Ezetimibe reduces intimal hyperplasia in rabbit jugular vein graft

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Background: **The selective cholesterol transport inhibitor ezetimibe is widely used to prevent development of atherosclerosis in patients with hypercholesterolemia. However, whether this agent inhibits intimal hyperplasia in autologous vein grafts is unknown. The present study was undertaken to clarify if ezetimibe reduces cell proliferation and intimal hyperplasia in vein grafts.**

Methods: **Forty-four rabbits were randomly divided into two groups: one group received ezetimibe (0.6 mg/kg/d), and the control group did not. Ezetimibe administration was started 1 week before rabbits underwent interposition reversed autologous jugular vein grafts. The proliferative cells and apoptotic cells were counted in the vein grafts 14 days after implantation, and changes in acetylcholine-induced relaxation and endothelial intracellular concentration of Ca^{2+} ($[Ca^{2+}]_i$) were examined at 28 days.**

Results: **Ezetimibe reduced serum cholesterol and triglyceride. There were fewer proliferating cells in the ezetimibe group (5.7% ± 0.2%, n = 7) than in the control group (12.8% ± 0.5%, n = 7; $P < .0001$) and more apoptotic cells in the ezetimibe group (5.3% ± 0.2%, n = 7) than in the control group (2.3% ± 0.2%, n = 7; $P < .0001$). Intimal hyperplasia was less in the ezetimibe group (46.1 ± 6.0 μm, n = 7) than in the control group (76.0 ± 2.5 μm, n = 7; $P < .01$). Acetylcholine-produced endothelium-dependent relaxation was observed only in the ezetimibe group, which was blocked by the nitric oxide (NO) synthase inhibitor N^{ω}-nitro-L-arginine. Acetylcholine increased $[Ca^{2+}]_i$ only in the ezetimibe group.**

Conclusions: **Ezetimibe reduced cell proliferation and enhanced cell apoptosis, thus inhibiting intimal hyperplasia in rabbit autologous vein grafts. Ezetimibe restored the acetylcholine-induced increase in $[Ca^{2+}]_i$ in endothelial cells and improved endothelium-dependent NO-mediated relaxation in the vein graft. Our results suggest that ezetimibe enhances the function of endothelial NO through an increase in endothelial $[Ca^{2+}]_i$, thus reducing vein graft intimal hyperplasia. (J Vasc Surg 2012;56:1689-97.)**

Clinical Relevance: **Intimal hyperplasia is a major obstacle to patency after vein grafting. Various treatments have been examined to reduce neointimal hyperplasia; however, a standard clinical treatment has not yet been established. We report here that ezetimibe inhibits intimal hyperplasia in rabbit autologous vein grafts. More importantly, we demonstrated that ezetimibe improves acetylcholine-induced endothelium-dependent relaxation by restoring its endothelial cell $[Ca2+]_i$–mobilizing activity in the grafts. The present results suggest that ezetimibe restores agonist-induced endothelial $[Ca2+]_i$ mobilization and enhances the function of endothelium-derived nitric oxide, thus reducing intimal hyperplasia in vein grafts. Our results support the notion that ezetimibe helps to prevent intimal hyperplasia in vein grafts.**

Surgical revascularization using autologous vein continues to be the most commonly used option for coronary artery bypass grafting and the surgical treatment of peripheral arterial disease, its main advantages being ready availability and suppleness.[1,2] Such venous implants are subject to increased shear stress, loss of endothelial cells, migration and invasion of inflammatory cells, and migration and proliferation of smooth muscle cells.[3,4] These changes lead to intimal hyperplasia and subsequently accelerate atherogenesis, being responsible for vein graft occlusion.[5-7] Endothelium, through the synthesis and release of vasorelaxing factors, such as nitric oxide (NO) and prostacyclin, is critical in the regulation of vascular tone and vascular wall structure changes.[8,9] Several studies have demonstrated the importance of preserving the function of endothelium-derived NO to inhibit intimal hyperplasia in autologous vein grafts.[10-13]

Ezetimibe is a potent inhibitor of sterol absorption, which selectively inhibits biliary and dietary cholesterol uptake in the small intestine by blocking the Niemann-Pick C1 like 1 sterol transporter activity,[14] thus lowering the serum concentration of total cholesterol and low-density lipoprotein cholesterol (LDL-C).[15-17] Ezetimibe was also found to reduce monocyte chemoattractant protein-1 expression and decrease macrophage/monocyte content in atherosclerotic plaque in an atherosclerosis rabbit model.[18] Furthermore, ezetimibe enhances acetylcholine-induced endothelium-dependent relaxation through an enhancement of the function of endothelium-derived NO in apolipoprotein E-deficient mice.[19] The Heart and Renal Protec-

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tion clinical trial[20] demonstrated that lowering of LDL-C with simvastatin plus ezetimibe safely reduced the incidence of major atherosclerotic events in a wide range of patients with advanced chronic kidney disease. Taken together, these results suggest that ezetimibe may reduce intimal hyperplasia seen in autologous vein grafts. However, this remains to be determined.

