of BAEC with statin (5–50 μM) did not detectably increase total levels of PKC-ζ protein, whereas it significantly increased PKC-ζ phosphorylation at Thr410/403 within 15 min of treatment (Fig. 4B). The statin-induced phosphorylation of PKC-ζ-Thr410/403 occurred in a dose-dependent manner (Fig. 4A). In addition, statin significantly (p < 0.01) increased PKC-ζ activity 2.1-fold relative to control groups (Fig. 4C), as determined by examining a PKC-ζ-specific substrate. Collectively, these results indicate that statin activated PKC-ζ prior to activating either LKB1 or AMPK in BAEC and suggest that PKC-ζ may be required for statin-induced activation of LKB1 and/or AMPK.

Inhibition of PKC-ζ Abolishes Statin-enhanced AMPK Activation—To define the role of PKC-ζ in statin-induced LKB1 and AMPK activation, PKC-ζ activity was suppressed by either pharmacological or genetic means. In order to suppress PKC-ζ activity, a dominant negative PKC-ζ mutant (Ad-PKC-ζ).
Statin via PKC-ζ Activates AMPK

A.

FIGURE 4. Statin activates PKC-ζ in cultured BAEC. A, time course of statin-enhanced PKC-ζ in BAEC. BAEC were incubated with statin (50 µM) for the indicated durations. Values represent mean ± S.E. from six independent experiments; *, p < 0.05 compared with control. B, dose-dependent PKC-ζ phosphorylation at Thr172 and ACC at Ser79 (Fig. 5A). Since overexpression of PKC-ζ-WT, which did not alter the basal levels of AMPK activity, abolished statin-enhanced AMPK activity (Fig. 6A). These data provide further proof that PKC-ζ is essential for statin-induced AMPK activation in endothelial cells.

Statin-induced LKB1 Translocation is PKC-ζ-dependent—Previous studies have shown that LKB1 is predominantly localized in the nucleus, whereas AMPK is mainly localized in the cytoplasm (32, 33). Therefore, we examined whether PKC-ζ is involved in the subcellular localization of LKB1 in HUVEC by immunohistochemical stainings in BAEC. Immunohistochemical stainings in BAEC, therefore, we examined whether PKC-ζ is involved in the subcellular localization of LKB1 in HUVEC by immunohistochemical stainings in BAEC. As expected, LKB1 was found predominantly localized in the nucleus of nonstimulated endothelial cells. Overexpression of PKC-ζ, either in the control GFP, blocked statin-induced AMPK at Thr172 and ACC at Ser79 (Fig. 6B). Statin increased the export of LKB1 from the nucleus to the cytosol in stimulated HUVEC (Fig. 6B). We further found that the PKC-ζ pseudosubstrate, a selective PKC-ζ inhibitor, abolished statin-induced LKB1 translocation to cytosol (Fig. 6B). In order to corroborate the immunofluorescent microscopy data, statin-enhanced LKB1 nuclear export was further examined in subcellular fractions. Immunoblot analysis of cellular fractions confirmed that statin treatment significantly increased cytoplasmic LKB1 protein levels and concurrently and reciprocally decreased nuclear LKB1 protein levels (Fig. 6C). Together, these data suggest that statin triggers LKB1 translocation from nucleus into cytosol by a pathway dependent on PKC-ζ activity.

PKC-ζ-dependent AMPK Activation in Vivo—We next determined if PKC-ζ was required for AMPK activation in mice. C57BL/6J mice were injected retroorbitaly with either mouse-specific PKC-ζ siRNA or control siRNA (1 mg/kg, diluted in 200 µl) every other 3 days for 6 days. As shown in Fig. 7A, in vivo transfection of PKC-ζ siRNA but not control siRNA significantly lowered the levels of PKC-ζ in isolated mouse aortas. In addition, statin significantly increased the phosphorylation of AMPK-Thr172 and ACC-Ser79 in C57BL/6J mice or C57BL/6J mice treated with control siRNA (Fig. 7, B and C). Compared with the levels of phosphorylated AMPK and ACC in mice treated with control siRNA, transfection of PKC-ζ siRNA significantly reduced statin-enhanced phosphorylation of AMPK-Thr172 (Fig. 7, B and C) and LKB1-Ser428 (Fig. 7D). These results suggest that PKC-ζ was required for statin-enhanced phosphorylation of [32P]ATP into the SAMS peptides. Statin was found to significantly increase AMPK activity in BAEC infected with either GFP or PKC-ζ-WT (Fig. 6A). However, overexpression of PKC-ζ-DN, which did not alter the basal levels of AMPK activity, abolished statin-enhanced AMPK activity (Fig. 6A). These data provide further proof that PKC-ζ is essential for statin-induced AMPK activation in endothelial cells.

