# Dipeptidyl peptidase 4 inhibitor reduces intimal hyperplasia in rabbit autologous jugular vein graft under poor distal runoff

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***Background:*** **Dipeptidyl peptidase 4 inhibitors are widely used in patients with type 2 diabetes mellitus to accomplish glycemic control through an increase in the blood glucagon-like peptide 1 (GLP-1) concentration. These agents also inhibit vascular inflammation (eg, in atherosclerosis). This study was undertaken to determine whether and how vildagliptin (a potent dipeptidyl peptidase 4 inhibitor) might reduce intimal hyperplasia in vein grafts.**

***Methods:*** **Twelve rabbits were randomly divided into two groups; one group received vildagliptin orally (10 mg/kg/d; n = 6), whereas the control group (n = 6) did not. Vildagliptin administration was started 7 days before rabbits underwent interposition reversed autologous jugular vein grafting and ended at graft harvesting (28 days after the operation). Histochemical changes in the vascular wall were examined, as were changes in the acetylcholine-induced effects on the endothelial $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) and endothelium-dependent relaxation.**

***Results:*** **Under fasting conditions, vildagliptin increased the plasma GLP-1 concentration, without affecting plasma glucose or insulin. Acetylcholine induced endothelium-dependent relaxation only in the vildagliptin group, and this was blocked by the nitric oxide synthase inhibitor $N^{\omega}$-nitro-L-arginine. Acetylcholine did not modify the endothelial $[Ca^{2+}]_i$ in either the control or vildagliptin group. Intimal hyperplasia was significantly less in the vildagliptin group (0.11 ± 0.02 mm, n = 5) than in the controls (0.31 ± 0.06 mm, n = 4; $P < .01$).**

***Conclusions:*** **Vildagliptin increased the plasma GLP-1 concentration. It also enhanced acetylcholine-induced $[Ca^{2+}]_i$-independent endothelial nitric oxide release and reduced vein graft intimal hyperplasia, independently of any glycemic control action. (J Vasc Surg 2015;■:1-11.)**

***Clinical Relevance:*** **Intimal hyperplasia is a major obstacle to patency after vein grafting. Although various treatments have been tested, a standard clinical treatment for intimal hyperplasia remains elusive. Here, in a rabbit model, long-term systemic administration of vildagliptin improved endothelial function, increased nitric oxide release, and reduced intimal hyperplasia in vein grafts. Our results support that dipeptidyl peptidase 4 inhibition is beneficial by limiting intimal hyperplasia in vein grafts.**

Vein bypass grafting is an effective and durable treatment for many patients with atherosclerotic occlusive diseases of the coronary or peripheral circulations.[1-3] However, in the lower extremity, failure of the vein graft has occurred in 30% to 40% of cases at 5 years, and >50% of coronary grafts have failed within 10 years, leading to significant morbidity and mortality.[4] During adaptation to the arterial hemodynamic environment, autologous vein grafts undergo wall thickening, with the formation of neointima, which is composed primarily of vascular smooth muscle cells (VSMCs) and extracellular matrix (ECM) components such as elastin and collagens.[1-3] During the healing of vein bypass grafts, collagen deposition plays a significant role in increased wall stiffness (which serves to resist arterial pressure) and is an important determinant of final lumen size.[1-3] However, in vein bypass grafts, the relationships among elasticity changes, matrix composition, and graft dimensions are yet to be fully clarified.

A new class of antitype 2 diabetes mellitus drugs, dipeptidyl peptidase 4 (DPP-4) inhibitors, reduce the blood glucose concentration by inhibiting the inactivation of glucagon-like peptide 1 (GLP-1), thereby increasing insulin secretion and suppressing glucagon secretion by the pancreas.[5] It has been found that DPP-4 inhibitors not only normalize the blood glucose concentration (glucose-lowering effects) but also impede atherosclerosis development (anti-inflammatory effects) in both animals and humans.[6-10] Atherosclerosis is an inflammatory disease of the arterial wall, and endothelial dysfunction has been shown to contribute to this condition.[11,12] However, it is unknown whether DPP-4 inhibitors might inhibit intimal hyperplasia in autologous vein grafts.

VSMC contraction is regulated in both membrane potential-dependent and membrane potential-independent manners.[13] Membrane depolarization in VSMC increases

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Author conflict of interest: none.

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The editors and reviewers of this article have no relevant financial relationships to disclose per the JVS policy that requires reviewers to decline review of any manuscript for which they may have a conflict of interest.

0741-5214



http://dx.doi.org/10.1016/j.jvs.2014.12.048

the open probability of the voltage-dependent L-type $Ca^{2+}$ channel and thereby increases the cellular concentration of $Ca^{2+}$ ($[Ca^{2+}]_i$) and tension.[13] Acetylcholine, an endothelium agonist, induces an endothelial $[Ca^{2+}]_i$ increase and causes productions of nitric oxide (NO) and endothelium-derived hyperpolarizing factor, thus causing endothelium-dependent relaxation.[13-16] NO induces a relaxation that is independent of the VSMC membrane potential, whereas endothelium-derived hyperpolarizing factor causes relaxation through hyperpolarization,[13-16] and the normalizing function of these factors has been suggested to inhibit intimal hyperplasia in vein grafts.[1-3,17-20] However, the effects of DPP-4 inhibitors on such functions in vein grafts remain unknown.

This study was undertaken to clarify whether long-term administration of the DPP-4 inhibitor vildagliptin[9,21] might enhance endothelial function and inhibit the intimal hyperplasia that occurs in rabbit autologous vein grafts under conditions of poor distal runoff. This rabbit model was chosen because it has been used in previous studies to examine the effects of agents that modulate vein graft hyperplasia, and we employed vascular ring or strip preparations because their use is well established in studies of functional changes in vein grafts under physiologic conditions.[17-20] To clarify the mechanism underlying the observed effects of vildagliptin, the changes in endothelial $[Ca^{2+}]_i$, VSMC membrane potential, and endothelium-dependent relaxation induced by acetylcholine in ring or strip preparations were compared between vein grafts from vildagliptin-treated and nontreated rabbits.

## METHODS

**Animals and vein graft implantation.** All experiments 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 they were approved by the Committee on the Ethics of Animal Experiments in those institutions.

