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Original Article

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

Introduction

Since endothelial function has been shown to be inversely associated with future cardiovascular events[1, 2], amelioration 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[3]. 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[4, 5]. 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[6, 7].

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[6]. It has been demonstrated that statins activate the phosphatidylinositol 3-kinase/serine/threonine-specific protein kinase (Akt) pathway[8] and enhance the expression and activity of eNOS[9, 10]. In addition, various cardiovascular protective effects of statins are abolished in *eNOS*-deficient mice[11]. 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[12, 13]. 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.)[12, 13] 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[12, 13]. 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℃. BP and heart rate were measured on two consecutive days, and at least 10 readings were taken for each measurement, as previously described[12, 14].

3. Measurement of Plasma Lipids and Glucose

After administration of pitavastatin for 4 weeks in *eNOS*$^{-/-}$ *ApoE*$^{-/-}$ mice (Study 1) or for 2 weeks in *eNOS*$^{-/-}$ *ApoE*$^{+/+}$ 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℃ 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 Antioxidant Potential Assay

After administration of pitavastatin for 4 weeks in *eNOS*$^{-/-}$ *ApoE*$^{-/-}$ mice (Study 1) or for 2 weeks in *eNOS*$^{-/-}$ *ApoE*$^{+/+}$ 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)[15, 16]. 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 Carratelli 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[17]. 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[18]. 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*$^{-/-}$ *ApoE*$^{-/-}$ mice (Study 1) and from the ischemic thigh muscles in *eNOS*$^{-/-}$ *ApoE*$^{+/+}$ mice on day 1 after surgery (Study 2) and subsequent cDNA synthesis were performed as previously described[18, 19]. 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^2 Profiler PCR Arrays PAMM-038Z (QIAGEN), Apoptosis RT^2 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® (TAKARA BIO INC. Ohtsu, Japan) for *Hif1a*, *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 $\Delta\Delta C_t$-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[18, 19]. 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[20]. 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™-P, 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℃ 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), p38MAPK, phosphorylated p38MAPK (Thr180/Tyr182), Akt, phosphorylated Akt (Ser473), AMPK α , 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 pitavasttain 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