In order to further confirm the essential role of PKC-ζ, endogenous PKC-ζ in endothelial cells was suppressed by transfection of PKC-ζ-specific siRNA. As expected, BAEC transfected with control siRNA showed no significant change in PKC-ζ levels, whereas PKC-ζ was undetectable in BAEC transfected with PKC-ζ-specific siRNA (Fig. 5C), confirming the reduction of PKC-ζ by siRNA transfection. Confirming the results using cells overexpressing the dominant negative form of PKC-ζ, we found that expression of PKC-ζ-specific siRNA, but not control siRNA, abolished statin-induced phosphorylation of both AMPK at Thr172 and ACC at Ser79, without altering the expression of AMPKα (Fig. 5D).

We next determined if PKC-ζ inhibition alters statin-enhanced AMPK activity. To investigate statin-induced AMPK activity, GFP, PKC-ζ-WT, or PKC-ζ-DN was transiently overexpressed in BAEC. Following mock or statin treatment, AMPK was immunoprecipitated with antibodies raised against AMPKα, which was then assayed for activity by the incorporation of [32P]ATP into the SAMS peptides. Statin was found to significantly increase AMPK activity in BAEC infected with either GFP or PKC-ζ-WT (Fig. 6A). However, overexpression of PKC-ζ-DN, which did not alter the basal levels of AMPK activity, abolished statin-enhanced AMPK activity (Fig. 6A). These data provide further proof that PKC-ζ is essential for statin-induced AMPK activation in endothelial cells.
Statin via PKC-ζ Activates AMPK

A. 

|          | Ad-GFP | Ad-PKC-ζ DN |
|----------|--------|-------------|
| p-AMPK   |        |             |
| AMPK     |        |             |
| p-ACC    |        |             |
| PKC-ζ    |        |             |
| Control  | Con    | Statin      |
|          |        |             |
|          | Con    | Statin      |

B. 

|          | Ad-GFP | Ad-PKC-ζ DN |
|----------|--------|-------------|
| p-LKB1   |        |             |
| LKB1     |        |             |
| Control  | Con    | Statin      |
|          |        |             |
|          | Con    | Statin      |

C. 

| PKC-ζ    |        | PKC-ζ siRNA |
|          |        |             |
| p-actin  | Con    | Statin      |
|          | Con    | Statin      |

D. 

|          | Con siRNA | PKC-ζ siRNA |
|----------|------------|-------------|
| p-AMPK   |            |             |
| AMPK     |            |             |
|          |            |             |
|          |            |             |

FIGURE 5. Genetic inhibition of PKC-ζ abolishes statin-induced AMPK activation. A, adenoviral overexpression of Ad-PKC-ζ-DN (but not Ad-GFP) attenuated statin-enhanced AMPK phosphorylation in both AMPK-Thr172 and ACC-Ser79 in BAEC. A, adenoviral overexpression of PKC-ζ-DN abolished AMPK phosphorylation in BAEC. Values represent mean ± S.E. from five independent experiments. B, inhibition of PKC-ζ abolished AMPK phosphorylation in BAEC. Values represent mean ± S.E. from five independent experiments. C, transfection of PKC-ζ siRNA, but not control siRNA, abolished statin-induced AMPK activation. D, transfection of PKC-ζ siRNA, but not Ad-GFP, attenuated statin-enhanced phosphorylation of AMPK-Thr172 and ACC-Ser79 (data not shown), indicating that hydrogen peroxide was not involved in statin-enhanced AMPK activation in BAEC.