The present study was undertaken to determine whether long-term administration of ezetimibe was able to inhibit intimal hyperplasia in rabbit autologous vein grafts. Rabbits were used because this model has been used to examine the effects of lipid-lowering agents in vein graft hyperplasia. Next, to clarify the underlying mechanism, acetylcholine-induced changes in endothelial intracellular Ca^{2+} ($[Ca^{2+}]_i$), and endothelium-dependent relaxation in grafted vein from rabbits treated with ezetimibe was compared with that of nontreated rabbits.

METHODS

Animals and vein graft implantation. All experiments performed in this study conformed to "Guidelines on the Conduct of Animal Experiments" issued by the Nagoya University Graduate School of Medicine and by the Graduate School of Medical Sciences in Nagoya City University and were approved by the Committee on the Ethics of Animal Experiments in that institution.

The study randomly divided 44 male Japanese albino rabbits (2.5 to 3.0 kg, supplied by Nippon SLC, Hamamatsu, Japan) into two groups. The control group was fed commercial rabbit chow (CR3, containing 4% crude fat from soybean oil; CLEA Japan Inc, Tokyo, Japan), and the ezetimibe group received the CR3 chow with 0.6 mg/kg/d ezetimibe, as previously described.[18] Treatment with ezetimibe was initiated 1 week before vein implantation and continued for 2 or 4 weeks until harvest of the vein graft. The amount of chow that remained in a cage in the morning was checked daily to ascertain the daily amount of chow ingested by a rabbit, and the rabbits that had ingested ≤50% of the food provided were omitted.

Carotid interposition reversed autologous jugular vein grafts were performed, as previously reported.[21] Anesthesia was induced intramuscularly with ketamine hydrochloride (50 mg/kg) and xylazine (10 mg/kg) and maintained with the intravenous (i.v.) administration of ketamine hydrochloride (10 mg/kg) and xylazine (10 mg/kg) whenever required. After a longitudinal neck incision, the right jugular vein and the right common carotid artery were exposed. The branches of the jugular vein were carefully ligated with 8-0 polypropylene sutures. An approximately 2.5-cm segment of the jugular vein was taken for the autologous reversed vein graft.

Graft harvesting was completed with meticulous care to avoid injuring the graft wall. The harvested graft was kept moistened in heparinized saline (5 IU/mL) at room temperature. The animals were systemically heparinized (200 IU/kg), and the internal carotid artery and two of the three branches of the external carotid artery were ligated. The most inferior branch of the external carotid artery served as the only outflow for the conditions modeling poor runoff. The common carotid artery was clamped distally and proximally, and a graft was anastomosed in an end-to-end fashion into the divided artery with interrupted 8-0 polypropylene sutures under a surgical microscope. The wound was closed layer to layer.

Harvest of implanted vein grafts. For histochemical examination, autologous vein grafts were harvested under general anesthesia at 14 and 28 days after implantation. In brief, rabbits were euthanized by an overdose of pentobarbital (50 mg/kg i.v.) and the graft was harvested after systemic heparinization (200 IU/kg i.v.). The harvested graft was fixed with 4% formaldehyde at 100 mm Hg for 30 minutes, perfused, and immersed in the same fixative overnight at room temperature. Each sample was embedded in paraffin and cut into 5-μm sections.

Intimal hyperplasia was assessed in the harvested vein grafts in each group (n = 7 in each group). Three sections were obtained from each vein graft, and each section was deparaffinized in a xylene/ethanol series and stained with hematoxylin and eosin or with elastica van Gieson. The intimal area and medial area were measured by MACSCOPE (Mitani Co, Fukui, Japan) at eight different randomly selected views per section and these were averaged. The three sections were examined in the same way by three observers who were blinded to the identity of the groups. Values were averaged to represent the intimal hyperplasia of the vein graft per rabbit and were used for statistical analysis.

Immunohistochemical staining. Vein grafts (n = 7 in each group) were harvested at 14 days after implantation and deparaffinized in a xylene/ethanol series. Proliferative cells in the neointima were identified by immunohistochemical staining with Ki-67 monoclonal antibody (DAKO Cytomation Inc, Carpenteria, Calif). To determine apoptotic cells, we performed terminal deoxynucleotidyl transferase-mediated deoxyuride-5'-triphosphate nick-end labeling (TUNEL) staining (Roche, Mannheim, Germany), as previously described.[22] The Ki-67-positive and TUNEL-positive nucleated cells in the neointima were counted (original magnification, ×400) by three observers who were blinded to the identity of the groups. The values obtained by the three observers were averaged for each section. The quantitative analysis was performed for eight independent sections in each rabbit. The number of Ki-67–positive and TUNEL-positive cells/total number of cells counted was defined as the Ki-67 index and TUNEL index of the vein graft.