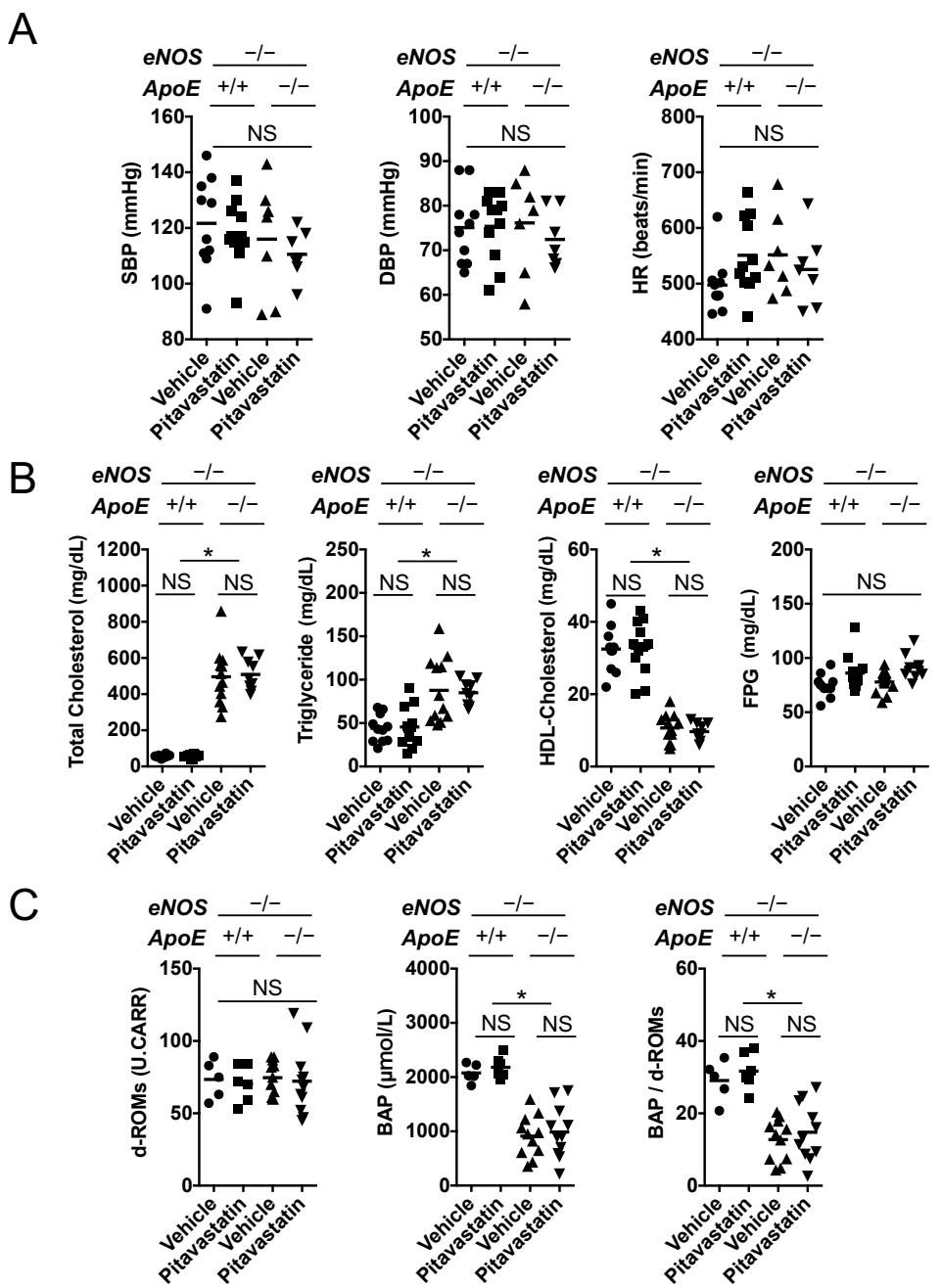

Fig. 1. Blood pressure, heart rate, plasma lipids, plasma glucose, plasma reactive oxygen metabolites and biological anti-oxidant potential in *eNOS*$^{-/-}$ *ApoE*$^{+/+}$ and *eNOS*$^{-/-}$ *ApoE*$^{-/-}$ mice

(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*$^{-/-}$ *ApoE*$^{+/+}$ mice group Vehicle: $n=10$, Pitavastatin: $n=11$, *eNOS*$^{-/-}$ *ApoE*$^{-/-}$ 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*$^{-/-}$ *ApoE*$^{+/+}$ mice ($n=11$) vs vehicle-treated *eNOS*$^{-/-}$ *ApoE*$^{-/-}$ mice ($n=12$) or pitavastatin-treated *eNOS*$^{-/-}$ *ApoE*$^{-/-}$ mice ($n=9$), pitavastatin-treated *eNOS*$^{-/-}$ *ApoE*$^{+/+}$ mice ($n=12$) vs vehicle-treated *eNOS*$^{-/-}$ *ApoE*$^{-/-}$ mice or pitavastatin-treated *eNOS*$^{-/-}$ *ApoE*$^{-/-}$ 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*$^{-/-}$ *ApoE*$^{+/+}$ mice ($n=5$) vs vehicle-treated *eNOS*$^{-/-}$ *ApoE*$^{-/-}$ mice ($n=11$) or pitavastatin-treated *eNOS*$^{-/-}$ *ApoE*$^{-/-}$ mice ($n=11$), pitavastatin-treated *eNOS*$^{-/-}$ *ApoE*$^{+/+}$ mice ($n=6$) vs vehicle-treated *eNOS*$^{-/-}$ *ApoE*$^{-/-}$ mice or pitavastatin-treated *eNOS*$^{-/-}$ *ApoE*$^{-/-}$ mice).

eNOS$^{-/-}$*ApoE*$^{-/-}$ mice. As is shown in **Fig. 2B**, the number of F4/80-positive cells in atherosclerotic plaque was markedly reduced in pitavastatin-treated *eNOS*$^{-/-}$*ApoE*$^{-/-}$ mice (**Fig. 2B**).