ONOO−-dependent Activation of AMPK—To further establish if ONOO− was involved in AMPK activation by statin in vivo, statin was given to eNOS−/− mice (attenuate ONOO− by lacking eNOS-derived NO) or to the wild type C57BL/6j mice. Mouse aortas were isolated for assaying 3-nitrotyrosine (3-NT), a footprint for reactive nitrogen species, including ONOO−. The specificity of 3-NT staining was confirmed by the absence of staining when the antibody was omitted or diluted in 10 mm 3-NT (data not shown). As shown in Fig. 8C, the positive staining with the 3-NT antibody was only weakly visible in the aortic tissues isolated from sham-treated C57BL/6j mice. The levels of 3-NT-positive proteins in aortas of C57BL/6j mice (Fig. 8C) were markedly increased by statin treatment (Fig. 8C). Compared with weak stainings of 3-NT in the aortas isolated from sham-treated eNOS−/− mice, statin did not increase 3-NT-positive proteins in the aortas from eNOS−/− mice (Fig. 8C), suggesting that NO from eNOS was required for statin-increased ONOO− in vivo.

We next determined the effects of statin in C57BL/6j and eNOS−/− mice. As shown in Fig. 8D, administration of statin significantly increased the phosphorylations of both AMPK-Thr172 and ACC-Ser79 in C57BL/6j mice but not in eNOS−/− mice (Fig. 8D). Consistently, statin increased the phosphorylation of PKC-ζ in the aortas of C57BL/6j wild-type mice but not in eNOS−/− mice (Fig. 8D). Taken together, these results suggested that the ONOO−-PKC-ζ-AMPK axis operates in statin-enhanced AMPK activation in vivo.

ONOO−-dependent and ONOO−-independent AMPK Activation in BAEC Exposed to High Glucose—We next determined if NO or ONOO− derived from eNOS caused a feedback activation of AMPK in BAEC. Since our earlier studies (1, 2) had demonstrated that prolonged exposure of human aortic endothelial cells to high glucose (HG) resulted in ONOO− formation, we used this model to dissect the contribution of ONOO− in high glucose-induced AMPK activation. As depicted in Fig. 9A, exposure of BAEC to 30 mmol glucose (HG) caused a biphasic increase of AMPK phosphorylation, which peaked at 2 and 48 h, respectively. In parallel, 3-nitrotyrosine, a footprint of ONOO−, was increased in BAEC treated with HG for 48 h. Uric acid (50 μM), a potent scavenger for ONOO−, significantly suppressed HG-enhanced 3-nitrotyrosine at 48 h (Fig. 9B) but did...
FIGURE 6. PKC-ζ is required for statin-induced LKB1 nucleus export and AMPK activation. 
A, inhibition of PKC-ζ blocks statin-enhanced AMPK activation. The infected cells were then treated with 
(50 μM) for 2 h. Values represent mean ± S.E. from five independent experiments. B, statin-induced 
nucleus export is PKC-ζ-dependent. LKB1 subcellular localization was detected by immunocytochemical stain-
ning in HUVEC. PKC-ζ-pseudosubstrate (10 μM, 30 min prior to statin) prevented statin-induced transloca-
tion to the cytosol in HUVEC. C, statin-induced subcellular distribution of LKB1 was not affected by the 
amount of LKB1 in cytosol, whereas it reciprocally decreased LKB1 in nucleus. D, LKB1 protein levels were 
determined if genetic inhibition of PKC-ζ abolishes statin-enhanced phosphorylation of 
AMPK, ACC, and LKB1. C57BL/6J mice were injected retroorbitally with either mouse-specific PKC-ζ siRNA or 
control siRNA (1 mg/kg, diluted in 200 μl) every other 3 days for 6 days. AMPK, PKC-ζ, and LKB1 as well as their 
phosphorylated forms were detected in Western blots by using the specific antibodies. A, transfection of PKC-ζ 
siRNA inhibits PKC-ζ in isolated mouse aortas. Shown is a representative blot from at least three blots from 
three independent experiments. n = 5; *p < 0.05. B and C, effects of PKC-ζ siRNA transfection on statin-
enhanced phosphorylation of AMPK-Thr172 and ACC-Ser79 in vitro. Shown is a representative blot from at least 
three blots from three independent experiments. n = 5; *p < 0.05 (control versus statin or statin plus control siRNA); †, p < 0.05 (statin plus control siRNA versus statin plus PKC-ζ-specific siRNA). D, effects of transfection of PKC-ζ siRNA on statin-enhanced LKB1 phosphorylation at Ser428 in vivo. The blot is a representative blot from at least 
three blots obtained from three independent experiments. 

