**Endothelial Nitric Oxide Synthase-Independent Pleiotropic Effects of Pitavastatin Against Atherogenesis and Limb Ischemia in Mice**

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**Aim:** Statins have a protective impact against cardiovascular diseases through not only lipid-lowering effects but also pleiotropic effects, including activation of the endothelial nitric oxide synthase (eNOS) system. We aimed to clarify the protective effects of a statin against atherogenesis and ischemia in eNOS⁻/⁻ mice.

**Methods:** Study 1. eNOS⁻/⁻ Apolipoprotein E (ApoE)⁻/⁻ mice were treated with a vehicle or pitavastatin (0.3 mg/kg/day) for 4 weeks. Study 2. eNOS⁻/⁻ mice were also treated with a vehicle or the same dose of pitavastatin for 2 weeks prior to hind-limb ischemia.

**Results:** In Study 1, pitavastatin attenuated plaque formation and medial fibrosis of the aortic root with decreased macrophage infiltration in eNOS⁻/⁻ ApoE⁻/⁻ mice. PCR array analysis showed reductions in aortic gene expression of proatherogenic factors, including Ccl2 and Ccr2 in pitavastatin-treated double mutant mice. In addition, pitavastatin activated not only atherogenic p38MAPK and JNK but also anti-atherogenic ERK1/2 and ERK5 in the aorta of the double mutant mice. In Study 2, pitavastatin prolonged hind-limb survival after the surgery with increased BCL2-to-BAX protein ratio and inactivated JNK. Enhanced expression of anti-apoptotic genes, including Vegf, Api5, Atf5, Prdx2, and Dad1, was observed in the ischemic limb of pitavastatin-treated eNOS⁻/⁻ mice. Furthermore, pitavastatin activated both aortic and skeletal muscle AMPK in the eNOS-deficient vascular injury models.

**Conclusion:** Pitavastatin exerts eNOS-independent protective effects against atherogenesis and hind-limb ischemia in mice, which may occur via modifications on key molecules such as AMPK and diverse molecules.

**Key words:** Pitavastatin, Pleiotropic effect, Atherogenesis, Ischemia, eNOS

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**Introduction**

Since endothelial function has been shown to be inversely associated with future cardiovascular events¹,², improvement and preservation of endothelial function are key objectives for the promotion of public health, especially in patients with accumulating cardiovascular risks. Endothelial function is greatly affected by nitric oxide (NO) bioavailability in vivo and NO bioavailability is extremely regulated by NO production in the vascular wall. NO has been shown to be generated through L-arginine metabolism by endothelial nitric oxide synthase (eNOS), and dysregulation of the

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eNOS system has been implicated in various vascular failures, including atherosclerosis, impaired angiogenesis, neointimal hyperplasia, and pulmonary hypertension. Therefore, up-regulation and activation of eNOS by appropriate physiological and/or pharmacological interventions are useful approaches for preventing cardiovascular diseases (CVDs).

It is well established that statins, inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A reductase, attenuate the development of atherosclerotic diseases, including stroke, coronary artery disease, and peripheral arterial disease. Accumulating evidence has demonstrated that the favorable effects of statins are exerted by not only lipid-lowering actions but also lipid-lowering-independent actions called pleiotropic effects.

The pleiotropic effects of statins have been recognized as various biological actions, including improvement of endothelial dysfunction, reduction of inflammatory responses, stabilization of atherosclerotic plaques, and attenuation of procoagulant activity and platelet function. It has been demonstrated that statins activate the phosphatidylinositol 3-kinase/threonine-specific protein kinase (Akt) pathway and enhance the expression and activity of eNOS in aortic endothelial cells.

In addition, various cardiovascular protective effects of statins are abolished in eNOS-deficient mice. Therefore, the pleiotropic actions of statins have been thought to be mostly mediated via eNOS activation. Taken together, a biologically available eNOS system seems to be needed to guarantee lipid-lowering-independent beneficial effects of statins on vascular protection.

On the other hand, regarding eNOS-independent pharmacological actions of statins, we previously reported that pitavastatin, a lipophilic statin, attenuates angiotensin II-induced cardiovascular insufficiency in eNOS-deficient mice. However, it remains unclear whether pitavastatin can exert eNOS-independent protective action against atherogenesis and critical ischemic stimuli. In order to elucidate this issue, we investigated eNOS-independent protective effects of pitavastatin in murine models of atherosclerosis and limb ischemia.

Methods

1. Animal Preparation

   Study 1

   C57BL/6J genetic background eNOS−/− mice and Apolipoprotein E (ApoE)−/− mice were crossed to generate double heterozygous mutant mice. These mice were then crossed and the offspring were genotyped for eNOS and ApoE gene deficiency by polymerase chain reaction. After genotyping, the obtained eNOS−/− ApoE−/− mice at the age of 12 weeks were divided into two groups for ad libitum administration of pitavastatin (NK 104 KOWA Pharmaceutical Co., Ltd.) or a vehicle for 4 weeks. Pitavastatin was dissolved in drinking water at a concentration of 2.5 mg/L. Average daily amounts of drinking water for eNOS−/− ApoE−/− mice and eNOS−/− ApoE−/− mice were about 3.0 mL and 2.5 mL, respectively. Average body weights were about 25.2 g and 21.1 g, respectively. Therefore, estimated intake of pitavastatin was approximately 0.3 mg/kg/d.