2.3 Pitavastatin Reduces Gene Expression Levels of Proatherogenic Factors in eNOS$^{-/-}$*ApoE*$^{-/-}$ *Mice*

In order to determine the atherogeneseis-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*$^{-/-}$*ApoE*$^{-/-}$ mice

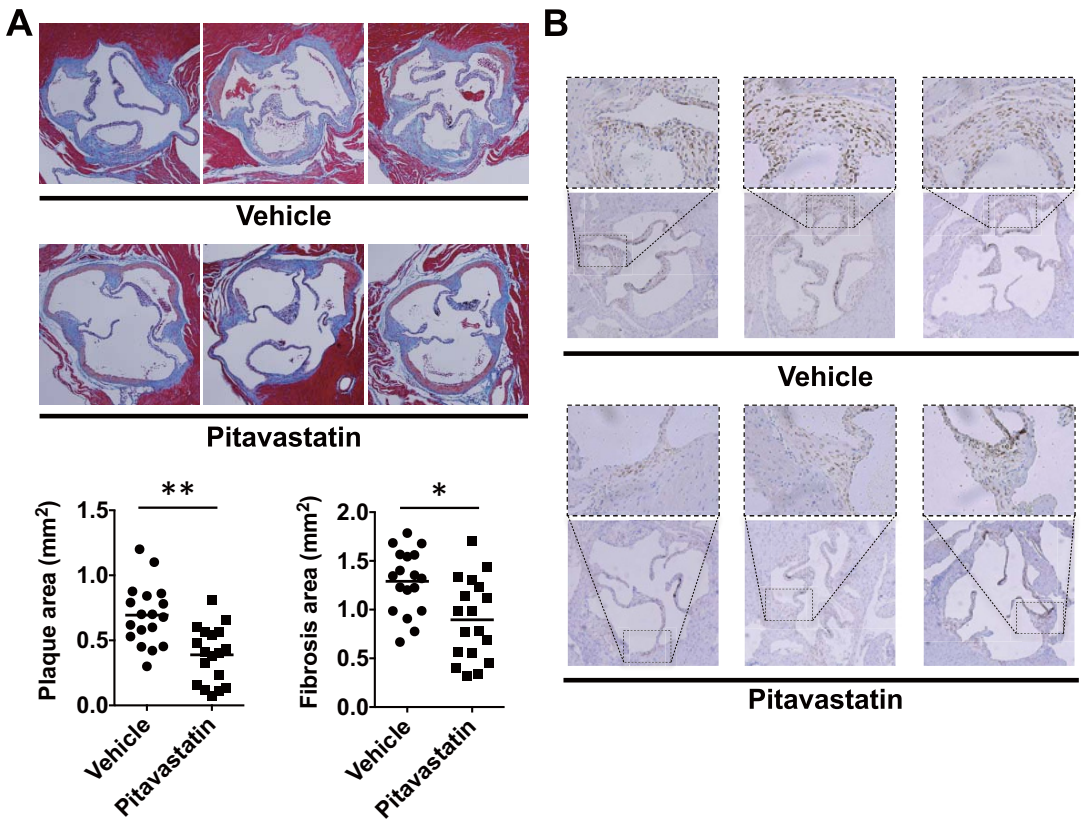

Fig. 2. Attenuation of atherosclerosis, medial fibrosis and macrophage infiltration in the aortic root of *eNOS*$^{-/-}$ *ApoE*$^{-/-}$ mice

(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 Pitavasatatin-Induced Anti-atherogenesis against Dyslipidemia in *eNOS*$^{-/-}$ Mice

Gene Symbol	Description	Fold Change (Pitavastatin/Vehicle)	95% CI	*p*-value
Ccl2	Chemokine (C-C motif) ligand 2	0.19	(0.03, 0.35)	0.006431
Ccr2	Chemokine (C-C motif) receptor 2	0.35	(0.07, 0.63)	0.024167
Cflar	CASP8 and FADD-like apoptosis regulator	0.41	(0.14, 0.68)	0.033197
Col3a1	Collagen, type Ⅲ, alpha 1	0.23	(0.05, 0.41)	0.000308
Nfkb1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1, p105	0.27	(0.04, 0.50)	0.018265
Pdgfa	Platelet-derived growth factor, alpha	0.29	(0.06, 0.52)	0.022822
Serpinb2	Serine (or cysteine) peptidase inhibitor, clade B, member 2	0.07	(0.03, 0.97)	0.028673

(**Table 1**, **Fig. 2B**).