The study protocol randomly divided 12 male Japanese albino rabbits (2.5-3.0 kg, supplied by Nippon SLC, Hamamatsu, Japan) into two groups. Each group (control group [n = 6] and vildagliptin group [n = 6]) was fed commercial rabbit chow (containing 4% crude fat from soybean oil), and rabbits were housed individually in a temperature- and light-controlled room (around 20°C ± 3°C, 12-hour light/dark cycle) with free access to water. Vildagliptin (10 mg/kg/d in drinking water) was used as reported previously,[9,21] with this treatment beginning 7 days before vein implantation and continuing until harvest of the vein graft (which was at 28 days after implantation).

The procedure to create the vein graft (namely, carotid interposition reversed autologous jugular vein graft under poor distal runoff) was performed as previously reported.[17-20] In brief, the right jugular vein and the right common carotid artery were exposed, and the branches of the jugular vein were carefully ligated. A segment of the jugular vein was taken to provide the autologous reversed vein graft. The most inferior branch of the external carotid artery served as the only outflow for the present conditions, modeling poor distal runoff. The common carotid artery was clamped distally and proximally, and under a surgical microscope the graft was anastomosed in an end-to-end fashion into the divided artery, using interrupted sutures.

**Biochemical analysis.** Blood samples were collected from rabbits (6 in the control group and 5 in the vildagliptin group) after an overnight fast at 28 days after vein graft implantation. Plasma glucose, total cholesterol, triglyceride, and free fatty acid levels were measured by enzymatic methods; low-density and high-density lipoprotein cholesterol concentrations were measured by direct methods.[20] Insulin and GLP-1 (active form) were determined by enzyme-linked immunosorbent assay with kits from Shibayagi (Shibukawa, Japan) and Immuno-Biological Laboratories (Fujioka, Japan), respectively.

**Tissue preparation.** At 28 days after implantation, the vein grafts were harvested under general anesthesia, as previously described.[17-20] In brief, the rabbits were killed by an overdose of pentobarbital (50 mg/kg intravenously). Vein grafts were immediately excised, placed in Krebs solution, and cleaned by removal of connective tissues. The middle portion of the harvested graft was used for all studies.

**Histochemical staining.** Four weeks after the operation, the harvested grafts were immersion fixed in 4% paraformaldehyde and embedded in optical cutting temperature compound (Tissue-Tek; SAKURA Finetechnical, Tokyo, Japan), then frozen and stored at −80°C. Sections were cut at 5-μm thickness on a cryostat,[17-20] and their intimal hyperplasia was assessed.

For immunohistochemistry, the sections were treated with 0.3% hydrogen peroxide in methanol for 30 minutes to inactivate endogenous peroxidase, then incubated overnight at 4°C with the primary antibody—antihuman matrix metalloproteinase (MMP) 2, MMP-9, or tissue inhibitor of metalloproteinase (TIMP) 2 monoclonal antibody (each at 1:100 dilution; Daiichi Fine Chemical Co, Takaoka, Japan). The sections were incubated sequentially with goat biotinylated antimouse immunoglobulin G polyclonal antibody (1:5000 dilution; Dako, Tokyo, Japan), as secondary antibody, and a 0.02% solution of 3,3′-diaminobenzidine tetrahydrochloride (DAB; Sigma-Aldrich, St. Louis, Mo).[17-20] Hematoxylin was used as the counterstain. Negative control data were obtained by application of second antibody only (ie, without application of primary antibody) to show background staining.

For examination of MMP-2 by immunofluorescence staining, the procedure was as follows. After incubation with the primary antibody, the sections were incubated with the secondary antibody (Alexa Fluor 488 antimouse immunoglobulin G antibody, 1:5000 dilution; Molecular Probes, Eugene, Ore). The fluorescence of Alexa Fluor 488 was detected by confocal laser scanning microscopy (LSM5 PASCAL; Carl Zeiss, Jena, Germany), and the acquisition of images from different groups of rabbits was

**Table I.** Effects of presence of endothelium and the nitric oxide (NO) synthase inhibitor $N^{\omega}$-nitro-L-arginine (*L-NNA*) on the contraction induced by prostaglandin $F_{2\alpha}$ (*$PGF_{2\alpha}$*) in vein grafts

| | *Concentration of $PGF_{2\alpha}$, μmol/L* | *Tension, mN/mm* | P *value* | *RT*[a] | P *value* |
|---|---|---|---|---|---|
| Effect of endothelium | | | | | |
| Control group, E(+) | 0.33 ± 0.10 | 0.03 ± 0.00 | | 0.03 ± 0.01 | |
| Vildagliptin group, E(+) | 0.53 ± 0.16 | 0.04 ± 0.01 | >.1 | 0.03 ± 0.01 | >.5 |
| Vildagliptin group, E(−) | 0.58 ± 0.17 | 0.03 ± 0.00 | >.1 | 0.03 ± 0.01 | >.5 |
| Effect of L-NNA[b] | | | | | |
| Vildagliptin group, E(+), L-NNA(−) | 1.43 ± 1.40 | 0.04 ± 0.00 | | 0.02 ± 0.00 | |
| Vildagliptin group, E(+), L-NNA(+) | 1.43 ± 0.81 | 0.04 ± 0.01 | >.5 | 0.03 ± 0.01 | >.5 |

*E(+),* Endothelium-intact preparation; *E(−),* endothelium-denuded preparation.
[a]Relative tension (*RT*) was normalized to the maximum contraction induced by 10 μmol/L 5-hydroxytryptamine (5-HT) in each preparation.
[b]Contractions were obtained to $PGF_{2\alpha}$ before and after application of L-NNA.

performed under identical conditions, as described previously.[14,15,18,19] The total fluorescence intensities obtained from eight areas (each 20 × 20 pixels square) in a given preparation were averaged, and this (ie, one value for each preparation) was used for the subsequent analysis.

**Assessment of vascular wall thickness.** For morphometric analysis, each vein graft frozen section, cut at 5-μm thickness, was stained by the elastica van Gieson or Masson trichrome method. Color images were taken by a DP70 digital camera (Olympus, Tokyo, Japan) set in an Olympus IX71 microscope. Vascular wall thickness and lumen area were measured with ImageJ software (National Institutes of Health; http://rsb.info.nih.gov/ij/). All sections, whether from control or vildagliptin-treated animals, were examined in the same way, and the values obtained from them were averaged to assess the effect of vildagliptin on the wall thickness of the vein graft. For collagen measurement, each color image obtained from Masson trichrome-stained sections was separated into RGB components by means of ImageJ software, and the area of the blue color image in the intima/media was calculated as a percentage of the total intima/media area.