   Study 2

   C57BL/6J genetic background eNOS−/− mice at the age of 12 weeks were divided into two groups for ad libitum administration of pitavastatin or a vehicle for 2 weeks. Pitavastatin was dissolved in drinking water at a concentration of 2.5 mg/L, and estimated intake of pitavastatin was approximately 0.3 mg/kg/d as in Study 1. After 2 weeks of pitavastatin administration, we performed surgery to induce left hind-limb ischemia. To induce hind-limb ischemia in these mice, the proximal portion of the femoral artery and the distal portion of the saphenous artery were ligated and then excised. Thereafter, pitavastatin administration was continued until the day of sacrifice of mice.

   These animals (Study 1 and Study 2) were housed in a specific pathogen-free facility under climate-controlled conditions with a 12-hour light/12-hour dark cycle and were provided standard diet chow (9% water, 5% crude ash, 23% crude protein, 5% crude fat, 3% crude fiber, 55% soluble nitrogen free extract, Oriental Yeast Co., Ltd. Tokyo, Japan). And these genetically-engineered mice were purchased from The Jackson Laboratory (Sacramento, CA, USA).

2. Measurement of Blood Pressure and Heart Rate

   After administration of pitavastatin for 4 weeks in eNOS−/− ApoE−/− mice (Study 1) or for 2 weeks in eNOS−/− ApoE−/− mice (Study 2), systolic and diastolic blood pressure (BP) levels and heart rate were measured using a noninvasive computerized tail-cuff system (BP98A Softron Corp., Tokyo, Japan). Unanesthetized mice from both groups were placed in a holding device mounted on a thermostatically controlled warming plate, maintained at 37°C. BP and heart rate were measured on two consecutive days, and at least 10 readings were taken for each measurement, as previously described.
3. Measurement of Plasma Lipids and Glucose

After administration of pitavastatin for 4 weeks in eNOS<sup>+/−</sup> ApoE<sup>−/−</sup> mice (Study 1) or for 2 weeks in eNOS<sup>+/−</sup> ApoE<sup>+/+</sup> mice (Study 2), blood was drawn through an inferior vena cava puncture and collected into a tube containing a 1/10 volume of heparin (10 U/ml) after overnight fasting and then centrifuged at 2000 g for 20 minutes. Plasma was stored at −80°C until use. Plasma levels of glucose were determined by an enzymatic method with Quickauto Neo GLU-HK (Shino-Test Corporation, Japan) using a Hitachi 7180 Clinical Analyzer (Hitachi, Tokyo, Japan). Plasma levels of lipids were also analyzed by an enzymatic method with type Wako CHO • H (Wako Pure Chemical Industries, Ltd., Tokyo, Japan) using the same analyzer as that for glucose.

4. Hydroperoxide Assay and Biological Anti-oxidant Potential Assay

After administration of pitavastatin for 4 weeks in eNOS<sup>+/−</sup> ApoE<sup>−/−</sup> mice (Study 1) or for 2 weeks in eNOS<sup>+/−</sup> ApoE<sup>+/+</sup> mice (Study 2), fasting plasma derivatives of reactive oxygen metabolite (d-ROMs), a marker of oxidant status, and the plasma biological anti-oxidant potential (BAP) were analyzed semi-automatically using a FREE carp diam (WISMERLL Co., Ltd. Japan)<sup>15, 36</sup>. Organic hydroperoxide serum measurements were performed to evaluate the levels of free radicals. The concentrations of radical species were measured by spectrophotometry (505 nm). The results for all d-ROM measurements were expressed in Carretelli units (U. CARR). Plasma BAP levels were also measured to determine the anti-oxidant potency. The BAP test is based on the ability of a colored solution containing a source of ferric ions bound to a chromogenic substrate to decolorize when ferric ions are reduced to ferrous ions, which occurs when a reducing/anti-oxidant source is added. The concentration is then measured by spectrophotometry (505 nm).

5. Macroscopic Evaluation of the Severity of Hind-Limb Ischemia

Study 2

After the ischemic operation, clinical outcomes of the mice were observed and recorded until 14 days after the operation. The ischemic limb was macroscopically evaluated, and diagnosis of hind-limb autoamputation was determined as extension of necrosis above the crus (knee loss and total hind-limb loss).