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[21]. 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 immunosuppression, 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[22, 23], 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 arteriectomy 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 (**Fig. 4B**) 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, *Api5*, *Atf5*, *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[24, 25], 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[26], 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[27-29]. In addition, Momi *et al.* showed that an NO-donating property confers enhanced anti-

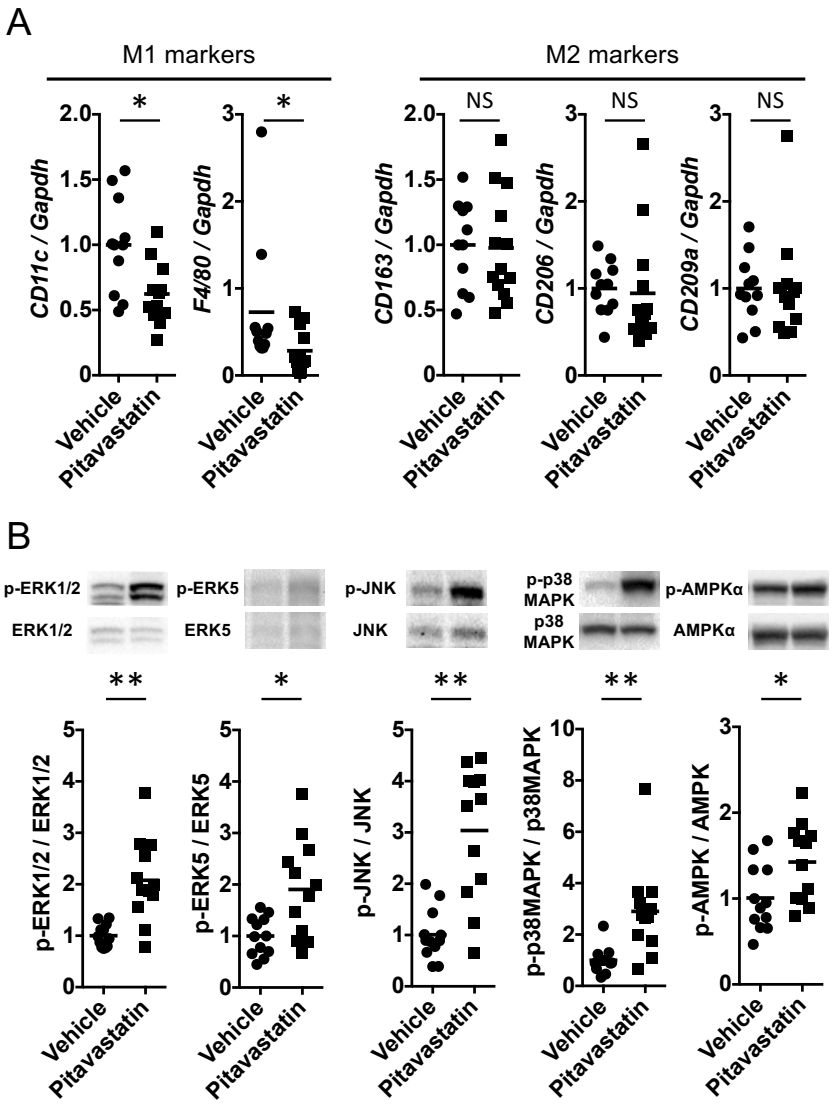

Fig. 3. Expression and activation of atherogenesis-associated factors in aortas of *eNOS*$^{-/-}$ *ApoE*$^{-/-}$ mice with or without pitavastatin treatment

(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. $^{*}P<0.05$, $^{**}P<0.01$.

atherogenic and anti-inflammatory effects of a statin against the development of atherosclerosis[30]. 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[12, 13]. However, interestingly, the present results proved that