**Isometric tension measurement.** A given ring preparation (~1 mm wide) was suspended for measurement of isometric tension (calculated per millimeter length of ring) in an organ chamber.[14,15,17-20] Resting tension was adjusted to obtain the maximum contraction to high $K^+$ solution (128 mmol/L). In some preparations, the endothelium was removed as described elsewhere.[17-20]

To obtain concentration-dependent effects, acetylcholine was cumulatively applied during the contraction induced by prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) in endothelium-intact or endothelium-denuded preparations. To examine the role played by endothelium-derived NO, the effect of acetylcholine was examined in the presence of $N^{\omega}$-nitro-L-arginine (L-NNA). The concentration of $PGF_{2\alpha}$ was modified in each preparation so as to obtain matched amplitudes of contraction (Table I). 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.

**Table II.** Metabolic parameters in nontreated control rabbits and in vildagliptin-treated rabbits

| | *Control group (n = 6)* | *Vildagliptin group (n = 5)* | P |
|---|---|---|---|
| Body weight, kg | 2.80 ± 0.05 | 2.75 ± 0.05 | .542 |
| $HbA_{1c}$, % | 2.88 ± 0.08 | 2.68 ± 0.10 | .135 |
| Glucose, mg/dL | 135.8 ± 5.9 | 136.8 ± 3.6 | .897 |
| Insulin, μU/mL | 5.80 ± 1.20 | 6.96 ± 1.23 | .520 |
| GLP-1, pmol/L | 5.13 ± 0.57 | 7.31 ± 0.63 | .031 |
| Triglyceride, mg/dL | 55.8 ± 14.4 | 53.4 ± 10.9 | .899 |
| Free fatty acid, mEq/L | 279.3 ± 27.3 | 243.8 ± 15.3 | .312 |
| Cholesterol, mg/dL | | | |
| Total | 38.5 ± 5.1 | 27.6 ± 5.4 | .177 |
| HDL | 21.3 ± 1.6 | 15.4 ± 2.9 | .095 |
| LDL | 11.7 ± 3.4 | 7.2 ± 1.8 | .306 |

*GLP-1,* Glucagon-like peptide 1; *$HbA_{1c}$,* glycosylated hemoglobin; *HDL,* high-density lipoprotein; *LDL,* low-density lipoprotein.
Data are shown as mean ± standard error of the mean.

**Measurement of $[Ca^{2+}]_i$.** The concentration of $[Ca^{2+}]_i$ was estimated using the ratiometric fluorescence $Ca^{2+}$ indicator Fura-2.[14,15,20] Endothelium-intact preparations were loaded with Fura-2-acetoxymethyl ester (5 μmol/L) in Krebs solution containing 0.0001% Pluronic F-127 for 4 hours at room temperature. The vein graft preparation was mounted on a fluorescence microscope for en face endothelial cell Fura-2 imaging, and it was superfused with warmed (37°C) Krebs solution, as described previously.[20] Acetylcholine was applied for 90 seconds. Fura-2 was excited by dual wavelengths of 340 nm ($F_{340}$) and 380 nm ($F_{380}$), and emissions were collected through a 510-nm emission filter (half-width, 20 nm) at 5-second intervals, as described previously.[15,16,20] The mean values of $F_{340}/F_{380}$ obtained from six endothelial cells in each preparation were averaged, and one value per preparation was used for the later analysis.

**Electrophysiologic study.** Membrane potentials were measured in VSMCs by a conventional microelectrode technique, as described previously.[15,19] Acetylcholine or 5-hydroxytryptamine (5-HT) was applied for 90 seconds in endothelium-intact preparations.

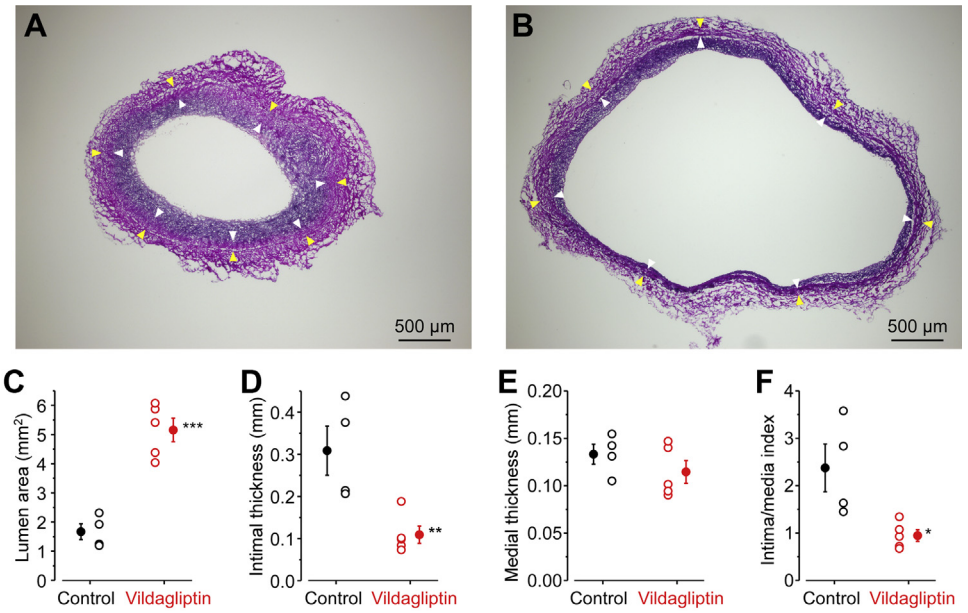


**Fig 1.** Effects of vildagliptin on morphometric changes in autologous vein grafts, as assessed at 28 days after the operation. **A** and **B**, Microscopic findings revealed by elastica van Gieson staining in control group **(A)** and vildagliptin group **(B)**. The *inner (white) arrowhead* indicates internal elastic lamina; the *outer (yellow) arrowhead* shows external elastic lamina. **C-F,** Morphometric analysis for lumen area **(C)**, intimal thickness **(D),** medial thickness **(E),** and intima/media index **(F)** (n = 4, control group; n = 5, vildagliptin group). Four sections were obtained from each vein graft, and each section was stained by the elastica van Gieson method. The intimal thickness was measured at eight randomly selected different sites per section, and these values were averaged. The four sections were each examined in the same way, and the values were averaged to represent intimal hyperplasia in that graft. The extent of intimal hyperplasia was expressed as an intima/media index (intima thickness divided by media thickness). Results are expressed as values for individual grafts and as mean ± standard error of the mean (*error bars*). $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$ vs control group. Measurements were made by blinded researchers who were not otherwise involved in the present study.