6. Histological and Immunohistochemical Studies

Study 1

Removed aortic roots were fixed in 10% neutral buffered formalin overnight. The aortic roots were then cut into subserial 5-μm-thick cross sections with intervals of 200 μm. The sections were stained with Masson trichrome and F4/80 antibody (abcam, Cambridge, USA) as previously described<sup>17</sup>. Plaque area was identified as the innermost part of the intimal area. Vascular medial fibrosis area was identified as the blue-stained vascular medial area. The average from 5 sections for each animal tissue was used to quantify each lesion area.

Study 2

Ischemic adductor muscles at day 1 were fixed in 10% paraformaldehyde overnight. After fixation, the muscles were embedded in paraffin and were serially cut into 3-μm-thick slices. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining was performed as previously described<sup>18</sup>. TUNEL-positive nuclei were counted in five randomly selected microscopic fields in each plate, and the percentages of apoptotic nuclei to all myocytes were expressed as ratio of TUNEL-positive nuclei-to-total myocytes. In addition, necrotic cells that were not TUNEL-stained and lucent cells were identified, and the ratio of necrotic myocytes to total myocytes was also calculated. These lesion quantifications were performed by the same researcher in a blind manner and each measurement was performed twice and averaged.

7. Quantitative RT-PCR Analysis and Gene Expression Profiling using PCR Arrays

Total RNA extraction from the descending aortas in eNOS<sup>+/−</sup> ApoE<sup>−/−</sup> mice (Study 1) and from the ischemic thigh muscles in eNOS<sup>+/−</sup> ApoE<sup>+/+</sup> mice on day 1 after surgery (Study 2) and subsequent cDNA synthesis were performed as previously described<sup>18, 19</sup>. Quantitative RT-PCR analysis of 84 atherosclerosis-related genes (Study 1), 84 apoptosis-related genes (Study 2), M1 and M2 marker genes (Study 1), and several angiogenesis-related genes (Study 2) was performed using Atherosclerosis RT<sup>2</sup> Profiler PCR Arrays PAMM-038Z (QIAGEN), Apoptosis RT<sup>2</sup> Profiler PCR Arrays PAMM-012Z (QIAGEN) and specific primers (Primer sequences for CD11c, F4/80, CD163, CD206, and CD209a are listed in Supplemental Table 1 and other commercially available PCR primers were purchased from Perfect real-time primer system<sup>®</sup> (TAKARA BIO INC. Ohtsu, Japan) for Hif1α, Vegfa, Fgf2, Kdr, and Gapdh) according to the manufacturer’s protocols, respectively.

Data analysis of each PCR array kit was performed using the manufacturer’s integrated web-based software for the PCR Array System (http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php) using ΔΔC<sub>t</sub>-based fold-change calculations.
8. Western Blot Analysis

For Western blotting analysis, the descending aortas of eNOS−/− ApoE−/− mice (Study 1) and the ischemic thigh muscle of eNOS−/− ApoE−/− mice on day 1 after surgery (Study 2) were prepared as previously described. Protein expression levels of B-cell lymphoma 2 (BCL2) and BCL2-associated X protein (BAX) and phosphorylation of extracellular signal-regulated kinase (ERK)1/2, ERK5, p38 mitogen-activated protein kinase (MAPK), c-jun N-terminal kinase (JNK), Akt and AMP-activated protein kinase α (AMPKα) were evaluated by Western blot analysis. Protein extraction and Western blot analysis were performed as described previously. In brief, 50-μg protein extracts from the aortas or thigh muscle in both groups were boiled for 5 min in Laemmli sample buffer and then run on SDS-PAGE. The protein extracts were then transferred to a chemical membrane (Amersham Hybond™, GE Healthcare, Buckinghamshire, UK). The membrane was blocked for 20 min at room temperature with SuperBlock T20 TBS Blocking Buffer (Thermo Scientific, Rockford, IL). The blots were incubated overnight at 4°C with each primary antibody, followed by incubation for 1 hr with anti-rabbit secondary antibody (horseradish peroxidase-conjugate). Immunoreactive bands were visualized using enhanced chemiluminescence with ECL Prime Western Blotting Detection Reagent (GE Healthcare, Buckinghamshire, UK) and were exposed and quantified by a lumino image analyzer (ChemiDoc™ XRS+ System) (BIO-RAD). We used primary antibodies against ERK1/2, phosphorylated ERK1/2 (Thr202/Tyr204), ERK5, phosphorylated ERK5 (Thr218/Tyr220), JNK, phosphorylated JNK (Thr183/Tyr185), p38 MAPK, phosphorylated p38 MAPK (Thr180/Tyr182), Akt, phosphorylated Akt (Ser473), AMPKα, phosphorylated AMPKα (Thr172), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Cell Signaling Technology, Beverly, MA), BCL2 (Santa Cruz Biotechnology, Inc., CA) and BAX (Santa Cruz Biotechnology, Inc., CA).

All experimental procedures were performed in accordance with the guidelines of the Animal Research Committee of the Tokushima University Graduate School of Biomedical Sciences.