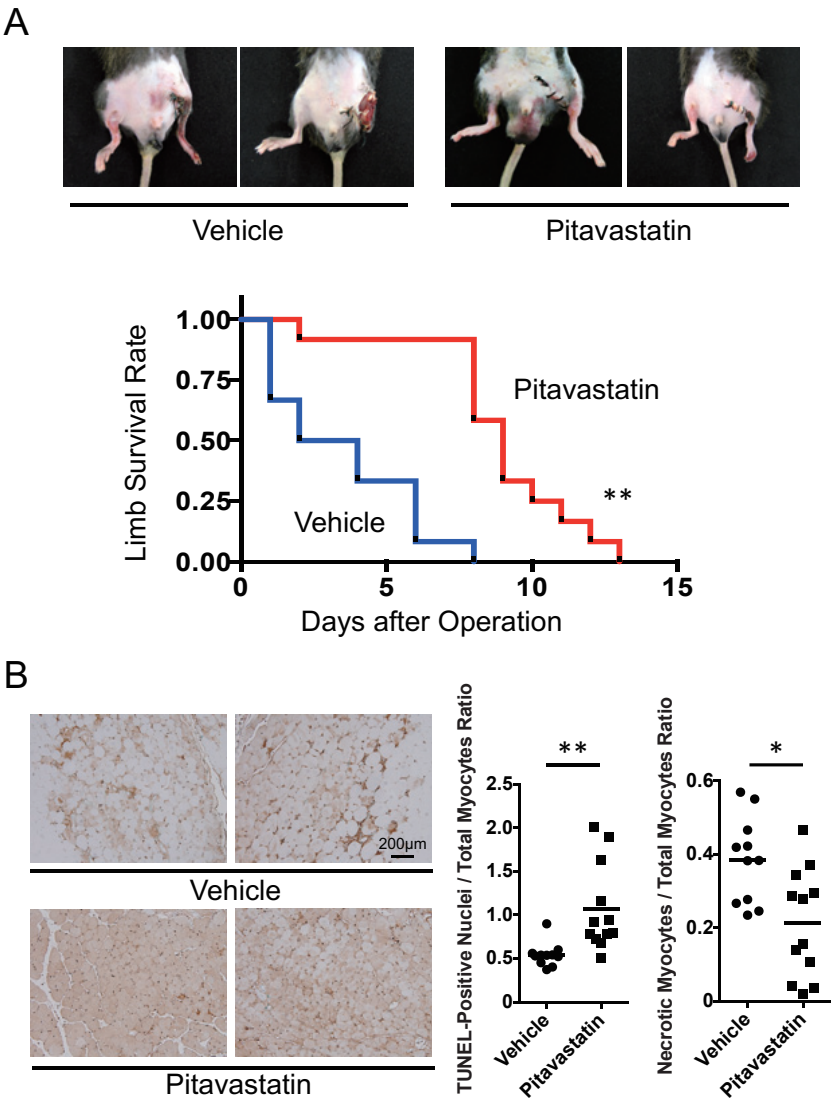

Fig. 4. Prolonged hind-limb survival by pitavastatin after femoral arteriectomy in *eNOS*$^{-/-}$ mice

(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 Pitavasatatin-Induced Anti-apoptosis against Limb Ischamia in *eNOS*$^{-/-}$ Mice

Gene Symbol	Description	Fold Change (Pitavastatin/Vehicle)	95% CI	*p*-value
Api5	Apoptosis inhibitor 5	1.46	(1.19, 1.73)	0.003328
Atf5	Activating transcription factor 5	2.03	(1.79, 2.27)	0.000002
Bcl2l1	Bcl2-like 1	2.67	(1.98, 3.36)	0.000021
Bcl2l2	Bcl2-like 2	2.04	(1.53, 2.55)	0.000544
Dad1	Defender against cell death 1	1.52	(1.35, 1.69)	0.000022
Prdx2	Peroxiredoxin 2	1.84	(1.60, 2.08)	0.000002