**Solutions.** The composition of the Krebs solution was as follows (mmol/L): $Na^+$, 137.4; $K^+$, 5.9; $Mg^{2+}$, 1.2; $Ca^{2+}$, 2.5; $HCO_3^-$, 15.5; $H_2PO_4^-$, 1.2; $Cl^-$, 134; glucose, 11.5. The solutions were bubbled with 95% oxygen and 5% carbon dioxide (pH, 7.3-7.4).

**Drugs.** The drugs used were 5-HT hydrochloride and diclofenac sodium (Sigma-Aldrich), acetylcholine hydrochloride (Daiichi Pharmaceutical, Tokyo, Japan), Fura-2-acetoxymethyl ester (Molecular Probes), guanethidine (Tokyo Kasei, Tokyo, Japan), L-NNA (Peptide Institute Inc, Osaka, Japan), and $PGF_{2\alpha}$ (Cayman Chemical Co, Ann Arbor, Mich). Vildagliptin was kindly provided by Novartis Pharma AG (Basel, Switzerland).

**Statistical analysis.** All values are expressed as means ± 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-measures analysis of variance, with post hoc comparisons made using the Scheffé procedure or Student unpaired *t*-test, was used for the statistical analysis. The level of significance was set at $P < .05$.

## RESULTS

**General properties.** All animals survived the operation, and all vein grafts were patent until the time of harvest. Long-term (35 days') administration of vildagliptin (10 mg/kg) increased the plasma concentration of GLP-1 without affecting the plasma insulin, glucose, or glycosylated hemoglobin ($HbA_{1c}$) concentrations under fasting conditions. Vildagliptin did not alter body weight or the plasma concentrations of total cholesterol, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, triglyceride, or free fatty acid (Table II).

**Intimal hyperplasia in vein graft.** Fig 1 shows effects of vildagliptin on morphometric changes in autologous vein grafts, as assessed at 28 days after the operation. The vascular wall was much thicker in the controls (n = 4) than in the vildagliptin group (n = 5; Fig 1, *A* and *B*), whereas lumen area was larger in the vildagliptin group than in the controls (Fig 1, *C*). The intimal thickness (Fig 1, *D*) and intima/media index (Fig 1, *F*) of the vein graft were each significantly less in the vildagliptin group than in the control group, but medial thickness was similar between the two groups (Fig 1, *E*).

**Effects of vildagliptin on acetylcholine-induced endothelial $[Ca^{2+}]_i$.** Under basal conditions, the Fura-2 ratio in the endothelial cells of the vein graft was 1.21 ± 0.05 in the control group (n = 4) and 1.25 ± 0.05 in the vildagliptin group (n = 4; $P > .1$; Fig 2, *A*). Acetylcholine (3 μmol/L) did not modify the endothelial $[Ca^{2+}]_i$ in either group (n = 4 for each group; $P > .1$; Fig 2, *B*).

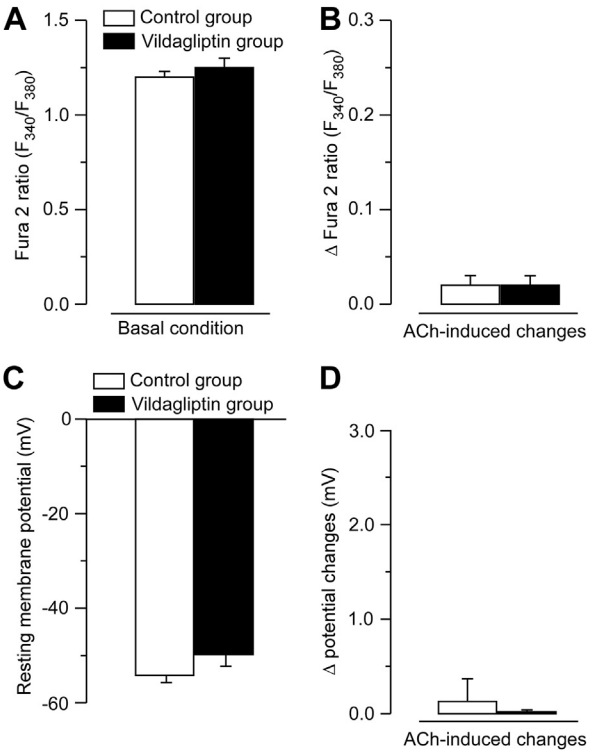


**Fig 2.** Effects of vildagliptin on endothelial cell $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) (**A** and **B**) and vascular smooth muscle cell (VSMC) membrane potential (**C** and **D**) in vein grafts. Neither the basal $[Ca^{2+}]_i$ **(A)** nor the resting membrane potential **(C)** in the vildagliptin group was significantly different from that in the control group. Acetylcholine (*ACh*, 3 μM) had no effect on endothelial cell $[Ca^{2+}]_i$ **(B)** or VSMC membrane potential **(D)** in either the control or vildagliptin group. $[Ca^{2+}]_i$ is expressed as the Fura-2 ratio ($F_{340}/F_{380}$). Data are shown as mean ± standard error of the mean (n = 4, in each case).

**Effects of vildagliptin on membrane potential in VSMCs.** In endothelium-intact vein graft preparations, the VSMC resting membrane potential was −54.2 ± 1.5 mV (n = 4) in the control group and −50.9 ± 2.6 mV (n = 4; $P > .05$) in the vildagliptin group (Fig 2, *C*). Acetylcholine (3 μmol/L) did not significantly modify these values in either the control group (Δ value, 0.1 ± 0.2 mV; n = 4) or the vildagliptin group (Δ value, 0.0 ± 0.0 mV; n = 4; Fig 2, *D*). 5-HT (0.1 μmol/L) depolarized the VSMC membrane by 19.8 ± 1.6 mV (n = 4) in the controls and by 21.4 ± 2.9 mV in the vildagliptin group (n = 4; $P > .5$; Fig 3).