9. Statistical Analysis

Values for each parameter within a group are expressed as dot plots with mean bars. For comparisons of quantitative data between groups, statistical significance was assessed by the Kruskal–Wallis test or Mann–Whitney U test. Limb survival rate was assessed by the log-rank test. These analyses were performed by using GraphPad Prism6 (GraphPad Software, San Diego, CA). Statistical significance was set at P<0.05.

Results

1. Common Experiments in both Study 1 and Study 2

1.1 Blood Pressure and Heart Rate

Pitavastatin treatment in eNOS−/− mice did not affect BP levels or heart rate regardless of ApoE deficiency (Fig. 1A).

1.2 Plasma Lipid Profiles and Glucose Levels

ApoE deletion caused significant elevations of plasma total cholesterol levels and triglyceride levels both in pitavastatin-treated and untreated mice (Fig. 1B). Conversely, ApoE disruption reduced plasma HDL-cholesterol levels in the eNOS−/− mice regardless of pitavastatin treatment. Pitavastatin did not have a statistically significant influence on glucose levels among the groups of mice (Fig. 1B).

1.3 Determination of Plasma Reactive Oxygen Metabolites and Biological Anti-oxidant Potential

As is shown in Fig. 1C, ApoE deficiency did not change plasma levels of d-ROMs, a biomarker of reactive oxygen metabolite, in eNOS−/− mice. However, marked reductions of plasma BAP levels and BAP-to-d-ROMs ratio, a biomarker of anti-oxidant potential, were found in eNOS−/−ApoE−/− mice compared to those in eNOS−/−ApoE−/− mice. Pitavastatin did not affect any values of those biomarkers in eNOS−/− mice regardless of ApoE deficiency (Fig. 1C).

2. Study 1

2.1 Pitavastatin Attenuates Plaque Formation and Medial Fibrosis of Aortic Roots in eNOS−/−ApoE−/− Mice

Pitavastatin treatment significantly reduced plaque formation of aortic roots in eNOS−/−ApoE−/− mice compared to that in vehicle-treated eNOS−/−ApoE−/− mice (Fig. 2A).

In addition, pitavastatin administration significantly attenuated medial fibrosis of aortic roots in eNOS−/−ApoE−/− mice compared to that in vehicle-treated eNOS−/−ApoE−/− mice (Fig. 2A).

2.2 Pitavastatin Inhibits Macrophage Infiltration into Atherosclerotic Lesions of Aortic Roots in eNOS−/−ApoE−/− Mice

Since macrophage recruitment plays a pivotal role in the development of atherosclerosis, we assessed the effect of pitavastatin on macrophage infiltration into atherosclerotic lesions of the aortic roots in
2.3 Pitavastatin Reduces Gene Expression Levels of Proatherogenic Factors in eNOS<sup>−/−</sup>ApoE<sup>−/−</sup> Mice

In order to determine the atherogenesias-associated gene expression profiling, we performed quantitative RT-PCR analysis using a commercially available PCR array kit for atherosclerosis. Supplemental Fig. 1 shows that pitavastatin treatment tended to reduce the gene expression levels of proatherogenic factors, and we found candidate genes that are accountable for pitavastatin-induced attenuation of atherosclerotic plaque formation (Table 1). In particular, decreased gene expression levels of Ccl2 and Ccr2 were thought to be consistent with the results of F4/80 staining of the aortic roots (Table 1, Fig. 2B). As for decreased medial fibrosis by pitavastatin treatment, reduced gene expression levels of Col3a seemed to be explicable for the phenotypic change in eNOS<sup>−/−</sup>ApoE<sup>−/−</sup> mice.

Fig. 1. Blood pressure, heart rate, plasma lipids, plasma glucose, plasma reactive oxygen metabolites and biological anti-oxidant potential in eNOS<sup>−/−</sup>ApoE<sup>−/−</sup> and eNOS<sup>−/−</sup>ApoE<sup>−/−</sup> mice

(A) Systolic blood pressure (SBP) (left), diastolic blood pressure (DBP) (middle) and heart rate (HR) (right) in the 4 groups of mice. Bars represent mean values in each group. (eNOS<sup>−/−</sup>ApoE<sup>−/−</sup> mice group Vehicle: n=10, Pitavastatin: n=11, eNOS<sup>−/−</sup>ApoE<sup>−/−</sup> mice group Vehicle: n=7, Pitavastatin: n=7) NS: not significant.

(B) From left to right, total cholesterol, triglycerides, HDL-cholesterol and fasting plasma glucose (FPG) levels in the 4 groups of mice. Bars represent mean values in each group. NS: not significant. *p<0.05 (vehicle-treated eNOS<sup>−/−</sup>ApoE<sup>−/−</sup> mice (n=11) vs vehicle-treated eNOS<sup>−/−</sup>ApoE<sup>−/−</sup> mice (n=12) or pitavastatin-treated eNOS<sup>−/−</sup>ApoE<sup>−/−</sup> mice (n=9), pitavastatin-treated eNOS<sup>−/−</sup>ApoE<sup>−/−</sup> mice (n=12) vs vehicle-treated eNOS<sup>−/−</sup>ApoE<sup>−/−</sup> mice or pitavastatin-treated eNOS<sup>−/−</sup>ApoE<sup>−/−</sup> mice).