FIGURE 7. In vivo gene silencing of protein kinase C-ζ abolishes statin-enhanced phosphorylation of 
AMPK, ACC, and LKB1. C57BL/6J mice were injected retroorbitally with either mouse-specific PKC-ζ siRNA or 
control siRNA (1 mg/kg, diluted in 200 μl) every other 3 days for 6 days. AMPK, PKC-ζ, and LKB1 as well as their 
phosphorylated forms were detected in Western blots by using the specific antibodies. A, transfection of PKC-ζ 
siRNA inhibits PKC-ζ in isolated mouse aortas. Shown is a representative blot from at least three blots from 
three independent experiments. n = 5; *p < 0.05. B and C, effects of PKC-ζ siRNA transfection on statin-
enhanced phosphorylation of AMPK-Thr172 and ACC-Ser79 in vitro. Shown is a representative blot from at least 
three blots from three independent experiments. n = 5; *p < 0.05 (control versus statin or statin plus control siRNA); †, p < 0.05 (statin plus control siRNA versus statin plus PKC-ζ-specific siRNA). D, effects of transfection of PKC-ζ siRNA on statin-enhanced LKB1 phosphorylation at Ser428 in vivo. The blot is a representative blot from at least 
three blots obtained from three independent experiments. n = 5; *p < 0.05 (control versus statin or statin plus control siRNA); †, p < 0.05 (statin plus control siRNA versus statin plus PKC-ζ-specific siRNA). 

not alter 3-nitrotyrosine in cells treated with normal glucose or HG for 2 h. These results suggest that 
HG increased ONOO− in BAEC at 48 h but not in BAEC exposed to normal glucose or HG for 2 h. 
We further determined if uric acid altered high glucose-enhanced AMPK activity. As shown in Fig. 9C, 
uric acid did not alter AMPK phosphorylation in BAEC exposed to normal glucose but markedly attenuated 
HG-enhanced AMPK activity at 48 h of exposure. Since the suppression of AMPK activity by uric 
acid was co-related with its inhibition on ONOO− formation in BAEC, these results strongly sug-
gest that ONOO− generated by HG might activate AMPK in BAEC.
Statin via PKC-ζ Activates AMPK

A.

B.

C.

D.

FIGURE 8. Activation of AMPK by statin is ONOO⁻/PKC-ζ-dependent. A, O₂⁻ generation. Intracellular O₂⁻ was detected by the DHE fluorescence as described in Materials and Methods. BAEC to 10 μM statin significantly increased DHE fluorescence, whereas TEMPOL suppressed O₂⁻ generation by statin (n = 6; p < 0.01), control versus statin). B, ONOO⁻/PKC-ζ-dependent activation of AMPK in BAEC (n = 6; p < 0.05, control versus statin). C, 3-NT-positive proteins were detected in the aortic homogenates of C57BL/6J mice but not in eNOS KO mice (n = 5; p < 0.05, WT control versus eNOS KO mice). Mice aortas were isolated and assayed as described under “Experimental Procedures.” Of note is that meformin increased AMPK-P and ACC-P in C57BL/6J mice but not in eNOS KO mice. The blot is a representative blot from at least three independent experiments. n = 5; p < 0.05.

DISCUSSION

Recent studies have suggested that AMPK is a therapeutic target for treating diabetes. Oral hypoglycemic agents, such as metformin and rosiglitazone, have been reported to exert their therapeutic effects by activating AMPK (for a review, see Ref. 18). In the present study, we observe that LKB1 Ser428 phosphorylation by atypical PKC-ζ is required for statin-stimulated AMPK activation. The key evidence can be summarized as follows. First, inhibition of LKB1 with D194A and S428A mutants or LKB1 siRNA effectively blocked AMPK activation induced by statin (Fig. 3, B and C). In addition, statin could not activate AMPK in either A549 or HeLa cells that lack LKB1, but wild type expression restored the stimulation of statin on AMPK activity (Fig. 3A). Second, STO, a potent CaMKKβ inhibitor, was found to inhibit statin-induced AMPK activation in BAEC (Fig. 2). Third, statin increased PKC-ζ activity prior to the phosphorylation of both LKB1 and AMPK in BAEC (Fig. 4C). Genetic inhibition of PKC-ζ effectively blocked statin-induced AMPK phosphorylation (Fig. 5A) and AMPK activity (Fig. 6A). Fourth, statin increased export of LKB1 from the nucleus to the cytosol, previously reported to be an important and essential step for LKB1 to activate AMPK (25), and PKC-ζ pseudosubstrate abolishes statin-induced LKB1 translocation (Fig. 6, B and C). It is noteworthy that statin-enhanced LKB1 phosphorylation in BAEC occurred within 30 min of treatment, which preceded the statin-induced increase in AMPK phosphorylation. These results suggest that LKB1 is an upstream activator of AMPK or a regulator of cellular signaling in concert with AMPK. Furthermore, the pleiotropic effects of statin thought to be beneficial to endothelial functions and antiatherogenesis might be mediated by PKC-ζ-dependent AMPK activation in endothelial cells.