**Effects of vildagliptin on acetylcholine-induced endothelium-dependent relaxation.** During the contraction induced by $PGF_{2\alpha}$ in endothelium-intact preparations, acetylcholine (0.1-10 μmol/L) failed to induce a relaxation in the control group (n = 4; Fig 4, *A*). In the vildagliptin group, however, (1) acetylcholine (0.3-10 μmol/L) concentration dependently induced relaxation in the presence of endothelium, but not in its absence (n = 5; $P < .001$; Fig 4, *A*), and (2) this endothelium-dependent relaxation was blocked by the NO synthase inhibitor L-NNA (0.1 mmol/L; $P < .001$ vs before L-NNA; Fig 4, *B*).

**Effects of vildagliptin on the contractions induced by high $K^+$ and 5-HT.** The contraction induced by high $K^+$ (128 mmol/L) was not significantly different in amplitude between the control and vildagliptin groups whether it was measured in the presence or absence of endothelium (n = 4 in each case; $P > .05$; Fig 5, *A-C*). L-NNA did not modify the high $K^+$-induced contraction in the presence or in the absence of endothelium in either group (n = 4 in each case; $P > .05$; Fig 5, *A-C*).

The amplitude of the contraction induced by 5-HT (10 μmol/L) in endothelium-intact preparations (but not in endothelium-denuded ones) was significantly enhanced by L-NNA in both the control group and the vildagliptin group (n = 4 in each case; $P < .05$), and the amplitude recorded in the presence of L-NNA was similar between the two groups (n = 4 in each case; $P > .05$; Fig 5, *D-F*). The $pD_2$ values obtained for 5-HT in endothelium-intact preparations from the control group were 6.78 ± 0.06 and 6.89 ± 0.06 before and after application of L-NNA, respectively (n = 4; $P > .1$). The corresponding values for the vildagliptin group were 6.84 ± 0.07 and 6.93 ± 0.04, respectively (n = 4; $P > .1$).

In endothelium-denuded preparations, the rate of relaxation after either removal of 5-HT (10 μmol/L) or removal of 128 mmol/L $K^+$ was slower in the control group than in the vildagliptin group (n = 3; $P < .05$ in each case; Fig 6). The times to half-relaxation after removal of 5-HT were 532 ± 64 seconds and 316 ± 42 seconds in the control group and vildagliptin group (n = 3 in each case; $P < .05$), respectively. The corresponding values for high $K^+$ were 131 ± 9 seconds and 79 ± 17 seconds (n = 3 in each case; $P < .05$), respectively.

**Histochemical examination.** Fig 7 shows elastica van Gieson (*A* and *B*) and Masson trichrome (*C* and *D*) staining of vein grafts from the control and vildagliptin groups. In each group, dark purple-colored (elastic) fibers were distributed within the intima (Fig 7, *A* and *B*), which was thinner in the vildagliptin group than in the controls (Fig 1, *D*). Blue-colored (collagen) fibers were diffusely and homogeneously distributed within the intima/media in each group, but the percentage of the intima/media area occupied by these blue fibers was smaller in the vildagliptin group than in the controls (n = 4; $P < .05$; Fig 7, *C-E*).

Fig 8 shows immunohistochemical staining of MMP-2, MMP-9, and TIMP-2 (an inhibitor of MMP) in the vascular wall of vein grafts. DAB staining revealed that TIMP-2 and MMP-9 were each mainly localized to the endothelial cell layer in both the control group and the vildagliptin group, and the intensity of the brown staining was not apparently different between the two groups (Fig 8, *B* and *C*). In contrast, MMP-2 was diffusely distributed within the neointimal region, and its staining intensity appeared to be greater in the controls than in the vildagliptin group (Fig 8, *D*). For MMP-2, the immunofluorescence intensity measured by confocal laser scanning microscopy of

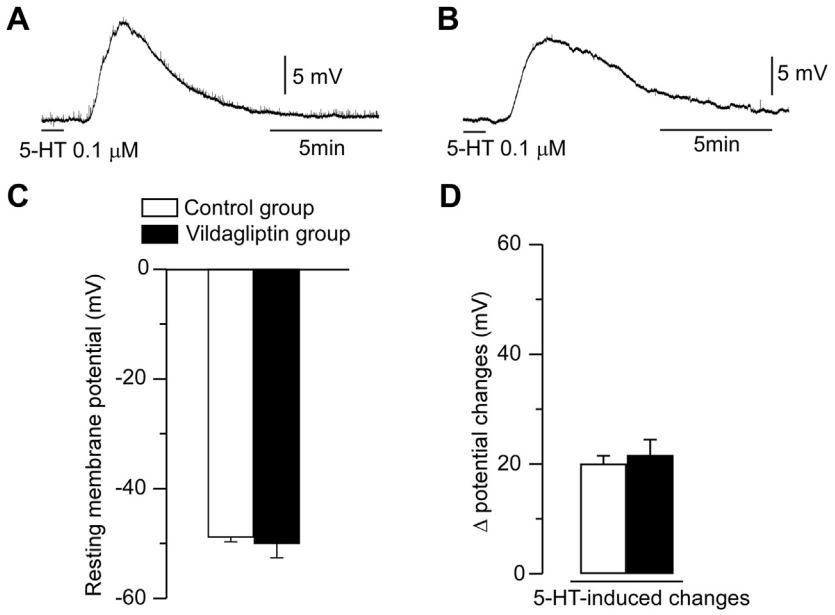


**Fig 3.** Effects of vildagliptin on resting membrane potential and 5-hydroxytryptamine (*5-HT*)-induced depolarization in vascular smooth muscle cells (VSMCs) in vein grafts. **A** and **B,** Actual traces of 5-HT (0.1 μM)-induced depolarization in control group **(A)** and vildagliptin group **(B)**. **C,** Resting VSMC membrane potential. **D,** 5-HT (0.1 μM)-induced depolarization in VSMCs. Data are shown as mean ± standard error of the mean (n = 4, in each case).

