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The Novel HMG-CoA Reductase Inhibitor, Pitavastatin, Induces a Protective Action in Vascular Endothelial Cells through the Production of Nitric Oxide (NO)

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(Received August 25, 2003; Accepted December 24, 2003)

This study sought to induce the effect of nitric oxide (NO) production in vascular endothelial cells by Pitavastatin, which is a novel HMG-CoA reductase inhibitor (statin). The growth capacity of vascular endothelial cells significantly ($p<0.01$) declined when stimulated with TNF-α (10 ng/ml). The growth capacity of the TNF-α treated cells recovered, when the TNF-α stimulation was performed after Pitavastatin (100 nM) pretreatment. The recovery of the growth capacity of the cells was suppressed by the presence of the NO synthase inhibitor, L-NAME. Pitavastatin increased NO production by the vascular endothelial cells in a dose and time dependent manner. The NO production was suppressed by the presence of mevalonic acid and geranylgeranyl pyrophosphate. In addition, the expression of endothelial nitric oxide synthase was strongly induced by Pitavastatin, and was suppressed by mevalonic acid and geranylgeranyl pyrophosphate by Western blot analysis. Our results show that Pitavastatin induces NO production by vascular endothelial cells, and protects vascular endothelial cells from injury due to the inflammatory reaction induced by TNF-α.

Key words——nitric oxide (NO); vascular endothelial cell; Pitavastatin

INTRODUCTION

Arteriosclerotic disease, such as myocardial infarction and cerebral apoplexy, occupies the epistasis of the cause of death within the Japanese population due to eating habits and in Europe and America due to life style. Arteriosclerotic disease may also appear in cases of hyperlipidemia, diabetes mellitus, and hypertension. The common mechanism by which arteriosclerosis develops from these diseases has been indicated to be the inflammatory reaction of blood vessels. Therefore, in drug therapy for the hyperlipidemia, drug development with vasculitis disease reaction inhibition has been required in addition to the improving the cholesterol level.

During the 1990's, large-scale clinical trials were conducted with the aim of evidence based medicine concerning hypocholesterolemic activity and ischemic heart disease crisis control by HMG-CoA reductase inhibitor (statin). These studies include the Scandinavian Simvastatin Survival Study (4S)[1] and the West of Scotland Coronary Prevention Study (WOSCOPS).[2] From the results of these studies, it became clear that the cardiovascular disease event crisis rate may be decreased by lowering the LDL-cholesterol. Especially from the result of WOSCOPS, by confirming a coronary artery event depression effect of statin (pravastatin) from an early stage (6 months),[2] the possibility that statin acts directly in the blood vessels and that it suppresses the vasculitis disease was proposed.

Recently, evidence has suggested a collected pleiotropic effect, which is a diverse effect of statin. The improvement of endothelial function, plaque stabilizing activity, effects on the blood coagulation fibrinolytic system,[3] an antioxidative effect, and an anti-inflammatory action have been reported. In addition, the following effects were also noticed: antiproliferative activity of smooth muscle cells,[4] promotion of the apoptosis of smooth muscle cells,[5] apoptosis control of vascular endothelial cells,[6] production control of free radical,[7] an inhibitory effect of diabetic vascular complication,[8] and an improvement in osteoporosis. Further, a nitric oxide (NO) production quantity increase in the vascular endothelial cell was reported as one of the pleiotropic effect of statin.[9]

In this study, we found that the effect of a novel statin, Pitavastatin,[10] induces a protective action in the vascular endothelial cell through the production of NO.

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MATERIAL AND METHOD

Materials Pitavastatin (trade name: LIVALO®, code name: NK-104), was kindly provided by Kowa Co., Ltd. (Nagoya, Japan) and Nissan Chemical Industries, Ltd. (Tokyo, Japan). Recombinant tumor necrosis factor-α (TNF-α) was purchased from CALBIOCHEM (Darmstadt, Germany). Mevalonic acid and geranylgeranyl pyrophosphate were purchased from SIGMA (St. Louis, MO, USA). N^G-nitro-L-arginine methyl ester hydrochloride (L-NAME) and dimethyl sulfoxide (DMSO) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Diaminofluoroscein-2 (DAF-2) was purchased from Daiichi Pure Chemicals Co., Ltd. (Tokyo, Japan). Krebs-Ringer phosphate buffer (KRP buffer) consists of NaCl (120 mM), KCl (4.8 mM), $CaCl_2$ (0.54 mM), $MgSO_4$ (1.2 mM), glucose (11 mM), and sodium phosphate (15.9 mM) pH 7.2.