Isometric tension measurement. The vein graft harvested at 4 weeks after implantation was excised and immediately placed in Krebs solution and thereafter cleaned by removing the connective tissues. The midportion of the harvested graft was used in the present experiments. In some preparations, the endothelium was removed as described elsewhere.[23] A ring preparation (~1-mm wide) was suspended for measurement of isometric contraction in an organ chamber. This was set between two stainless steel wires inserted into the lumen of the ring; one wire was

connected to a force transducer (UL-2 GR; Minebea Co, Nagano, Japan) and then a carrier amplifier (AS2101; NEC-San-ei Instruments, Tokyo, Japan).[24] The output signal was fed into an IBM/AT-compatible personal computer through an analog-to-digital PowerLab converter (AD Instruments Pty Ltd, Bella Vista, Australia). The organ chamber was filled with 3 mL Krebs solution at 37°C and gassed with 95% oxygen and 5% carbon dioxide. Resting tension was adjusted to obtain maximum contraction induced by K^+ solution (128 mmol/L).

To obtain concentration-dependent responses, acetylcholine (10 nmol/L to 3 μmol/L) was cumulatively applied in an ascending order during the contraction-induced prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, 1 μmol/L) in endothelium-intact strips. To study the influence of endothelium-derived NO, the effects of acetylcholine were examined in the presence and absence of the NO-synthase inhibitor N^{ω}-nitro-L-arginine (L-NNA, 0.1 mmol/L), which was applied as pretreatment for 60 minutes and was present thereafter. The concentration of $PGF_{2\alpha}$ was reduced to 0.3 μmol/L in the presence of L-NNA to obtain matched amplitudes of contraction.

The concentration–response relationship for the NO donor NOC-7 (0.1 nmol/L to 0.1 μmol/L) was obtained by its cumulative application during the contraction induced by $PGF_{2\alpha}$ in endothelium-denuded strips. Guanethidine (5 μmol/L, to prevent effects due to release of sympathetic transmitters) and diclofenac (3 μmol/L, to inhibit the production of cyclooxygenase products) were present throughout the experiments.

Measurement of [Ca2+]$_i$. The concentration of $[Ca^{2+}]_i$ was estimated using the ratiometric fluorescence Ca^{2+}-indicator Fura 2.[25] Endothelium-intact strips were loaded with Fura 2-acetoxymethyl ester (Fura 2-AM; 5 μmol/L) in Krebs solution containing 0.001% Pluronic F-127 for 4 hours at room temperature under dark conditions. After loading, the strip was mounted on a fluorescence microscope and superfused with warmed (37°C) Krebs solution. Next, acetylcholine (10 nmol/L to 3 μmol/L) was applied for 2 minutes in ascending order with a 10-minute interval. Fura 2 was excited by dual wavelengths (340 nm [F_{340}] and 380 nm [F_{380}]) for 182 ms and collected through a 510-nm emission filter (half-width, 20 nm) at 15-second intervals. The $[Ca^{2+}]_i$ was expressed as the fluorescence ratio F_{340}/F_{380}. The mean values of F_{340}/F_{380} obtained from six endothelial cells in each strip were averaged, and one value per strip was used for the later analysis.

Solutions. The composition of the Krebs solution was (mmol/L) NaCl, 122; KCl, 4.7; $MgCl_2$, 1.2; $CaCl_2$, 2.5; $NaHCO_3$, 15.5; KH_2PO_4, 1.2; and glucose, 11.5. The solution was bubbled with 95% oxygen and 5% carbon dioxide (pH, 7.3-7.4).

Drugs. The drugs used were L-NNA (Peptides Institute Inc, Osaka, Japan), acetylcholine-HCl (Daiichi-seiyaku, Tokyo, Japan), diclofenac sodium (Sigma, St. Louis, Mo), guanethidine (Tokyo Kasei, Tokyo, Japan), $PGF_{2\alpha}$ (Cayman Chemical Co, Ann Arbor, Mich), and Fura 2-AM (Molecular Probes, Eugene, Ore).

Stock solutions were made of Fura 2-AM (1 mmol/L in dimethylsulfoxide) and $PGF_{2\alpha}$ (10 mmol/L in ethanol). All other drugs were dissolved in ultrapure Milli-Q water (Japan Millipore Corp, Tokyo, Japan). The stock solutions were stored at −80°C and diluted in Krebs solution just before use.

Table I. Body weight and metabolic parameters in nontreated rabbits and in rabbits treated with ezetimibe[a]

Variable	*Control (n = 7)*	*Ezetimibe (n = 7)*	P
Body weight, kg	2.75 ± 0.08	2.80 ± 0.03	.54
Triglyceride, mg/dL	68.33 ± 5.30	53.83 ± 3.48	<.04
Cholesterol, mg/dL			
Total	32.83 ± 1.54	23.75 ± 1.24	.0006
HDL	15.33 ± 0.94	13.17 ± 1.15	.15
LDL	9.00 ± 0.69	4.33 ± 0.45	<.0001
HbA_{1C}, %	2.59 ± 0.05	2.63 ± 0.05	.53

HbA_{1c}, Glycosylated hemoglobin; *HDL*, high-density lipoprotein; *LDL*, low-density lipoprotein.
[a]Blood samples were collected from nontreated rabbits and rabbits treated with ezetimibe 4 weeks after the operation. Data are shown as mean ± standard error of the mean.