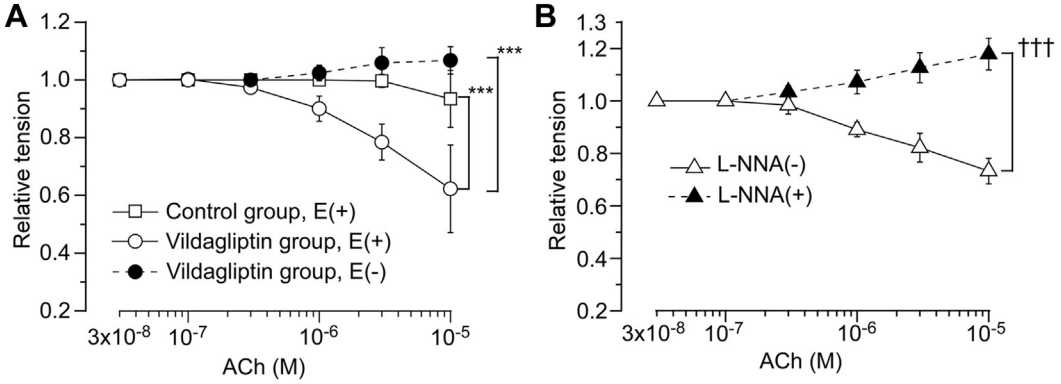


**Fig 4.** Effects of vildagliptin on acetylcholine (*ACh*)-induced endothelium-dependent relaxation in vein grafts. **A,** Effects of ACh (30 nM to 10 μM) on the contraction induced by prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) in endothelium-intact preparations [*E(+)*] from control group (n = 4) and vildagliptin group (n = 6) and also in endothelium-denuded preparations [*E(−)*] from vildagliptin group (n = 5). **B,** Effects of $N^{\omega}$-nitro-L-arginine (*L-NNA*) on ACh-induced response in endothelium-intact preparations from vildagliptin group. After ACh-induced responses had been recorded before application of L-NNA [*L-NNA(−)*], L-NNA was applied for 60 minutes, and ACh-induced responses were again obtained in the presence of L-NNA [*L-NNA(+)*] (n = 3). The concentration of $PGF_{2\alpha}$ was adjusted to obtain similar amplitudes of contraction before and after application of L-NNA (Table I). The contraction induced by $PGF_{2\alpha}$ before application of ACh was normalized as a relative tension of 1.0 for each preparation. Data are shown as mean ± standard error of the mean. $^{***}P < .001$ vs vildagliptin group E(+), $^{\dagger\dagger\dagger}P < .001$ vs L-NNA(−).

the vascular wall was significantly greater in the control group than in the vildagliptin group (Fig 8, *E* and *F*).

## DISCUSSION

We found here, in rabbits, that long-term systemic administration of the potent DPP-4 inhibitor vildagliptin increased the plasma GLP-1 concentration without altering the plasma glucose, $HbA_{1c}$, or insulin concentrations. We also found that MMP-2 expression in autologous jugular vein grafts under poor distal runoff was reduced when the rabbits had been orally treated (for 35 days) with vildagliptin. Most important, we found that this treatment reduced intimal hyperplasia in the vein grafts. These results suggest that vildagliptin inhibits vascular wall remodeling in such vein grafts. It was recently