Cell Culture Human Umbilical Vein Endothelial Cells (HUVEC) were purchased from Takara Bio Inc. (Otsu, Japan). The cells were cultured in endothelial cell basal medium-2 (EBM®-2) with 2% fetal bovine serum (FBS), 0.4% human fibroblast growth factor-B (hFGF-B), 0.1% human epidermal cells growth factor (hEGF), 0.1% heparin, 0.1% ascorbic acid, 0.1% vascular endothelial cells growth factor (VEGF), 0.1% R3-insulin-like growth factor (R3-IGF), 0.1% gentamycin and 0.1% amphotericin-B. The cultures were incubated in 5% CO_2 /95% air at 37°C and passaged twice a week by using trypsin EDTA. The 3-6 subculture cells were used for the experiment.

MTT Cellular Proliferation Assay and Cell Viability We used the 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cellular proliferation assay[11] to investigate the viability of HUVEC. We have previously described the methods for the MTT assay.[12] In brief, cells were seeded onto 96-well flat-bottomed culture plates. The cells were incubated with Pitavastatin (10 nM—1 mM) and/or TNF-α (0.1—10 ng/ml), L-NAME (10 mM) at 37°C for 48 hours. Ten mls of filtered MTT (Sigma) in PBS was added to a final concentration of 5 μg/μl. Cells treated with the reagents were incubated at 37°C for 4 hours. One hundred μl of acidic isopropanol was added and the plates were shaken at room temperature for 20 minutes. Solubilized formazan was quantified and the absorbance ratio (570 nm) was determined.

Determination of NO from HUVEC Diaminofluoresceins (DAFs) are fluorescent NO indicators. The reaction of NO and DAFs yields the corresponding bright green-fluorescent triazolofluoresceins.[13] Using these NO reactive fluorescent indiators, it is feasible to detect the generation of NO from HUVEC by means of a simple protocol.

Determination of NO was performed as described by Nakatsubo, N. *et al.*[14] Briefly, the HUVEC were seeded onto 96-well flat-bottomed culture plates, and were cultured for 6 hours to make the HUVEC adherent. Subsequently, the cells were cultured for 6 hours in 200 μl of fresh medium containing Pitavastatin (10 nM—1 μM) alone, or Pitavastatin (100 nM) and either mevalonate (1 mM), geranylgeranyl pyrophosphate (10 μM), or L-NAME (10 mM) at 37°C for 6 hours. The adherent cells were washed twice with KRP buffer, and then DAF-2 (10 μM), L-arginine (1 mM), and L-NAME (10 mM) dissolved in 200 μl of KRP buffer was added. After incubation for a further 2 hours, the supernatants were transferred to black microplates and the fluorescence was measured with a fluorescence microplate reader calibrated for excitation at 485 nm and emission at 538 nm.

Western Blot Cellular proteins were prepared and separated by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE) on an 8.5% acrylamide gel and transferred to PVDF membranes. Immunoblotting was performed with a rabbit anti-nitric oxide synthase III polyclonal antibody (1 : 200 dilution, CHEMICON, Temecula, CA, USA). Immunodetection was accomplished with an anti-rabbit IgG, horseradish peroxidase linked whole antibody (1 : 5000 dilution, Amersham Biosciences Corp., Piscataway, NJ, USA) and immunoreactive protein were visualized using an ECL kit (Amersham Pharmacis Biotech, Inc., Piscataway, NJ, USA)..