(C) Left: plasma reactive oxygen metabolites (d-ROMs), Middle: biological anti-oxidant potential (BAP), Right: BAP-to-d-ROMs ratio in the 4 groups of mice. Bars represent mean values in each group. NS: not significant. *p<0.05 (vehicle-treated eNOS<sup>−/−</sup>ApoE<sup>−/−</sup> mice (n=5) vs vehicle-treated eNOS<sup>−/−</sup>ApoE<sup>−/−</sup> mice (n=11) or pitavastatin-treated eNOS<sup>−/−</sup>ApoE<sup>−/−</sup> mice (n=11), pitavastatin-treated eNOS<sup>−/−</sup>ApoE<sup>−/−</sup> mice (n=6) vs vehicle-treated eNOS<sup>−/−</sup>ApoE<sup>−/−</sup> mice or pitavastatin-treated eNOS<sup>−/−</sup>ApoE<sup>−/−</sup> mice).

eNOS<sup>−/−</sup>ApoE<sup>−/−</sup> mice. As is shown in Fig. 2B, the number of F4/80-positive cells in atherosclerotic plaque was markedly reduced in pitavastatin-treated eNOS<sup>−/−</sup>ApoE<sup>−/−</sup> mice (Fig. 2B).
Fig. 2. Attenuation of atherosclerosis, medial fibrosis and macrophage infiltration in the aortic root of eNOS−/− ApoE−/− mice

(A) Upper panels: Representative findings of Masson Trichrom-stained aortic roots in eNOS−/− ApoE−/− mice with (n=18) or without (n=18) pitavastatin treatment.
Lower panels: Quantitative results of plaque area (left) and fibrotic area (right). Bars represent mean values in each group. N=18 in each group. *P<0.05, **P<0.01.

(B) Representative findings of F4/80 antibody-stained aortic roots of eNOS−/− ApoE−/− mice with or without pitavastatin treatment.

Table 1. Candidate Genes for Pitavastatin-Induced Anti-atherogenesis against Dyslipidemia in eNOS−/− Mice

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Description</th>
<th>Fold Change (Pitavastatin/Vehicle)</th>
<th>95% CI</th>
<th>p-value</th>
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<tr>
<td>Ccl2</td>
<td>Chemokine (C-C motif) ligand 2</td>
<td>0.19</td>
<td>(0.03, 0.35)</td>
<td>0.006431</td>
</tr>
<tr>
<td>Ccr2</td>
<td>Chemokine (C-C motif) receptor 2</td>
<td>0.35</td>
<td>(0.07, 0.63)</td>
<td>0.024167</td>
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<td>Cflar</td>
<td>CASP8 and FADD-like apoptosis regulator</td>
<td>0.41</td>
<td>(0.14, 0.68)</td>
<td>0.033197</td>
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<tr>
<td>Col3a1</td>
<td>Collagen, type III, alpha 1</td>
<td>0.23</td>
<td>(0.05, 0.41)</td>
<td>0.000308</td>
</tr>
<tr>
<td>Nfkb1</td>
<td>Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1, p105</td>
<td>0.27</td>
<td>(0.04, 0.50)</td>
<td>0.018265</td>
</tr>
<tr>
<td>Pdgfa</td>
<td>Platelet-derived growth factor, alpha</td>
<td>0.29</td>
<td>(0.06, 0.52)</td>
<td>0.022822</td>
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<tr>
<td>Serpinb2</td>
<td>Serine (or cysteine) peptidase inhibitor, clade B, member 2</td>
<td>0.07</td>
<td>(0.03, 0.97)</td>
<td>0.028673</td>
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2.4 Pitavastatin Reduces Gene Expression Levels of M1 Markers but not M2 Markers in the Aorta of eNOS−/− ApoE−/− Mice

Macrophage polarization is recognized as the ability of differentiated macrophages to respond to external stimuli by changing their phenotypic and functional characteristics. Since it is known that the role of the classically activated (M1) macrophage is as an effector cell in TH1 cellular immune responses and pro-inflammation, whereas the alternatively activated (M2) macrophage appears to be involved in immune-suppression, anti-inflammation and wound healing/tissue repair, macrophage polarization plays an important role in the development of atherosclerosis. In the present study, we found that pitavastatin attenuated the gene expression levels of M1 markers, including Ccl2, CD11c, and F4/80, but not M2 markers, including CD163, CD206, and CD209a (Fig. 3A). These results are consistent with the results of F4/80 staining of the aortic root (Fig. 2B).