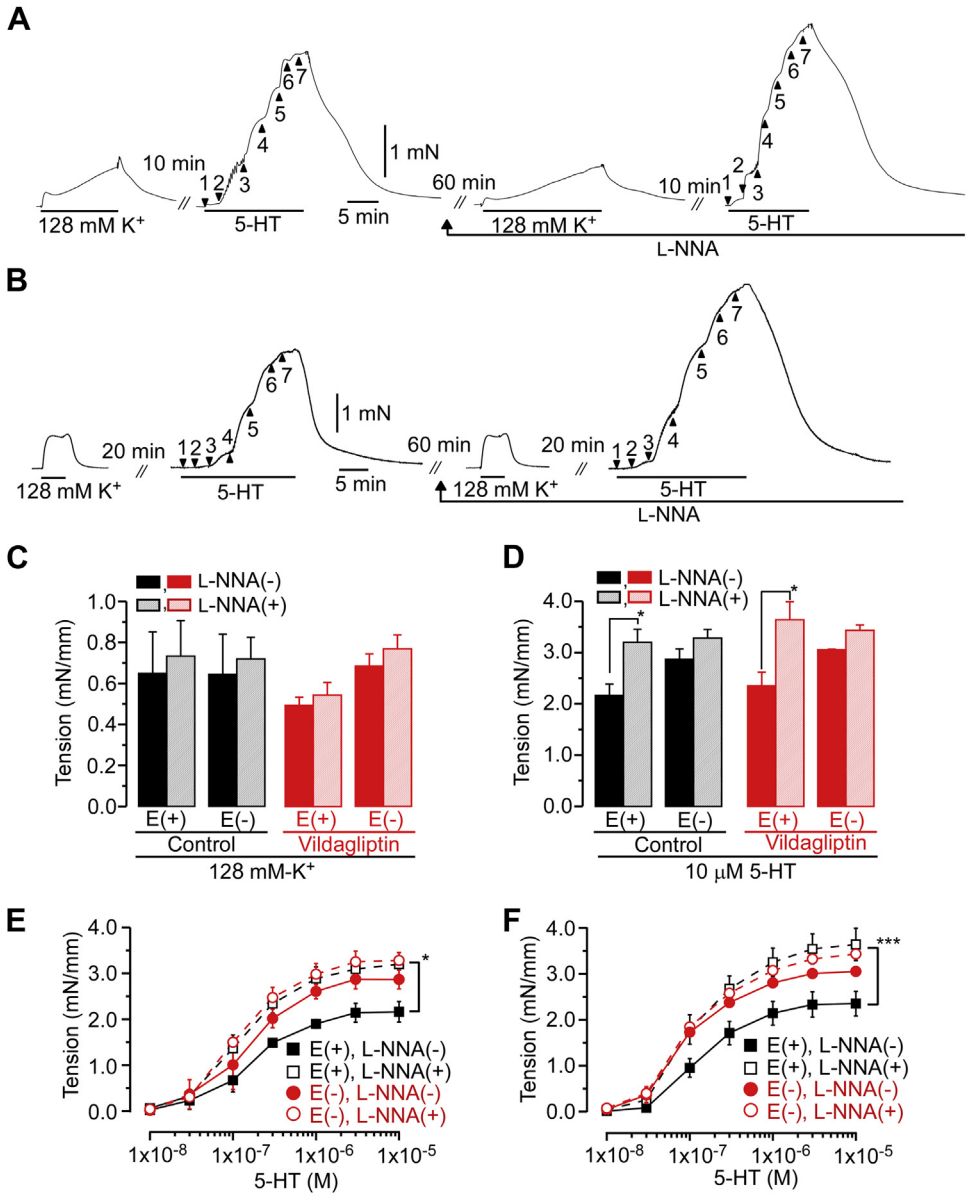


**Fig 5.** Effects of $N^{\omega}$-nitro-L-arginine (*L-NNA*) on the contractions induced by high $K^+$ and 5-hydroxytryptamine (*5-HT*) in vein grafts. **A** and **B,** Actual traces recorded from endothelium-intact vein grafts from control group **(A)** and vildagliptin group **(B)**. After the responses induced by high $K^+$ (128 mM) and 5-HT (10 nM to 10 μM) had been recorded, L-NNA (0.1 mM) was applied for 60 minutes and high $K^+$ and 5-HT were again applied in the presence of L-NNA. 5-HT was applied at concentrations of (1) 10 nM, (2) 30 nM, (3) 0.1 μM, (4) 0.3 μM, (5) 1 μM, (6) 3 μM, and (7) 10 μM. **C** and **D,** Absolute tension induced by 128 mM $K^+$ **(C)** or 10 μM 5-HT **(D)** before [*L-NNA(−)*] and after [*L-NNA(+)*] application of L-NNA in endothelium-intact [*E(+)*] or endothelium-denuded [*E(−)*] preparations in control group (*black*, n = 4) and vildagliptin group (*red*, n = 4). $^{*}P < .05$ vs L-NNA(−). **E** and **F,** Summary of the effects of L-NNA on 5-HT-induced contraction in endothelium-intact [*E(+)*] or endothelium-denuded [*E(-)*] preparations in control group (**E,** n = 4) and vildagliptin group (**F,** n = 4). Data are shown as mean ± standard error of the mean. $^{*}P < .05$, $^{***}P < .001$ vs L-NNA(−).

found that in apoE-deficient mice fed a high-fat diet, another DPP-4 inhibitor, sitagliptin, reduced atherosclerosis formation, independently of the fasting glucose and lipid profile.[7] Taken together, these results suggest that vildagliptin inhibits vascular growth development independently of the blood glucose and insulin levels and thereby reduces the intimal hyperplasia that occurs in vein grafts.

**Effects of vildagliptin on endothelial cells and VSMCs in vein grafts.** In line with our previous finding,[19] the NO synthase inhibitor L-NNA significantly enhanced

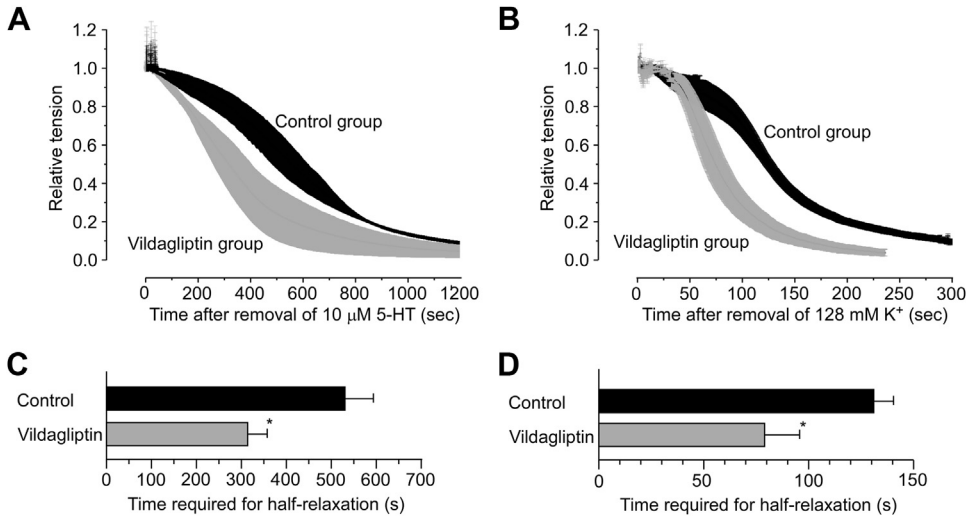


**Fig 6.** Rate of relaxation after removal of 10 μM 5-hydroxytryptamine (*5-HT*) or 128 mM $K^+$. Endothelium-denuded preparations were treated with 5-HT (n = 3, **A**) or high $K^+$ (n = 4, **B**) to obtain the maximum contraction, followed by rapid washout to induce relaxation. Tension was normalized to the maximum tension in each case. Time required for half-relaxation for 5-HT **(C)** and high $K^+$ **(D)**. Data are shown as mean ± standard error of the mean. $^*P < .05$ vs "Control."

the contraction induced by 5-HT in the rabbit vein grafts in the presence of endothelium. We also found that this L-NNA response was not modified by long-term administration of vildagliptin, suggesting that such DPP-4 inhibition did not enhance the function of the NO spontaneously released from the endothelium in vein grafts.

Endothelial cell-stimulating agonists (such as acetylcholine) increase $[Ca^{2+}]_i$ and activate endothelial NO synthase (eNOS) through $Ca^{2+}$-calmodulin binding and thereby induce NO production.[22-24] In addition, eNOS activity is regulated by phosphorylation at Ser-1177 and at Thr-495 by multiple protein kinases and phosphatases.[23,24] In previous studies, we found that in rabbit jugular vein grafts, (1) acetylcholine neither modified the endothelial $[Ca^{2+}]_i$ nor induced endothelium-dependent relaxation,[17-19] but (2) under long-term administration of ezetimibe (a cholesterol-lowering agent), acetylcholine induced both of these responses (namely, an endothelial $[Ca^{2+}]_i$ increase and an endothelium-dependent, NO-mediated relaxation).[20] Here, we found that although long-term administration of vildagliptin did not modify the acetylcholine-induced endothelial $[Ca^{2+}]_i$ response (Fig 2, *B*), it did enhance the acetylcholine-induced endothelial NO-mediated relaxation in the vein grafts (Fig 4, *A* and *B*). These results indicate that vildagliptin enhances acetylcholine-induced endothelial NO release through activation of an endothelial $[Ca^{2+}]_i$-independent mechanism. We speculate that an as yet unidentified role is played by eNOS phosphorylation in this vildagliptin-induced effect.