RESULT

1. Pitavastatin Protects Endothelial Cells Injury by TNF-α The effect of Pitavastatin on endothelial cell proliferation was examined by the MTT assay. The HUVEC were cultured in medium containing Pitavastatin (10 nM—1 mM) for 2 days. The growth capacity of the HUVEC declined in TNF-α administration in a concentration dependent manner (Fig. 1). TNF-α (10 ng/ml) stimulation significantly depressed the growth capacity of the cells ($p<0.01$).

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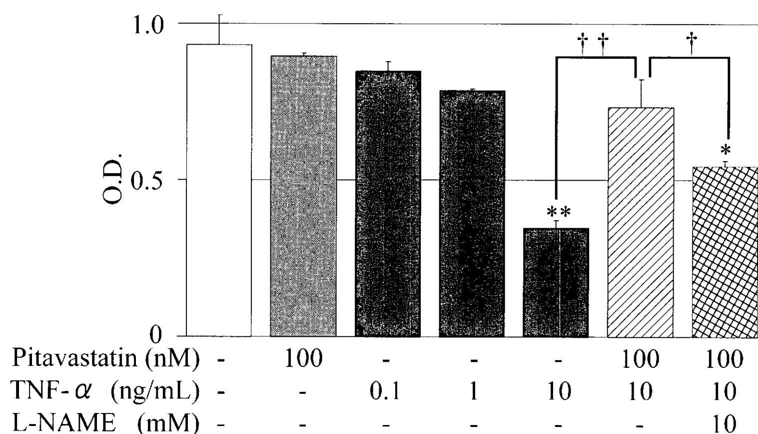

Fig. 1. Pitavastatin Protects HUVEC Injury by TNF-α

The cells were incubated with Pitavastatin (100 nM) and/or TNF-α (0.1—10 ng/ml), L-NAME (10 mM). MTT assay was performed for 48 hr. The data represents mean±SD ($n=3$) $^{*}p<0.05$, $^{**}p<0.01$ vs control. $^{\dagger}p<0.05$, $^{\dagger\dagger}p<0.01$ vs pitavastatin and TNF-α treated.

In the cultures treated with 1 μM Pitavastatin, the growth capacity of the cells was depressed significantly ($p<0.05$, data not shown). Pitavastatin (100 nM) did not show the growth capacity depression when HUVEC were pretreated for 6 hours with Pitavastatin, followed by TNF-α (10 ng/ml) ($p<0.01$). This recovery in the presence of Pitavastatin was suppressed by the presence of the NO synthase inhibitor, L-NAME (Fig. 1).

2. Pitavastatin Induces NO Production of HUVEC In HUVEC treated with Pitavastatin for 6 hours, an increase in NO in culture supernatants to 100 nM in a concentration dependent manner was detected using DAF-2. At over 100 nM, NO production decreased (Fig. 2A). The NO production increase depended on the time course of treatment. The NO production by Pitavastain (100 nM) was significant when the HUVEC were treated for over 6 hours (Fig. 2B). Mevalonic acid and geranylgeranyl pyrophosphate which inhibit statin formation in the mevalonate pathway were administered in order to clarify the specificity of the effect by Pitavastain. NO production by Pitavastain was significantly suppressed by both agents ($p<0.01$). Moreover, NO production by Pitavastain was significantly suppressed by L-NAME ($p<0.05$) (Fig. 2C).

3. Pitavastatin Induces Endothelial NO Synthase The expression of endothelial NO synthase (eNOS, NOS III) was examined by Western blot analysis. Pitavastatin (100 nM—10 μM) strongly induced the expression of eNOS (Fig. 3A). The induced expression of eNOS was suppressed by the presence of mevalonic acid or geranylgeranyl pyrophosphate (Fig. 3B). Mevalonic acid treatment almost completely suppressed expression, while in the geranylgeranyl pyrophosphate treatment there was a depression effect of approximately 70%. And, when it was stimulated by TNF-α, eNOS expression bit have. However, eNOS expression was recovered by applying Pitavastatin (Fig. 3C).