2.5 Pitavastatin Activates Phosphorylation of MAPKs and AMPK in the Aorta of eNOS−/− ApoE−/− Mice

Since MAPKs and AMP-activated protein kinase (AMPK) are involved in the development of atherosclerosis, we examined the phosphorylation of MAPKs and AMPKα in the aorta of eNOS−/− ApoE−/− mice. Fig. 3B shows that pitavastatin activated all MAPKs, including ERK1/2, ERK5, JNK and p38 MAPK, and AMPKα in the aorta of eNOS−/− ApoE−/− mice.

3. Study 2

3.1 Pitavastatin Increases Survival Rate of Hind-limbs after Ischemia in eNOS−/− Mice

To elucidate the pleiotropic effects of pitavastatin in ischemic response, we created a model of hind-limb ischemia induced by surgical arterectomy of the left femoral artery in eNOS−/− mice. Log-rank analysis revealed that pitavastatin administration significantly prolonged hind-limb survival after critical ischemia (Fig. 4A). In TUNEL staining, more prominently accelerated necrosis of the skeletal muscle at 1 day after ischemia was observed in vehicle-treated eNOS−/− mice than in pitavastatin-treated eNOS−/− mice (Fig. 4B). Taken together, we speculated that the larger number of TUNEL-positive nuclei means slow cell death in skeletal muscle of pitavastatin-treated eNOS−/− mice, in contrast to the rapid cell death (necrotic cells) in skeletal muscle of vehicle-treated eNOS−/− mice.

3.2 Pitavastatin Increases Gene Expression Levels of Anti-apoptotic Factors in eNOS−/− Mice after Limb Ischemia

We then performed PCR array analysis to determine the factors involved in the process of apoptosis in the ischemic hind-limb of eNOS−/− mice with or without pitavastatin treatment. Supplemental Fig. 2 shows the results of heat mapping with dendrograms of apoptosis-associated genes, and Table 2 shows candidate anti-apoptotic genes up-regulated by pitavastatin administration including, Apo5, Afp5, Bcl2l1, Bcl2l2, Dad1, and Prdx2. In addition, real-time PCR analysis demonstrated that mRNA levels of Vegfa, but not those of Fgf2, Hif1a, and Kdr, were up-regulated by pitavastatin treatment (Fig. 5A).

3.3 Pitavastatin Increases BCL2-to-BAX Ratio, Activates AMPK Phosphorylation and Suppresses JNK Phosphorylation in the Ischemic Hind-limb of eNOS−/− Mice

In order to determine whether pitavastatin alters apoptosis-associated protein expression and phosphorylation after ischemia in eNOS−/− mice, we performed Western blot analysis using tissue homogenates of ischemic adductor muscles obtained from vehicle-treated and pitavastatin-treated eNOS−/− mice at 1 day after ischemia. Since the expression ratio of BCL2 to BAX plays a pivotal role in the apoptotic signal pathway, we analyzed expression levels of the molecules in ischemic skeletal muscle of the mice. As shown in Fig. 5B, we found that BCL2-to-BAX protein ratio was higher in pitavastatin-treated eNOS−/− mice than in vehicle-treated eNOS−/− mice.

Since activation of AMPKα has also been shown to promote cellular survival against ischemia, we examined these factors and found that phosphorylation of AMPKα was prominently enhanced by pitavastatin treatment than in eNOS−/− mice (Fig. 5B). Furthermore, it has been reported that myocyte apoptosis following ischemia-reperfusion occurs at least in part through activation of the JNK pathway, and we therefore investigated JNK phosphorylation and found that pitavastatin significantly attenuated JNK phosphorylation in the ischemic skeletal muscle of eNOS−/− mice (Fig. 5B).

Discussion

Statins have been clearly shown to exert pharmacological effects for cardiovascular protection through not only lipid-lowering effects but also activating the eNOS system that leads to improvement of endothelial function. In addition, Momi et al. showed that an NO-donating property confers enhanced anti-
atherogenic and anti-inflammatory effects of a statin against the development of atherosclerosis. Taken together, eNOS activation during statin treatment in subjects with serious endothelial dysfunction is a crucial factor for prevention of CVDs.

The present study demonstrated that pitavastatin has eNOS-independent protective actions via regulation of the expression and phosphorylation of various endogenous molecules against dyslipidemia-induced atherogenesis and ischemia-induced apoptosis and necrosis in mice. In our previous studies, we reported that pitavastatin counteracts angiotensin II-induced cardiovascular remodeling and renal insufficiency via reducing oxidative stress and suppressing transforming growth factor β/Smad signaling in eNOS−/− mice. However, interestingly, the present results proved that

![Graphs and figures showing expression and activation of atherogenesis-associated factors in aortas of eNOS−/− ApoE−/− mice with or without pitavastatin treatment.](image)

Fig. 3. Expression and activation of atherogenesis-associated factors in aortas of eNOS−/− ApoE−/− mice with or without pitavastatin treatment

(A) Evaluation of M1 and M2 marker gene expression of macrophages that have infiltrated into aortas of eNOS−/− ApoE−/− mice with or without pitavastatin treatment. Bars represent mean values in each group. Vehicle group: n = 11, Pitavastatin group: n = 13. NS: not significant. *P<0.05