Sample size calculation. Bypass operations were done in 44 rabbits: 22 in the control group and 22 in the ezetimibe group. The 22 rabbits in the control group were further separated into two groups. One group consisted of seven rabbits from which the vein grafts were harvested at 14 days after implantation for examination of cell proliferation and apoptosis, and the vein grafts in the remaining 15 rabbits were taken at 28 days histochemical examination of intimal thickness (n = 7) and for tension and endothelial $[Ca^{2+}]_i$ measurement (n = 8). Similar sample size calculation was made in the ezetimibe group.

Statistical analysis. All results are expressed as mean ± standard error of the mean, with n values representing the number of rabbits used (each rabbit provided one segment for a given experiment). A one-way or two-way repeated-measure analysis of variance, with post hoc comparisons made using the Scheffé procedure or the Student unpaired *t*-test, was used for the statistical analysis. The level of significance was set at $P < .05$.

RESULTS

Intimal hyperplasia in vein graft. All animals survived the operations, and all vein grafts were patent until the time of harvest. Oral administration of ezetimibe for 28 days had no effects on body weight and serum concentration of high-density lipoprotein cholesterol or glycosylated hemoglobin (HbA_{1c}), but serum concentrations of total cholesterol, LDL-C, and triglyceride were reduced (Table I).

Fig 1, *A* shows the whole-mount photomicrographs of vein grafts at 28 days after implantation obtained from a control rabbit (*left panel*) and a ezetimibe-treated rabbit (*right panel*). Although both veins remain widely patent

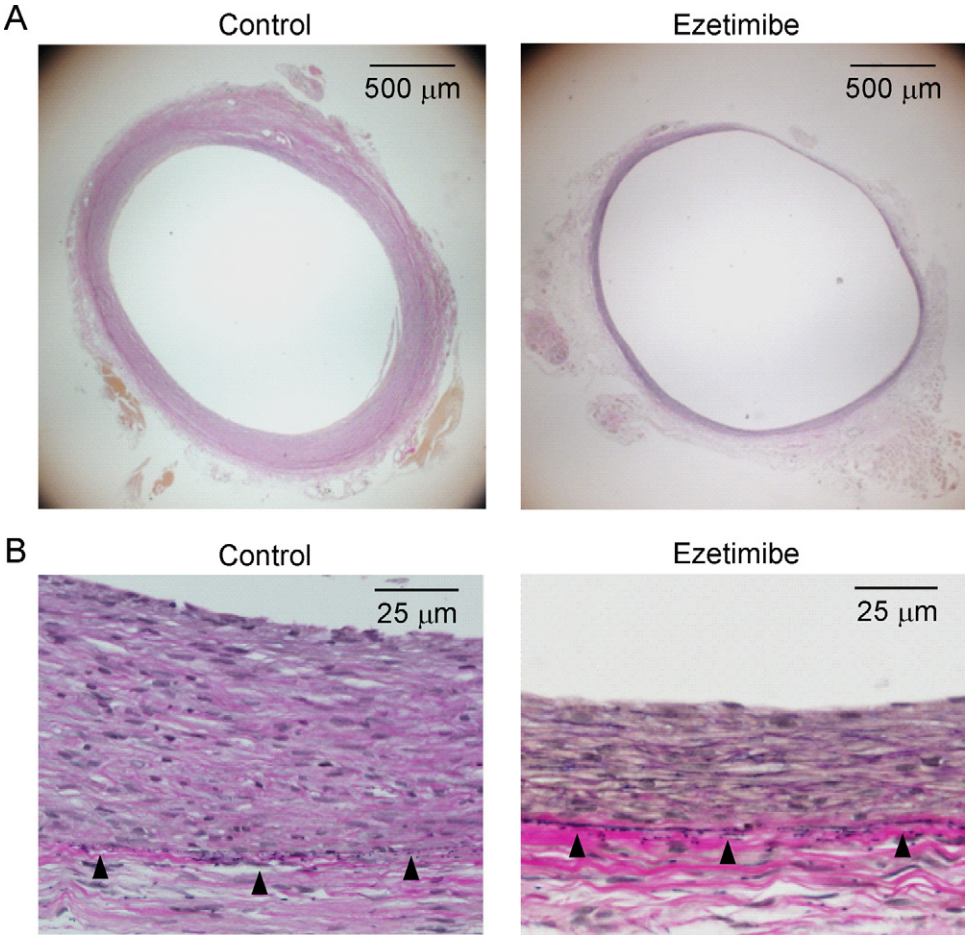

Fig 1. Effects of ezetimibe are shown on the development of intimal hyperplasia in autologous vein grafts at 28 days after operation. **A,** Microscopic findings in the middle portion of the vein graft from the control group (*left panel*) and the ezetimibe group (*right panel*; elastica van Gieson staining [original magnification, ×20]). **B**, The *arrowheads* indicate the internal elastic lamina (elastica van Gieson staining [original magnification, × 400]).