DISCUSSION

By treating vascular endothelial cells with statin, an increase in NO concentration in the culture solution, in eNOS gene expression, and in eNOS activity was detected.[9] NO of the eNOS derivation has a vascular protection, a vasorelaxation action and an anti-thrombus action, all of which normalizes the vascular function. However, the vascular endothelial cells are injured by diseases such as hyperlipidemia, diabetes mellitus, and hypertension. As a result, the endothelial cell function is affected, and the NO production quantity also decreases. In this study, we demonstrated increases in NO production and in the expression of eNOS by the novel HMG-CoA reductase inhibitor, Pitavastatin.

Of the pleiotropic effect of statin, the effects seem to depend on the isoprenoid production control, which exists in the mevalonate pathway. Statin inhibits the generation of mevalonic acid from HMG-CoA in the mevalonate pathway, as shown in Fig. 4. Simultaneously, the generation of isoprenoid such as farnesyl pyrophosphate and geranylgeranyl pyrophosphate which exists downstream of the mevalonate pathway, is suppressed. These isoprenoids make the protein combine to lipid, which are

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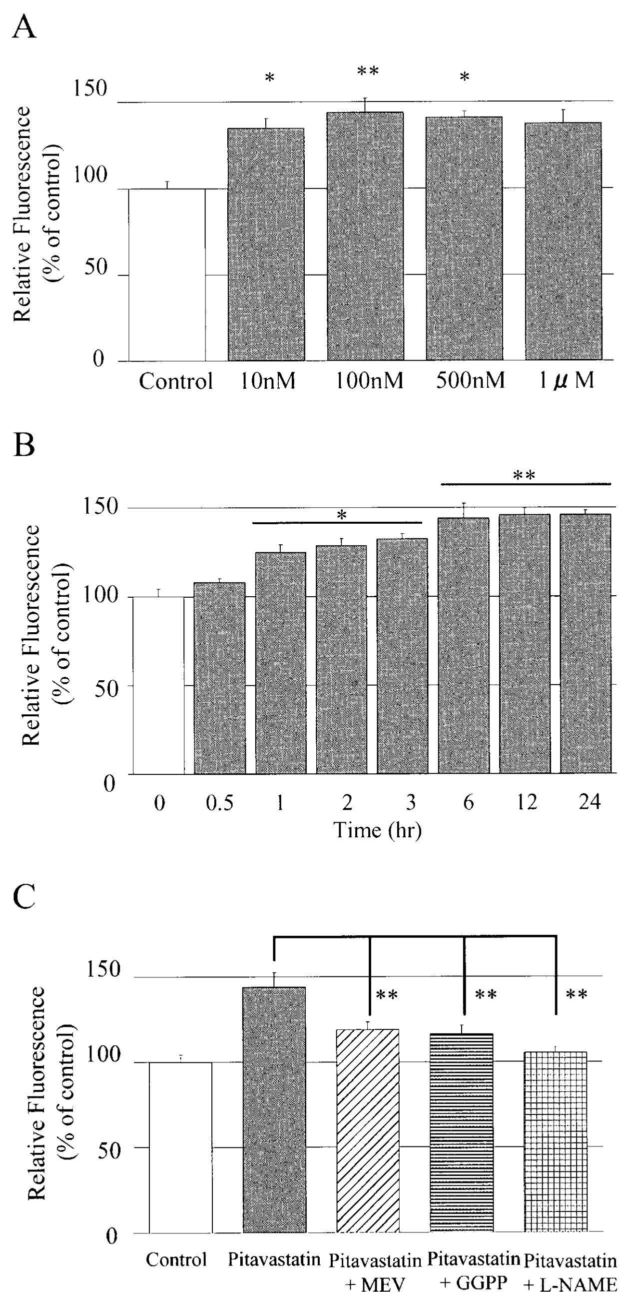