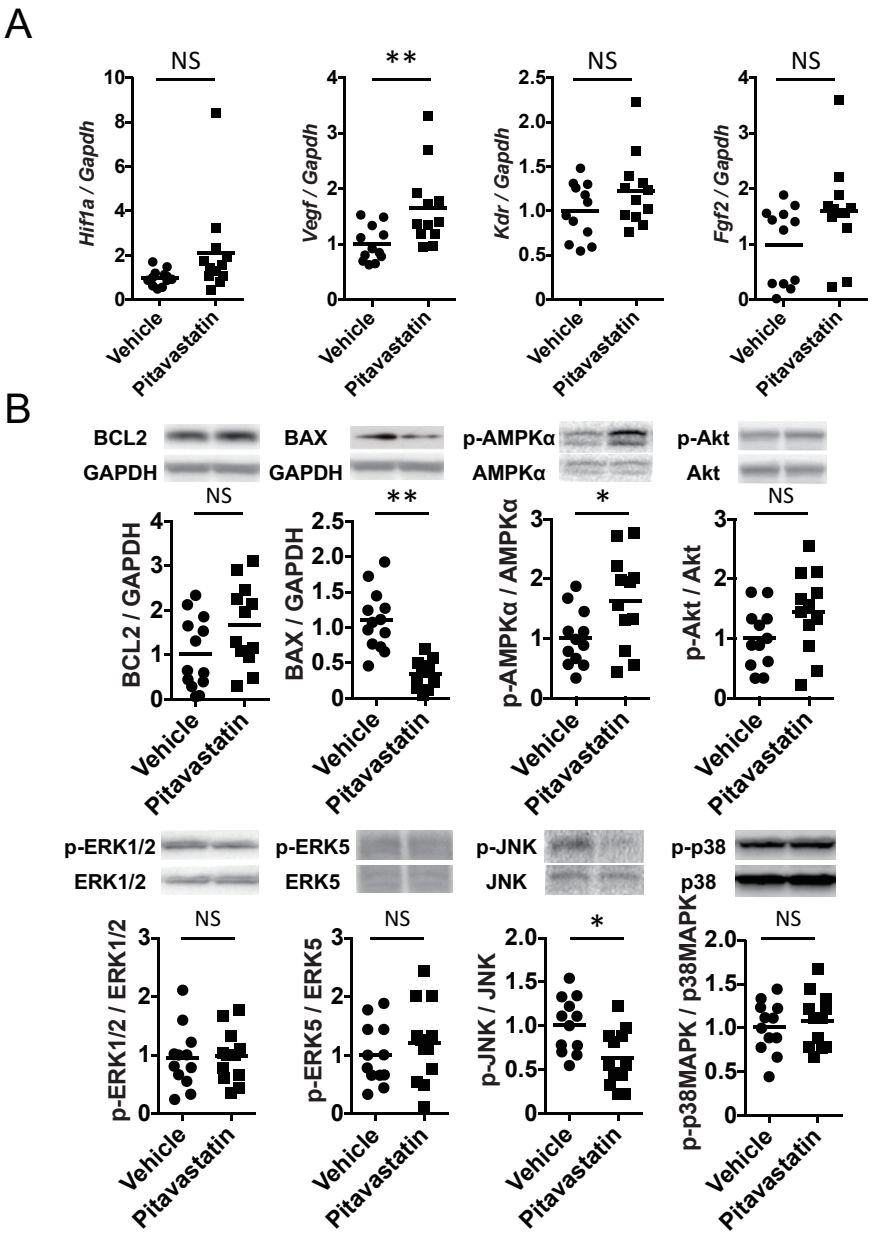

Fig. 5. Expression and activation of apoptosis and angiogenesis-associated factors in ischemic hind-limbs of *eNOS*$^{-/-}$ mice with or without pitavastatin treatment

(A) Evaluation of angiogenesis-associated gene expression in hind-limbs of vehicle or pitavastatin-treated *eNOS*$^{-/-}$ mice. Bars represent mean values in each group. $N=12$ in each group. NS: not significant. $^{**}P<0.01$.

(B) Protein expression of BCL2 and BAX and BCL2-to-BAX ratio and phosphorylation of ERK1/2, ERK5, JNK, p38MAPK and AMPK in ischemic thigh muscle of *eNOS*$^{-/-}$ 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. NS: not significant. $^{*}P<0.05$, $^{**}P<0.01$.

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*$^{-/-}$ *MCP-1*(*Ccl2*)$^{-/-}$ mice had 83% less lipid deposition throughout their aortas compared to *LDL receptor*$^{-/-}$ *MCP-1* (*Ccl2*)$^{+/+}$ mice[31], pitavastatin-induced reduction of the gene expression levels of both *Ccl2* and its receptor, *Ccr2* axis, might be one of the major causes of suppressed atherogenesis in *eNOS*$^{-/-}$ *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], and Kim *et al.* demonstrated that laminar flow-induced activation of the ERK5-Nrf2 signal pathway plays a critical role in the anti-inflammatory and anti-apoptotic mechanism in endothelial cells[35]. From these results, the pitavastatin-induced ERK5 activation in *eNOS*$^{-/-}$ *ApoE*$^{-/-}$ mice in the present study may have potency to exert an anti-vascular inflammatory effect leading to suppression of atherosclerotic lesion formation via an eNOS-independent manner. In contrast, although several studies showed that statins attenuate phosphorylation of p38-MAPK and JNK in *ApoE*$^{-/-}$ mice, our results are not consistent with those studies. Although we are not able to provide a plausible explanation for the discrepancy, there is a possibility that the difference between the diet in our study (normal diet chow) and that in previous studies (western diet chow) reflects the phosphorylation of MAPKs. Further studies are needed to clarify this issue.