Table II. Morphometric analysis[a]

Variable	*Control (n = 7)*	*Ezetimibe (n = 7)*	P
Lumen area, mm^2	9.5 ± 0.7	9.9 ± 0.7	.73
Intimal thickness, μm	76.0 ± 2.5	46.1 ± 6.0	<.01
Medial thickness, μm	65.2 ± 3.9	58.3 ± 1.0	.11
Intima/media index	1.2 ± 0.1	0.8 ± 0.1	<.01

[a]Data are shown as mean ± standard error of the mean.

after 28 days, the vascular wall was much thicker in a control group than in the ezetimibe-treated group (Fig 1, *B*). Intimal thickness and the intima/media index of the vein graft were significantly less in the ezetimibe group than in the control group (n = 7; $P < .01$; Table II), but medial thickness and total lumen area did not differ significantly between the two groups (Table II).

Cell proliferation and apoptosis in vein graft. In the neointimal region, the Ki-67 index was significantly smaller in the ezetimibe group (5.7% ± 0.2%) than in the control group (12.8% ± 0.5%, n = 7 in each group; $P < .0001$; Fig 2, *A* and *B*). The TUNEL index in the neointimal region was significantly bigger in the ezetimibe group (5.3% ± 0.2%) than in the control group (2.3% ± 0.2%, n = 7 in each group; $P < .0001$; Fig 2, *C* and *D*).

Effects of ezetimibe on acetylcholine-induced relaxation. There was no significant difference in the amplitude of contraction induced by K^+ (128 mmol/L) in endothelium-intact strips between the control group (n = 7) and the ezetimibe group (n = 7; $P > .05$; Fig 3, *A* and *B*). The NO synthase inhibitor L-NNA significantly enhanced the contraction induced by K^+ (128 mmol/L) to the same extent in the two groups, suggesting that the function of spontaneously released endothelial NO is not modified by ezetimibe (Fig 3, *B*).

In endothelium-intact strips, acetylcholine (10 nmol/L to 3 μmol/L) did not produce a relaxation during the contraction induced by $PGF_{2\alpha}$ in the control group (n = 8), but acetylcholine induced concentration-depen-

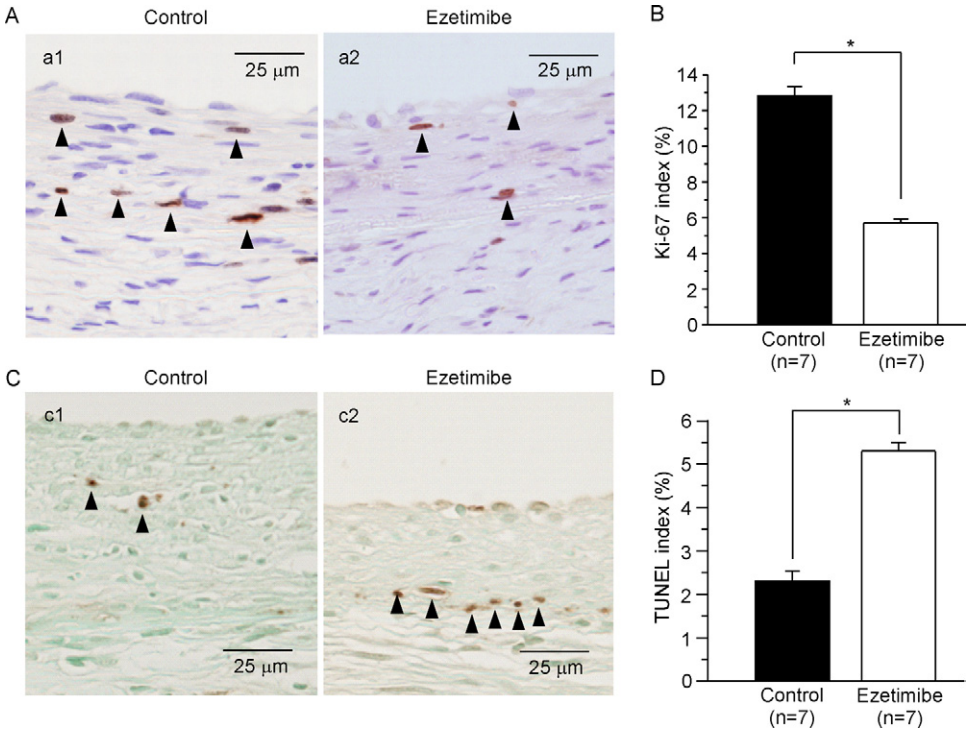

Fig 2. Effects of ezetimibe on proliferative activity and apoptosis in neointima of autologous vein grafts at 14 days after operation. **A**, Representative Ki-67 staining in the middle portion of the vein graft from nontreated control rabbits (*left panel*) or rabbits treated with ezetimibe (*right panel*; original magnification, × 400). The *arrowheads* indicate Ki-67–positive cells in the neointima. **B**, Graph shows Ki-67 index in vein grafts from nontreated control rabbits or rabbits treated with ezetimibe (n = 7 in each group). **C**, Representative photomicrographs stained with terminal deoxynucleotidyl transferase-mediated deoxyuride-5'-triphosphate nick-end labeling (*TUNEL*) in the middle portion of the vein graft from nontreated control rabbits (*left panel*) or rabbits treated with ezetimibe (*right panel*; original magnification, × 400). The *arrowheads* indicate TUNEL-positive cells in the neointima. **D**, The TUNEL index is shown in vein grafts from nontreated control rabbits or rabbits treated with ezetimibe. Results are expressed as mean ± standard error of the mean (*error bars*). **P* < .05 vs control.

dent relaxation in the ezetimibe group (n = 6; *P* < .0001; Fig 3, *C*). The acetylcholine-induced relaxation in the ezetimibe group (n = 6) was blocked by the NO synthase inhibitor L-NNA (0.1 mmol/L; *P* < .0001) vs the ezetimibe group before L-NNA (*L-NNA(−)* Fig 3, *C*).