Fig. 2. Piavastatin Induces NO Production

(A) The cells were incubated with Pitavastatin (10 nM—1 μM). The DAF-2 assay was performed for 6 hr. The data represents mean ± SD ($n=3$) *$p<0.05$, **$p<0.01$ vs control. (B) The cells were incubated with Pitavastatin (100 nM). The DAF-2 assay was performed for 0—24 hr. The bar graph is a value of NO production quantity after the Pitavastatin administration. The data represents mean ± SD ($n=3$) *$p<0.05$, **$p<0.01$ vs control. (C) The cells were incubated with Pitavastatin (100 nM) alone, or Pitavastatin (100 nM) and either mevalonic acid (MEV: 1 mM), geranylgeranyl pyrophosphate (GGPP: 10 μM), or L-NAME (10 mM). The DAF-2 assay was performed for 6 hr. The data represents mean ± SD ($n=3$) **$p<0.01$ vs Pitavastatin treated.

farnesyl or geranylgeranyl units, by what is called isoprenylation, the following are being adjusted intracellular localization and functional expression of the small G protein, Rho or Ras.[15] In the pleiotropic effect of statin, the mechanism may act through such isoprenylation. We demonstrated that the expression of eNOS by Pitavastatin was suppressed by the presence of mevalonic acid. That is to say, mevalonic acid is produced from HMG-CoA, and this pathway is blocked by statin. In the meantime, taking the partial expression of eNOS as a control, the expression of eNOS persisted in the presence of geranylgeranyl pyrophosphate (Fig. 3(B)). This persistence may reflect mevalonic acid metabolism under farnesyl pyrophosphate.

Recently, TNF-α was reported to suppress the expression of eNOS in vascular endothelial cells[16] and to induce the expression of iNOS.[17,18] Celivastatin, known as "Vascular Statin", can generate a recovery from cell injury induced by TNF-α, similar to results obtained using Pitavastatin (data not shown). Therefore, the cytotoxicity of TNF-α may be reduced by the eNOS activation increase induced by statins. It is known that Atorvastatin counters the induction of iNOS by TNF-α, while Pitavastatin does not.[19] In addition, other statins (Lovastatin, Fluvasatatin and Pravastatin) suppress the expression of iNOS.[20] Pitavastatin induces eNOS expression and TNF-α suppresses it in HUVEC (Fig. 3). Then, Pitavastatin was used to recover the eNOS expression suppressed by TNF-α. This is similar to the result of the MTT assay (Fig. 1). These results suggest that Pitavastatin induces eNOS expression that was suppressed by TNF-α, and protects the cell from injury by TNF-α. In the future, we will examine whether Pitavastatin affects iNOS expression in the endothelial cells.

We also examined whether the NO donor affected the endothelial cell injury induced by TNF-α. This time, we used SNAP (N-(acetyloxy)-3-nitrosothiovaline), which generates NO. However, SNAP did not reduce the cytotoxicity of TNF-α (data not shown). Moreover, as far as NO production, the effect was increased by TNF-α with SNAP, and NO production was increased. By measuring NO production using DAF-2, we showed that NO production by SNAP is greater than twice that induced by Statin (data not shown). Therefore, excess NO production *in vitro* decreases the cytotoxicity of TNF-α.

eNOS seems to exist locally in an inner layer of the cell membrane in the vascular endothelial cell locally, and in the inactivation condition, it combines with caveolin-1. eNOS is combined with Heat shock protein 90 (HSP90) and calmodulin, is phosphorylated,

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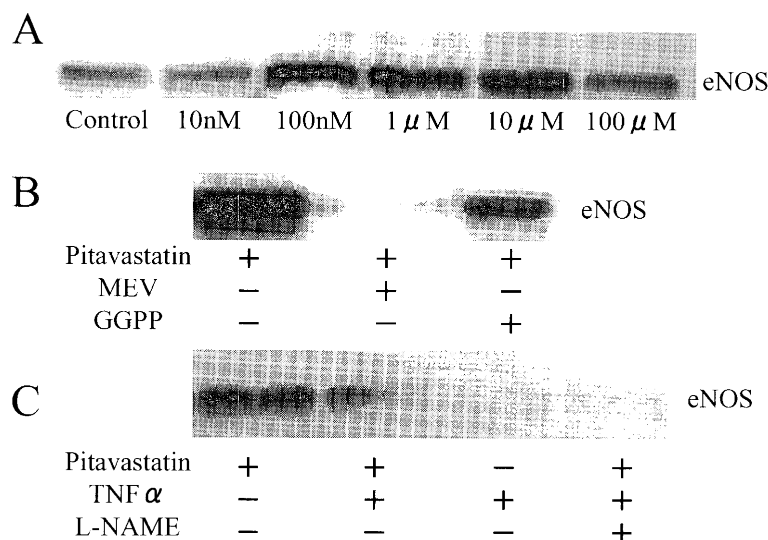