We previously observed that inhibition of c-Src or phosphatidylinositol 3-kinase activity by pharmacologic agents (PKC-ζ siRNA) or genetic suppression (PKC-ζ-DN) of PKC-ζ abolishes AMPK stimulation by metformin or by ONOO⁻ in endothelial cells (25). In this study, we have extended these observation by showing that PKC-ζ is also required for statin-enhanced AMPK activation. Furthermore, we have for the first time demonstrated that in vivo inhibition of PKC-ζ was required for statin-enhanced...
FIGURE 9. Prolonged exposure of bovine aortic endothelial cells to 30 mmol/l glucose causes increased ONOO− formation. Confluent BAEC were exposed to normal glucose (NG; 5 mmol/liter glucose) or high glucose (30 mmol/liter D-glucose) at the times indicated. A, time course of high glucose increased ONOO− formation in BAEC; n = 3. ✴, p < 0.01 (control plus uric acid). B, uric acid suppressed high glucose-enhanced AMPK phosphorylation in BAEC. The blot is representative of three blots from three independent experiments. C, AMPK activation by statin suppresses Ang-II-induced endothelial ROS. Since eNOS−/− mice did not generate ONOO− in response to statin, the data strongly suggest that ONOO− is required for AMPK activation by statin. These results strongly suggest that NO-derived oxidants, such as ONOO−, might be required for AMPK activation by statin.

Numerous studies from us and others (1, 2, 5) have demonstrated antioxidant effects of statin and AMPK in vivo. This apparently contradictory observation might be similar to ROS in ischemic preconditioning, in which low levels of ROS precondition the tissues to prevent massive production of reactive species in index hypoxia. Our results suggest that statin, like ischemic preconditioning, via the generation of low levels of oxidative stress by statin, “preconditions” the cells or tissues to alleviate oxidant production by activating AMPK. In line with this conclusion, we found that statin suppressed ROS triggered by Ang-II and high glucose. Further, we found that AMPK activation by statin is required phosphorylation of AMPK and LKB1. These important findings imply that PKC-ζ-LKB1-AMPK is a common pathway for AMPK activation in vivo.

The present study has, for the first time, demonstrated that statin released O2− and the O2− or its derived oxidant, such as ONOO−, was required for statin-enhanced AMPK activation. The evidence supporting activation of AMPK by the increased formation of ROS is severalfold. First, exposure to statin significantly increased intracellular ROS. In addition, the concentrations of statin (10–50 μmol/liter) triggering ROS were similar to those required for the minimally effective concentrations required for phosphorylation and activation of AMPK-Thr172. This is supported by the fact that mito-

FIGURE 10. AMPK activation by statin suppresses endothelial ROS and apoptosis. A and B, AMPK activation suppresses endothelial ROS. Values represent mean ± S.E. from three independent experiments. ✴, p < 0.05 (control versus statin); ✴, p < 0.05 (statin versus statin plus AMPK-DN); ✴, p < 0.05 (AMPK-CA versus AMPK-CA plus statin). C, AMPK activation by statin suppresses Ang-II-induced endothelial ROS. ✴, p < 0.05 (control versus Ang-II); ✴, p < 0.05 (Ang-II versus Ang-II plus statin); ✴, p < 0.05 (Ang-II plus Ang-II plus compound C). D, AMPK activation by statin suppresses endothelial cell apoptosis triggered by high glucose (n = 5; ✴, p < 0.01 (control versus HG); ✴, p < 0.01 (HG versus HG plus statin); ✴, p < 0.01 (HG plus statin versus HG plus AMPK-DN)). Control, normal glucose, 5 mmol/liter D-glucose; OG, osmotic control glucose (5 mmol/liter D-glucose + 25 mmol/liter L-glucose); HG, 30 mmol/liter D-glucose; statin, 5 μmol/liter statin; AMPK-DN, dominant negative mutants of AMPK.
Statin via PKC-ζ Activates AMPK

for the reduction of reactive nitrogen species (ONOO −). Our earlier work (39, 40) has also demonstrated that metformin, one of the most used antidiabetic drugs, activates AMPK by increasing ONOO −, and AMPK activation suppresses oxidant production. Thus, we consider that AMPK might function as a redox sensor, and AMPK activation might reduce oxidant stress by attenuating oxidant stress by other sources or by enhancing antioxidant potentials.