Effects of ezetimibe on NOC-7-induced relaxation. In endothelium-denuded preparations, NOC-7 (0.1 nmol/L to 0.1 μmol/L) induced a concentration-dependent relaxation during the contraction induced by $PGF_{2\alpha}$; however, there was no significant difference between the control group and the ezetimibe group (n = 5 in each group; *P* > .05; Fig 3, *D*).

Effects of ezetimibe on acetylcholine-induced $[Ca^{2+}]_i$ increase in endothelial cells. Under basal conditions, the Fura-2 ratio in endothelial cells of the vein grafts was 1.19 ± 0.03 in the control group (n = 5) and 1.21 ± 0.07 in the ezetimibe group (n = 8; *P* > .05; Fig 4, *Bb1*). Acetylcholine (10 nmol/L to 3 μmol/L) did not modify the endothelial $[Ca^{2+}]_i$ in the control group but concentration-dependently increased the $[Ca^{2+}]_i$ in endothelial cells in the ezetimibe group (n = 5-8; *P* < .0001; Fig 4, *Bb2*).

DISCUSSION

We found that long-term administration of ezetimibe reduced intimal hyperplasia in rabbit autologous vein grafts. Acetylcholine did not induce relaxation in the vein grafts of the control group. However, acetylcholine did induce the endothelium-dependent relaxation in the ezetimibe group, which was blocked by the NO synthase inhibitor L-NNA. More importantly, we found that acetylcholine increased $[Ca^{2+}]_i$ in endothelial cells in the ezetimibe group but not in the control group. In addition, ezetimibe treatment did not modify smooth muscle relaxation induced by the NO donor NOC-7 in the graft. These results indicate that sustained administration of ezetimibe improves acetylcholine-induced endothelium-dependent NO-mediated relaxation through an enhancement of acetylcholine-induced endothelial $[Ca^{2+}]_i$ mobilization in rabbit autologous vein grafts.

Venous adaptation to the arterial environment is characterized by thickening of the intima, media, and adventitia.[26,27] These modifications, which result from deposition of smooth muscle cells and extracellular matrix compo-