Fig. 3. (A) HUVEC were incubated with Pitavastatin (10 nM— 100 μM). Western blot analysis for the presence of eNOS (130 kDa) was performed. (B) HUVEC were incubated with Pitavastatin (100 nM) alone, or Pitavastatin (100 nM) and either mevalonic acid (MEV: 1 mM) or geranylgeranyl pyrophosphate (GGPP: 10 μM). Western blot analysis for the presence of eNOS (130 kDa) was performed. (C) HUVEC were incubated with Pitavastatin (100 nM) alone, or Pitavastatin (100 nM) and either TNF-α (10 ng/ml) or L-NAME (10 mM). Western blot analysis for the presence of eNOS (130 kDa) was performed.

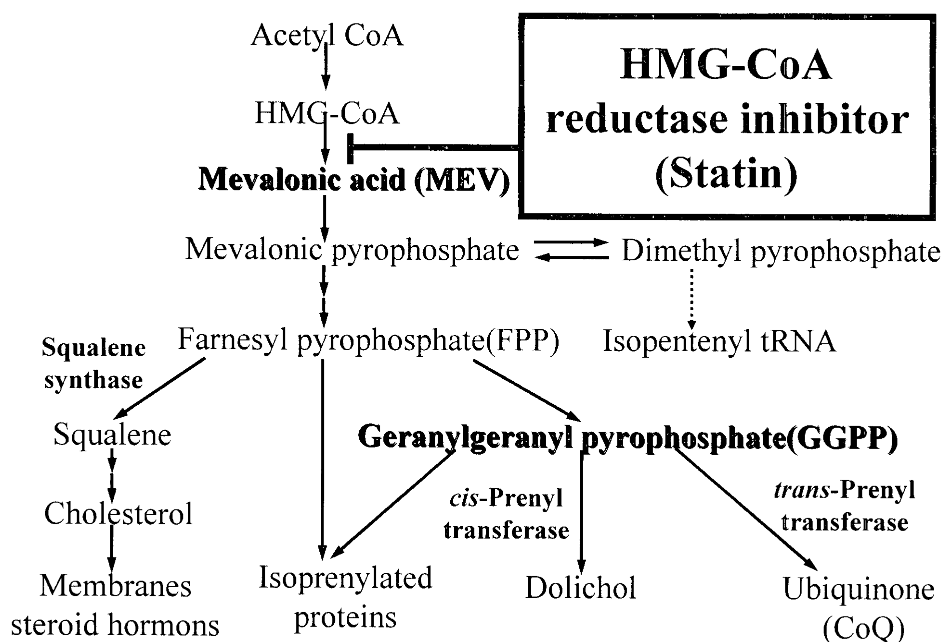

Fig. 4. Scheme of Mevalonate Pathway
Statin inhibits the generation of mevalonic acid from HMG-CoA.

and becomes activated.[21] eNOS reacts with L-arginine, which is a substrate of NO, and NO is produced. It has been reported that statin increases expression of eNOS and/or decreases the expression of caveolin-1.[22] Further experiments are requires to determine whether the eNOS expression induction effect or a posttranscriptional modification action through the caveolin-1 decrease is a main factor in the NO production mechanism by Pitavastatin.

In this study, we demonstrated that Pitavastatin acts directly in the vascular endothelial cells, and that it has a vascular protection action for the inflammatory reaction of TNF-α by inducing NO production in the endothelium.

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ACKNOWLEDGMENTS

We express special thanks to Kowa Co., Ltd. (Nagoya, Japan) for advising, supporting and providing us with Pitavastatin.

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