Several genes of pro-atherogenic factors, including *Pdgfa*, *Serpinb2*, *Nfkb1*, *Cflar*, and *Col3a*, 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 eNOS system, our results showed for the first time that pitavastatin can reduce the expression of *Pdgfa*, *Serpinb2*, *Nfkb1*, *Cflar*, and *Col3a*, in atherogenesis regardless of the presence or absence of the 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[36]. 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 *eNOS*$^{-/-}$ mice, and therefore inactivated JNK by pitavastatin treatment may promote cellular survival after critical ischemia regardless of eNOS expression.

PCR array analysis and real-time PCR analysis demonstrated that pitavastatin up-regulates the expression of several anti-apoptotic genes, including *Api5*, *Atf5*, *Dad1*, *Prdx2*, and *Vegf*, after ischemia in *eNOS*$^{-/-}$ mice. Except for *Vegf*, there has been no report on the relationships between statins and *Api5*, *Atf5*, *Dad1*, and *Prdx2* with or without the 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 atherogenesis and ischemia-induced apoptosis in *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-κB 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 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[44, 45]. 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[46, 47]. 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[48]. Budzyn *et al*. demonstrated that mevastatin causes eNOS-independent attenuation of vascular contraction through the suppression of RhoA and Rho kinase[49]. 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[13]. 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 pitavastain 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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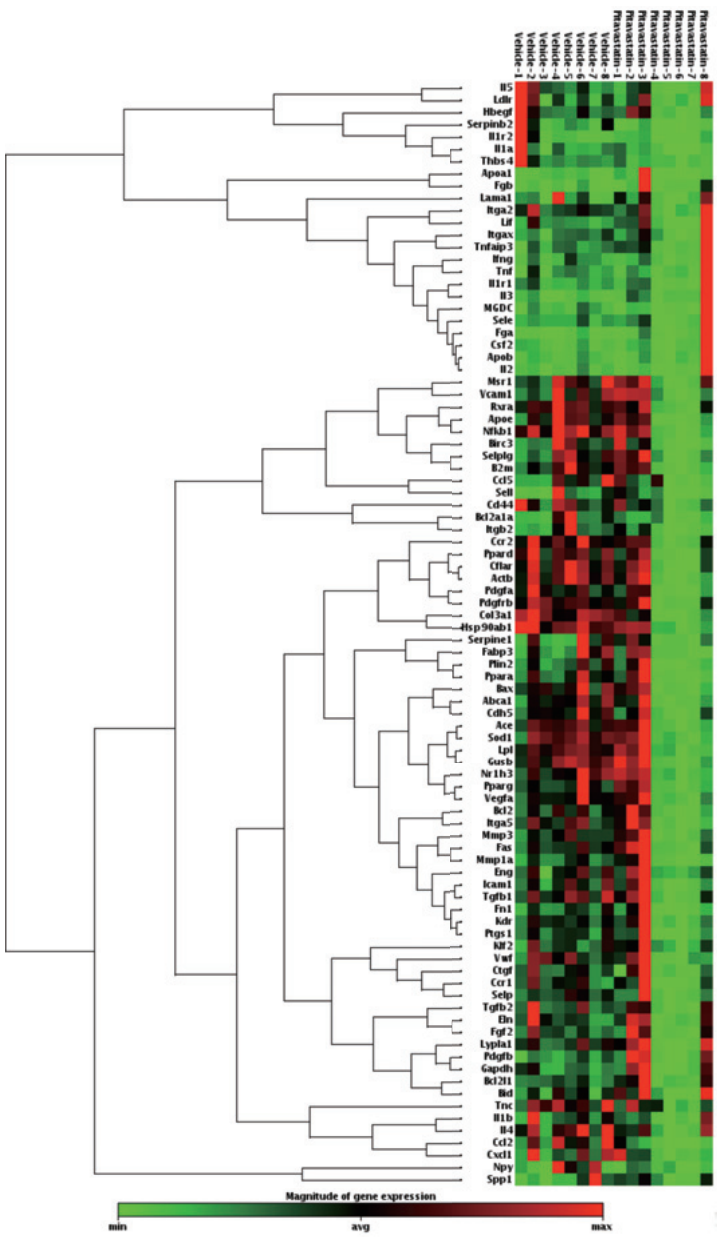
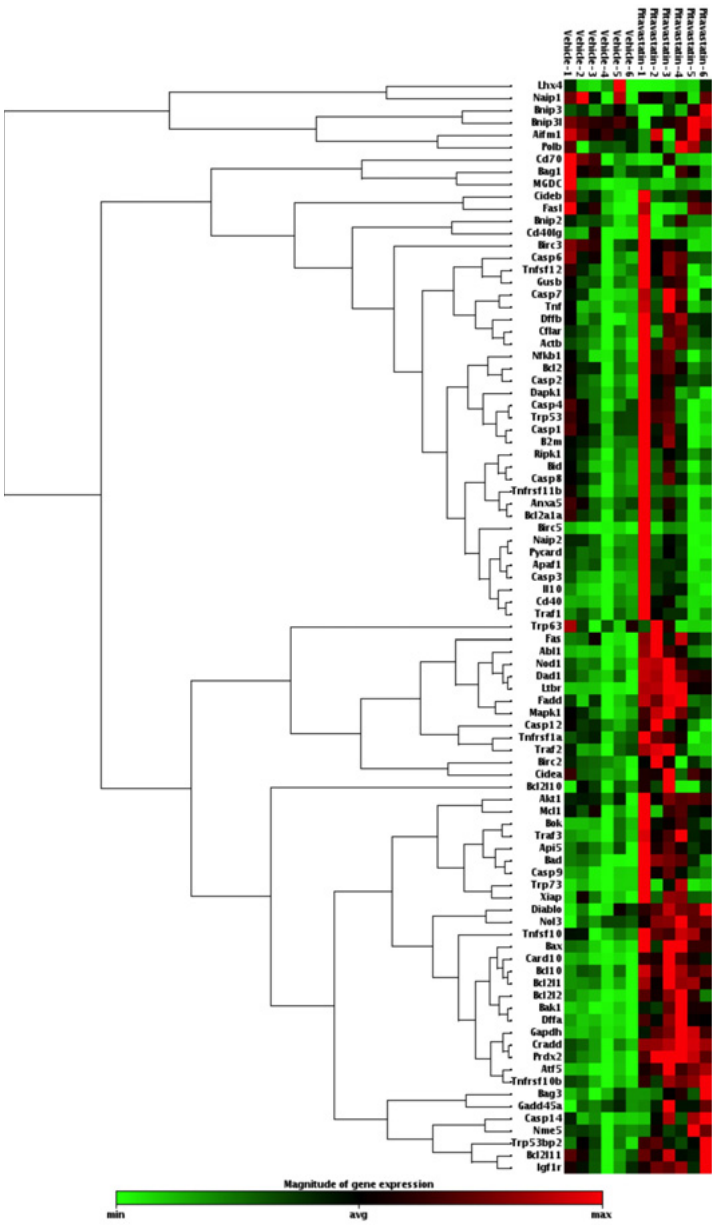
Supplemental Table 1.

Gene Symbol	Description	Forward	Reverse
CD11c	Integrin, alpha X (complement component 3 receptor 4 subunit)	atttctgagacccagacga	ccatttgcttcctccaacat
F4/80	EGF-like module-containing mucin-like hormone receptor-like 1	tgcatctagcaatggacagc	gccttctggatccatttgaa
CD163	Cluster of Differentiation 163 antigen	tggtgtgcagggaattacaa	atccctgctgtgggtacaag
CD206	mannose receptor, C type 1	caaggaaggttggcatttgt	cctttcagtcctttgcaagc
CD209a	Cluster of Differentiation 209a antigen	tgctggttgtcatccttgtc	gatctacgccagccttcaac



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