(B) Western blot analysis of phosphorylation of ERK1/2, ERK5, JNK, p38MAPK and AMPK in aortas of eNOS−/− ApoE−/− mice with or without pitavastatin treatment. Representative blots and quantitative results are shown. Bars represent mean values in each group. N = 12 in each group. *P<0.05, **P<0.01.
Fig. 4. Prolonged hind-limb survival by pitavastatin after femoral arterectomy in eNOS−/− mice

(A) Limb survival rate after ischemic surgery in vehicle or pitavastatin-treated eNOS−/− mice. Autoamputation of the hind-limb was defined as extended necrosis above the knee. n=24 in each group. **P<0.01.

(B) Representative photos of TUNEL staining (left panel), quantitative result of TUNEL-positive nuclei-to-total myocytes number ratio (middle panel) and quantitative result of necrotic cells-to-total myocytes number ratio (right panel) in hind-limbs of vehicle or pitavastatin-treated eNOS−/− mice on day 1 after ischemic surgery. Bars represent mean values in each group. Vehicle group: n=11, Pitavastatin group: n=12. *P<0.05, **P<0.01.

Table 2. Candidate Genes for Pitavastatin-Induced Anti-apoptosis against Limb Ischemia in eNOS−/− Mice

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Description</th>
<th>Fold Change (Pitavastatin/Vehicle)</th>
<th>95% CI</th>
<th>p-value</th>
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<td>Bcl2-like 1</td>
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<td>(1.98, 3.36)</td>
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<tr>
<td>Bcl2l2</td>
<td>Bcl2-like 2</td>
<td>2.04</td>
<td>(1.53, 2.55)</td>
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<td>Dad1</td>
<td>Defender against cell death 1</td>
<td>1.52</td>
<td>(1.35, 1.69)</td>
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<td>Prdx2</td>
<td>Peroxiredoxin 2</td>
<td>1.84</td>
<td>(1.60, 2.08)</td>
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amelioration of dyslipidemia-induced atherogenesis and ischemic tolerance ability by pitavastatin in eNOS−/− mice is independent of the redox state.

As for the pathogenesis of atherogenesis, macrophage recruitment is a very important issue because macrophages can be found in all stages of atherosclerosis and because continuous recruitment of macrophages drives chronic vascular inflammation that leads
to the development of atherosclerotic plaque formation. Since Gu et al. demonstrated that Low-density-lipoprotein (LDL) receptor \(^{-/-}\) \(\text{MCP}-1\) \((\text{Ccl2})^{-/-}\) mice had 83% less lipid deposition throughout their aortas compared to LDL receptor \(^{-/-}\) \(\text{MCP}-1\) \((\text{Ccl2})^{+/-}\) mice, pitavastatin-induced reduction of the gene expression levels of both \text{Ccl2} and its receptor, \text{Ccr2} axis, might be one of the major causes of suppressed atherogenesis in \(\text{eNOS}^{-/-}\) \(\text{ApoE}^{-/-}\) mice.

We found that pitavastatin activates ERK1/2 and ERK5 in the aorta of the double mutant mice. Previous studies have shown that a steady laminar flow activates both ERK1/2 and ERK5 in human umbilical vascular endothelial cells, leading to preservation of endothelial function\(^{32, 33}\). Furthermore, ERK5 has been reported to regulate endothelial integrity and protect against vascular dysfunction and disease\(^{34}\). Recent findings have demonstrated that AMPK is a common molecule for attenuation of both atherosclerosis and ischemia-induced apoptosis in \(\text{eNOS}^{-/-}\) mice. In the present study, we identified AMPK as a key mediator of cell survival.

Several genes of pro-atherogenic factors, including \text{Pdgfa}, \text{Serpinb2}, \text{Nfkb1}, \text{Cflar}, and \text{Co3a}, that were significantly down-regulated by pitavastatin treatment were identified by PCR array analysis. Although it has been shown that statins reduce the expression of almost all of those genes in a preserved \text{eNOS} system, our results showed for the first time that pitavastatin can reduce the expression of \text{Pdgfa}, \text{Serpinb2}, \text{Nfkb1}, \text{Cflar}, and \text{Co3a}, in atherosclerosis regardless of the presence or absence of the \text{eNOS} system.

The pro-apoptotic protein BAX plays a critical role in the intrinsic apoptotic pathway, and BCL2 forms heterodimers with BAX and prevents its insertion into the mitochondrial membrane. Therefore, the BCL2-to-BAX ratio is critical for determination of the apoptotic threshold\(^{30}\). Although there is little information concerning the relationship between statin treatment and apoptosis, lovastatin has been shown to protect chondrocytes against hydrogen-peroxide-induced cellular apoptosis with an increased ratio of BCL2-to-BAX\(^{37}\). In addition, since it has been reported that hydrogen-peroxide-induced vascular smooth muscle cellular apoptosis was accompanied by increased activation of JNK, regulation of JNK activity plays a significant role during the process of apoptosis after cellular injuries. In the present study, we observed that pitavastatin reduced phosphorylation of JNK in ischemic skeletal muscle of \(\text{eNOS}^{-/-}\) mice, and therefore inactivated JNK by pitavastatin treatment may promote cellular survival after critical ischemia regardless of \text{eNOS} expression.