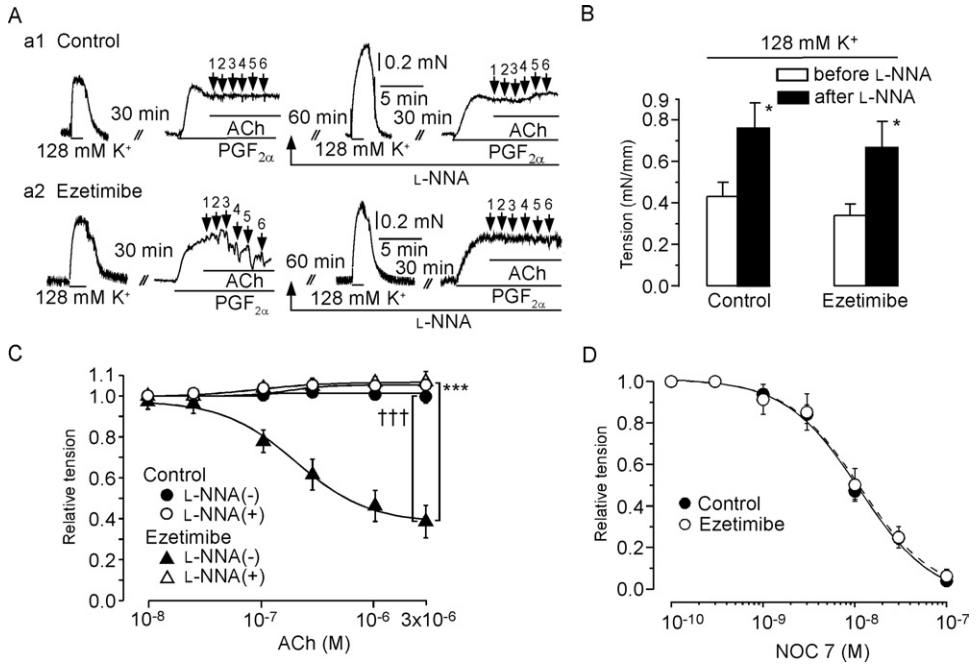

Fig 3. Effects of ezetimibe on high K^+-induced contraction and acetylcholine (*ACh*)-induced relaxation in autologous vein grafts and the effects on NOC-7–induced relaxation in endothelium-denuded vein grafts. **A**, After recording of the mechanical responses induced by high K^+ (128 mM) and acetylcholine (10 nM to 3 μM), N^{ω}-nitro-L-arginine (*L-NNA*) was applied for 60 minutes, and high-K^+ and ACh-induced responses were again obtained in the presence of L-NNA in endothelium-intact strips from nontreated control rabbits (**a**) or rabbits treated with ezetimibe (**a2**). Acetylcholine, at concentrations of (1) 10 nM, (2) 30 nM, (3) 0.1 μM, (4) 0.3 μM, (5) 1 μM, and (6) 3 μM, was applied during a contraction induced by prostaglandin $F_{2\alpha}$ (*$PGF_{2\alpha}$*). **B**, The absolute tension induced by 128 mM K^+ before and after application of L-NNA in the vein graft from nontreated control rabbits (n = 7) or rabbits treated with ezetimibe (n = 7). **C**, Summary of the effects of ezetimibe on ACh-induced responses. The tonic contraction induced by $PGF_{2\alpha}$ before application of acetylcholine was normalized as the relative tension of 1.0 for each strip (n = 8, control group; n = 6, ezetimibe group). *L-NNA(−)*, Before application of L-NNA; *L-NNA(+)*, after application of L-NNA. Results are expressed as mean ± standard error of the mean. ***$P < .001$ L-NNA(−) in ezetimibe vs L-NNA(+) in ezetimibe; †††$P < .001$ L-NNA(−) in control vs L-NNA(−) in ezetimibe.

nents, stimulate remodeling and reduce compliance.[3-7,26,27] This remodeling appears to involve at least two distinct temporal phases: lumen-outward remodeling at an earlier phase and wall-stiffness changes at the later phase.[27] The earlier phase is suggested to be important for successful adaptation to increase diameter and wall thickness in human vein grafts. We found that ezetimibe not only reduced the number of proliferating cells but also increased the number of apoptotic cells, thus inhibiting intimal hyperplasia in autologous vein grafts at 14 days after implantation. Furthermore, in the vein grafts at 28 days after implantation, intimal thickness was less in the ezetimibe group than in the control group, although the total lumen area and medial thickness were comparable between the two groups. These results indicate that long-term administration of ezetimibe selectively inhibits the development of intimal hyperplasia in rabbit autologous vein grafts.

Endothelial cells are suggested to play an essential role in regulation of intimal growth in vein grafts through the synthesis and release of NO.[28] The NO synthesis by endothelial NO synthase is activated through an increase in $[Ca^{2+}]_i$.[29] We previously found that the increased intimal thickness in autologous vein grafts was closely related to the loss of acetylcholine-induced endothelium-dependent NO-mediated relaxation, although changes in agonist-induced endothelial $[Ca^{2+}]_i$ mobilization were not measured.[2,10-13] We also found that such hyperplasia was reduced by endothelial NO synthase gene transfer[13] or oral administration of the NO precursor L-arginine.[12] Interestingly, endothelium-derived NO inhibited leukocyte-endothelial interaction,[30,31] platelet adhesion and aggregation,[32] and smooth muscle cell proliferation.[33] Here, we found that in endothelium-intact vein grafts, the NO synthase inhibitor L-NNA enhanced the high K^+-induced contraction in the control and ezetimibe groups, and the magnitude of the response was similar between the two groups (Fig 3, *B*). Furthermore, resting endothelial $[Ca^{2+}]_i$ was similar between the two groups (Fig 4, *Bb1*), suggesting that long-term administration of ezetimibe may not modify the function of spontaneously released endothelial NO in rabbit autologous vein grafts. Most importantly, we found that long-term administration of