Overwhelming evidence suggests that in humans, improved endothelial functions are one of the earliest observed clinical effects, following the initiation of statin treatment (7, 8). Most importantly, statin therapy improves endothelial function by virtue of its antioxidant (8, 9) and anti-inflammatory (8, 9) effects as well as its ability to up-regulate eNOS (10, 11). In the present study, we have for the first time shown that AMPK might be implicated in the antiapoptosis effects of statin in diabetes. This finding is in line with the clinical studies. Several clinical trials, including the Heart Protection Study (3) and the Collaborative Atorvastatin Diabetes Study (4), have shown significant benefits with low to moderate dose statin therapy in diabetic patients with and/or without overt cardiovascular disease. In addition, overwhelming evidence suggests a beneficial effect of statin on these systems independent of vascular system. Activation of AMPK could therefore explain the beneficial effects of AMPK in both endothelial functions and the cardiovascular system. Activation of AMPK in the endothelium functions are one of the earliest observed clinical potentials.

Acknowledgment—We thank Dr. Balaraman Kalyanaraman (Medical College of Wisconsin) for providing mito-TEMPOL.

REFERENCES

1. Zou, M. H., Cohen, R., and Ullrich, V. (2004) Endothelium 11, 89–97
2. Zou, M. H. (2007) Prostaglandins Other Lipid Mediat. 82, 119–127
3. Collins, R., Armitage, J., Parish, S., Leigh, P., and Peto, R. (2003) Lancet 361, 2005–2016
4. Collignon, H. M., Betteridge, D. J., Durrington, P. N., Hitman, G. A., Neil, H. A., Livingstone, S. J., Thomason, M. J., Mackness, M. I., Charlton-Menys, V., and Fuller, I. H. (2004) Lancet 364, 685–696
5. Wenzel, P., Daiber, A., Oelze, M., Brandt, M., Closs, E., Xu, J., Thum, T., Bauersachs, J., Ertl, G., Zou, M. H., Forsterrmann, U., and Munzel, T. (2008) Atherosclerosis, 198, 65–76
6. Vaughan, C. J., and Delanty, N. (1999) Stroke 30, 1969–1973
7. Nissen, S. E., Tuzcu, E. M., Schoenhagen, P., Crowe, T., Sasiela, W. J., Tsai, J., Orazen, J., Magoni, R. D., O'Shaughnessy, C., and Ganz, P. (2005) N. Engl. J. Med. 353, 29–38
8. Cahoon, W. D., Jr., Crouch, M. A. (2007) Ann. Pharmacother. 41, 1687–1693
9. Nilaveris, P., Giannopoulous, G., Riga, M., Syntos, A., and Stefanadis, C. (2007) Curr. Vasc. Pharmacol. 5, 227–237
10. Laufs, U., Endres, M., Staglano, N., Amin-Hanjani, S., Chui, D. S., Yang, S. X., Simoncini, T., Yamada, M., Kakin, E., Allen, P. G., Huang, P. L., Bohm, M., Schoen, F. J., Moskowitz, M. A., and Liao, J. K. (2000) J. Clin. Invest. 106, 15–24
11. Laufs, U., Gertz, K., Huang, P., Nickenig, G., Bohm, M., Dirnagl, U., and Endres, M. (2000) Stroke 31, 2442–2449
12. Wassmann, S., Laufs, U., Baumer, A. T., Muller, K., Konkoli, C., Sauer, H., Bohm, M., and Nickenig, G. (2001) Mol. Pharmacol. 59, 646–654
13. Wagner, A. H., Kohler, T., Schloss, U., Just, I., and Hecker, M. (2000) Arterioscler. Thromb. Vasc. Biol. 20, 61–69
14. Warnholtz, A., Bonneau, F., and Munzel, T. (2004) Biochem. Soc. Symp. 71, 121–133