PCR array analysis and real-time PCR analysis demonstrated that pitavastatin up-regulates the expression of several anti-apoptotic genes, including \text{Api5}, \text{Atf5}, \text{Dad1}, \text{Prdx2}, and \text{Vegf}, after ischemia in \(\text{eNOS}^{-/-}\) mice. Except for \text{Vegf}, there has been no report on the relationships between statins and \text{Api5}, \text{Atf5}, \text{Dad1}, and \text{Prdx2} with or without the \text{eNOS} system. Although there is a possibility that those anti-apoptotic factors synergistically cooperate to prolong cellular survival, further examinations are needed to clarify this issue in order to understand the detailed anti-apoptotic effects of pitavastatin.

In the present study, we identified AMPK as a common molecule for attenuation of both atherosclerosis and ischemia-induced apoptosis in \(\text{eNOS}^{-/-}\) mice. Recent findings have demonstrated that AMPK is present in the endothelium and maintains endothelial homeostasis in response to various stimuli, including pro-inflammatory cytokines and oxidative damage\(^{38, 39}\). For more details, Zhang et al. demonstrated that AMPK reduces the endothelial inflammatory response leading to attenuation of the development of atherosclerosis by blocking p300-HAT-dependent NF-\(\kappa\)B acetylation\(^{40}\). In addition, Wang et al. demonstrated that cardiomyocyte-specific AMPK deficiency significantly enhanced post-ischemic myocardial apoptosis, increased infarct size, and worsened cardiac functional recovery after myocardial ischemia/reperfusion injury\(^{41}\). Therefore, activation of AMPK may hold great potential in preserving cell viability and protecting tissues from fatal ischemia. The results of the present study showing enhancement of AMPK by pitavastatin under a condition of serious cardiovascular stress such as \text{eNOS} deficiency are consistent with the results of previous studies showing that statins exert protective effects on various tissues, including cardiac myocytes and skeletal muscle, from harmful stress via activation of AMPK\(^{42, 43}\).

The Rho-associated coiled-coil-containing kinases (ROCKs) are members of the serine/threonine
protein kinase family and are downstream targets of the small GTP-binding protein Ras homolog gene family, member A (RhoA). There is growing evidence that activation of ROCKs contributes to the development of CVDs. Therefore, regulation of the RhoA/ROCKs pathway plays a pivotal role in the prevention of CVDs. In addition, Rac1-GTPase is a major regulator of the generation of reactive oxygen species through activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity. The suppression of Rac1-GTPase activity has thus been thought to be a therapeutic target for CVDs. Kobayashi et al. showed that phosphorylation of RhoA and expression levels of RhoA and ROCKs in failing hearts of male Dahl salt-resistant rats were attenuated by pitavastatin treatment. Budzyn et al. demonstrated that mevastatin causes eNOS-independent attenuation of vascular contraction through the suppression of RhoA and Rho kinase. Moreover, we previously reported that pitavastatin ameliorates atrial remodeling along with attenuation of cardiac Rac1-GTPase activity regardless of the presence or absence of the eNOS system. Taken together, the results indicate the possibility that those effects of pitavastatin contribute to protection from atherogenesis and peripheral ischemia in a condition with severe endothelial dysfunction such as eNOS deficiency.

Therefore, the pleiotropic effects of pitavastatin via synergistic regulation of common and diverse molecules should be able to counteract various cardiovascular stresses in patients with severe endothelial dysfunction.

In conclusion, in addition to the known pleiotropic effects of statins including inactivation of RhoA/ROCKs and Rac1-GTPase, the present study provides novel findings between pharmacological effects of pitavastatin and atherogenesis or ischemia-induced apoptosis. Since we did not identify the cell and molecule specific effects of pitavastatin, further examinations are needed to clarify the detailed molecular mechanisms.

Disclosures

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49) Budzyn K, Marley PD, Sobey CG: Chronic mevastatin modulates receptor-dependent vascular contraction in eNOS-deficient mice. Am J Physiol Regul Integr Comp Physiol, 2004; 287: R342-8
Supplemental Table 1.

<table>
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<th>Gene Symbol</th>
<th>Description</th>
<th>Forward</th>
<th>Reverse</th>
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Supplemental Fig. 1. The heat map of PCR array for atherosclerosis with dendrograms indicating co-regulated genes across groups or individual samples.
**Supplemental Fig. 2.** The heat map of PCR array for apoptosis with dendrograms indicating co-regulated genes across groups or individual samples.