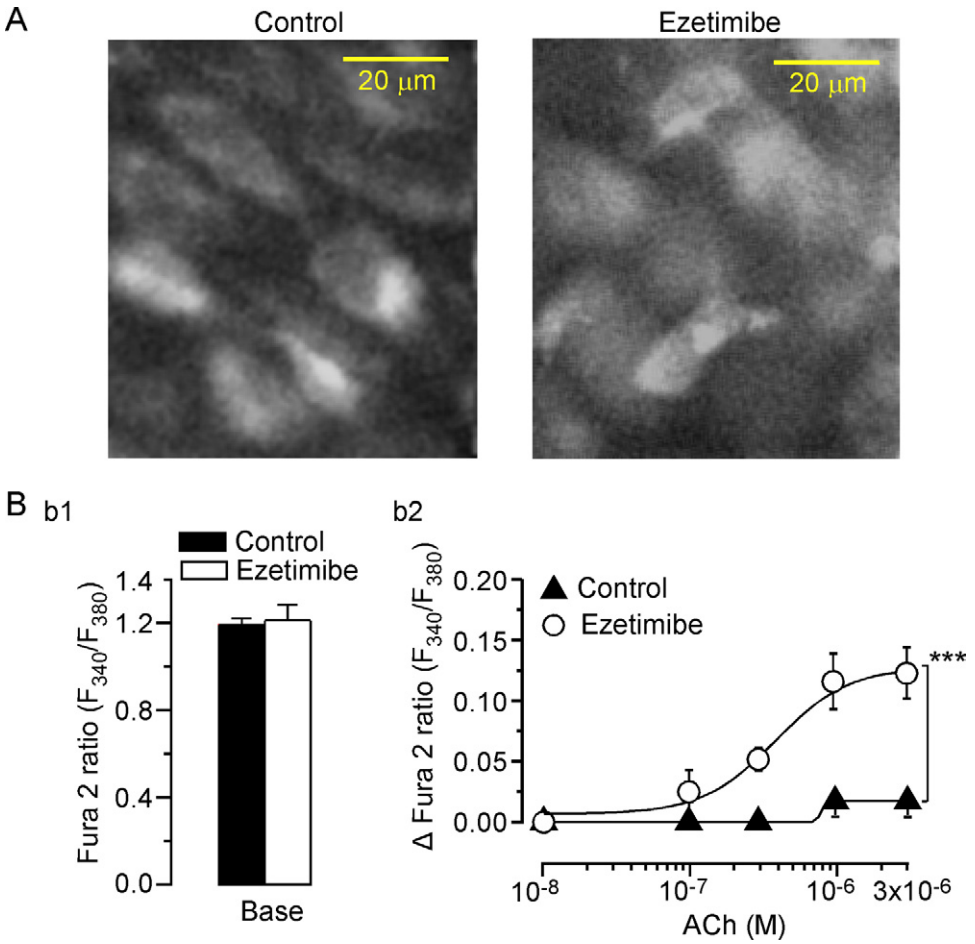

Fig 4. Effects of ezetimibe on acetylcholine (*ACh*)-induced increase in intracellular $[Ca^{2+}]_i$ in endothelial cells of vein grafts. **A**, Fluorescence ratio (F_{340}/F_{380}) images of the Ca^{2+}-sensitive dye Fura 2 in endothelial cells in the control group (*left panel*) and in the ezetimibe group (*right panel*). **B,** Effects on $[Ca^{2+}]_i$: **(b1)** the two groups did not differ significantly in basal $[Ca^{2+}]_i$; (**b2**) concentration-dependent effect of ACh 10 nM to 3 μM on endothelial $[Ca^{2+}]_i$. The change in $[Ca^{2+}]_i$ is expressed as ΔFura 2 ratio (F_{340}/F_{380}) (n = 5, control group; n = 8, ezetimibe group). Data are shown as mean ± standard error of the mean. ***$P < .001$ vs control.

ezetimibe restored acetylcholine-induced endothelial cell $[Ca^{2+}]_i$ increase in rabbit autologous vein grafts. Ezetimibe also greatly improved acetylcholine-induced endothelium-dependent NO-mediated relaxation in the vein graft.

These results are the first to suggest that long-term administration of ezetimibe restores agonist-induced endothelial cell $[Ca^{2+}]_i$ mobilization and thereafter enhances the function of endothelium-derived NO, thus reducing intimal hyperplasia in rabbit autologous vein grafts. However, whether ezetimibe enhances acetylcholine-induced endothelium-dependent vascular relaxation under the condition in which the synthesis of NO is blocked in vivo remains to be clarified.

Ezetimibe is thought to selectively block uptake of biliary and dietary cholesterol in the small intestine and decrease circulating levels of atherogenic lipoproteins, thus reducing atherosclerosis.[34] Furthermore, in addition to this beneficial lipid-lowering effect, the other direct or indirect vascular protective actions of ezetimibe have been suggested in apolipoprotein E-deficient mice.[19] Here, we found that ezetimibe reduced serum concentrations of total cholesterol, LDL-C, and triglyceride and restored acetylcholine-induced increase of endothelial cells $[Ca^{2+}]_i$ in autologous vein graft. Because the amount of ezetimibe used in the present experiments (0.6 mg/kg/d) is slightly greater than that used in patients (10 mg/d), future study should clarify whether this higher concentration may play a significant role in the ezetimibe-induced beneficial vascular effects.

Hypercholesterolemia was found to impair agonist-induced endothelial $[Ca^{2+}]_i$ mobilization and endothelium-dependent relaxation in rabbit femoral artery.[35] At present, however, we do not know whether ezetimibe improves acetylcholine-induced endothelial cell $[Ca^{2+}]_i$ mobilization simply due to its cholesterol-lowering effect in rabbit

autologous vein grafts. This remains to be clarified in future studies.

CONCLUSIONS

Long-term administration of ezetimibe inhibited intimal hyperplasia due to reduction of cell proliferation and enhancement of cell apoptosis in rabbit autologous vein graft. This ezetimibe treatment restored acetylcholine-induced endothelial cell $[Ca^{2+}]_i$ increase and endothelium-dependent NO-mediated relaxation in the vein graft. Thus, we suggest that the increased function of endothelium-derived NO may play a role in ezetimibe-induced inhibition of intimal hyperplasia in the vein graft. This novel finding may facilitate the clinical usage of ezetimibe to prevent the late graft failure after bypass grafting.

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AUTHOR CONTRIBUTIONS

Conception and design: TM, KK, TI
Analysis and interpretation: TM, KK, TI
Data collection: TM, KM, TI
Writing the article: TM, KK, TI
Critical revision of the article: TM, KK, TI
Final approval of the article: KK, TI
Statistical analysis: TM, KM
Obtained funding: Not applicable
Overall responsibility: KK

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