15. Kesteloot, H., Chen, Z. P., Murphy, S., Walter, H., Hinder, J. A., Anderen, B., Jennings, I. G., Iseli, B., and Hainfellner, J. A. (2005) Biochem. Soc Trans. 31, 59–66
16. Winder, W. W., and Hardie, D. G. (1999) J. Biol. Chem. 274, 3032–3035
17. Hattori, Y., Suzuki, K., Hattori, S., and Kasai, K. (2006) Circ. Res. 98, 621–630
18. Kobayashi, H., Ouchi, N., Kihara, S., Walsh, K., Umeki, R., Kuriyama, S., and Funahashi, T., and Matsuzawa, Y. (2005) Circulation 111, 2442–2448
19. Wagner, A. H., Kohler, T., and Steffes, J. T. (2008) Circ. Res. 102, 1585–1595
20. Cacicedo, J. M., Yagihashi, N., Keaney, J. F., Jr., Ruderman, N. B., and Ido, Y. (2003) J. Clin. Invest. 112, 952–962
21. Hattori, Y., Suzuki, K., Hattori, S., and Kasai, K. (2006) Hypertension 47, 1183–1188
22. Sun, W., Lee, T. S., Zhu, M., Gu, C., Wang, Y., Zhu, Y., and Shyy, J. Y. (2006) Circulation 114, 2655–2662
23. Xenos, E. S., Stevens, S. L., Freeman, M. B., Cassada, D. C., and Goldman, M. H. (2005) Ann. Vasc. Surg. 19, 386–392
24. Xie, Z., Dong, Y., Zhang, M., Cui, M. Z., Cohen, R. A., Riek, U., Neumann, D., Schlattner, U., and Zou, M. H. (2006) J. Biol. Chem. 281, 6366–6375
25. Xie, Z., Dong, Y., Scholz, R., Neumann, R., and Zou, M. H. (2008) Circulation 117, 952–962
26. Xie, Z., Dong, Y., Scholz, R., Neumann, R., and Zou, M. H. (2008) Circulation 116, 944–953
27. Song, P., Wu, Y., Xu, J., Xie, Z., Dong, Y., Zhang, M., and Zou, M. H. (2007) Circulation 116, 1585–1595
28. Hawley, S. A., Pan, D. A., Mustard, K. J., Ross, L., Bain, J., McElduff, P., and Dyck, J. R. (2007) Proc. Natl. Acad. Sci. U. S. A. 104, 9248–9251
29. Sanchez-Cespedes, M., Parrella, P., Esteller, M., Nomoto, S., Trink, B., Engles, J. M., Westra, W. H., Herman, J. G., and Sidransky, D. (2002) Cancer Res. 62, 3659–3662
30. Tiainen, M., Ylikorkala, A., and Makela, T. P. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 9248–9251
31. Tiainen, M., Ylikorkala, A., and Makela, T. P. (2002) Hum. Mol. Genet. 11, 1497–1504
32. Smith, C. M., Radzio-Andzel, E., Madhusudan, N., Akamine, P., and Taylor, S. S. (1999) Prog. Biophys. Mol. Biol. 71, 313–341
33. Han, S. P., Leiper, F. C., Woods, A., Carling, D., and Carlson, M. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 8839–8843
34. Noga, A. A., Soltys, C. L., Barr, A. I., Kovacic, S., Lopaschuk, G. D., and Dyck, J. R. (2007) Am. J. Physiol. 292, H1460–H1469

M.-H. Zou, unpublished data.
36. Delbosc, S., Cristol, J. P., Descomps, B., Mimran, A., and Jover, B. (2002) *Hypertension* **40**, 142–147
37. Stefanec, T. (2000) *Chest* **117**, 841–854
38. Kureishi, Y., Luo, Z., Shiojima, I., Bialik, A., Fulton, D., Lefer, D. J., Sessa, W. C., and Walsh, K. (2000) *Nat. Med.* **6**, 1004–1010
39. Zou, M. H., Hou, X. Y., Shi, C. M., Kirkpatrick, S., Liu, F., Goldman, M. H., and Cohen, R. A. (2003) *J. Biol. Chem.* **278**, 34003–34010
40. Davis, B., Wiles, W. G., IV, Xie, Z., Viollet, B., and Zou, M. H. (2006) *Diabetes* **55**, 496–505
41. Zang, M., Xu, S., Maitland-Toolan, K. A., Zuccollo, A., Hou, X., Jiang, B., Wierzbicki, M., Verbeuren, T. J., and Cohen, R. A. (2006) *Diabetes* **55**, 2180–2191