5-HT induces a VSMC contraction through activation of both L-type $Ca^{2+}$ channels and Rho kinase in rabbit jugular vein grafts.[19] We found here that vildagliptin modified neither the resting VSMC membrane potential nor the 5-HT (0.1 μmol/L)-induced VSMC membrane depolarization in the vein grafts (Fig 3). Furthermore, in endothelium-denuded preparations, neither the contraction induced by 128 mmol/L $K^+$ nor that induced by 10 μmol/L 5-HT differed between the control and vildagliptin groups (Fig 5, *C* and *D*). These results indicate that long-term administration of vildagliptin has no effects on the electrical and mechanical properties of differentiated VSMCs in rabbit jugular vein grafts. Moreover, we found that intimal thickness was less in the vildagliptin group than in the control group. Using antibodies against myosin heavy chain isoforms, we previously found that in rabbit vein grafts, intimal VSMCs appeared to be proliferating, as evidenced by proliferative cell nuclear antigen immunostaining, and that they retained the dedifferentiated phenotype.[17,19] These results suggest that although the number of differentiated VSMCs (mainly located in the medial region) may be similar between the control and vildagliptin groups, the synthetic type of VSMCs (located in the intima region) was less numerous in the vildagliptin group than in the controls.

In both the control and vildagliptin groups, L-NNA failed to modify the contractions induced by high $K^+$ and 5-HT in endothelium-denuded preparations. This suggests that the functional role played by inducible NOS in inhibiting contraction in dedifferentiated (synthetic) VSMCs or macrophages may be minimal in rabbit jugular vein grafts under the present experimental conditions.

**Effects of vildagliptin on MMP expression and stiffness in vein grafts.** Venous adaptation to the arterial environment is characterized by thickening of the intima, media, and adventitia.[1,25] These modifications, which result from deposition of VSMCs and ECM components,

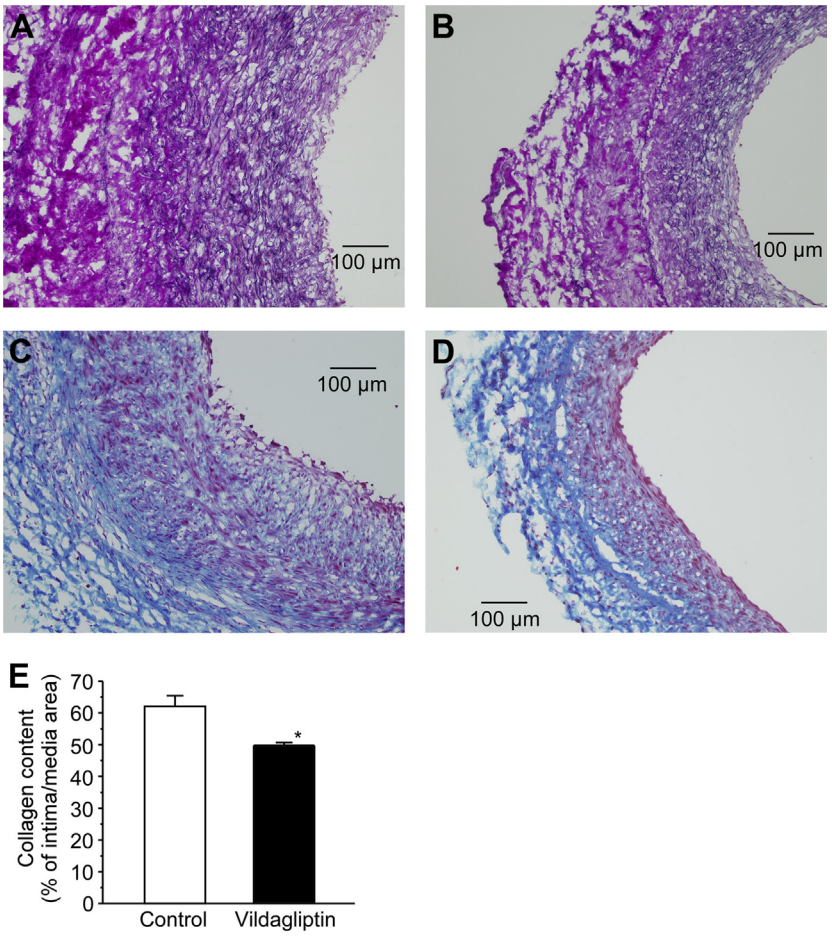


**Fig 7.** Histologic images of elastica van Gieson (**A** and **B**) and Masson trichrome (**C** and **D**) staining in vein grafts from control group (**A** and **C**) and vildagliptin group (**B** and **D**). Similar observations were made in three other preparations. **E,** Area occupied by collagen within intima/media was calculated and is shown as a percentage of the total intima/media area. The measurement was made at eight randomly selected different sites per section, and these values were averaged. The four sections were each examined in the same way, and the values were averaged for that graft. Data are shown as mean ± standard error of the mean for four samples from each group. *$P < .05$ vs "Control." Measurements were made by blinded researchers who were not otherwise involved in the present study.

stimulate remodeling.[2,3,26] Such vein graft remodeling appears to involve at least two distinct temporal phases: lumen outward remodeling at an earlier phase and wall stiffness changes at a later phase.[3] We previously suggested that these adaptive changes in rabbit vein grafts may be responsible for "physiologic adaptation" and may help the graft resist the higher pressure present on the arterial side of the circulation.[19,20] However, vein graft remodeling can have detrimental consequences; indeed, it is known that when late vein graft failure occurs, it is often due to massive intimal hyperplasia.[1,25]

The ECM protein-degrading activities of MMPs are regulated by TIMPs,[27,28] and dysregulation of MMPs promotes VSMC migration or proliferation. Indeed, injury to rat carotid arteries[29] and human saphenous veins[30] leads to (1) increased expressions of basement membrane-degrading MMP-2 and MMP-9 and (2) intimal thickening. Collagen is the only major component in the vein graft wall with an elastic modulus substantially greater than that of the original wall.[2,31] Here, we found that in the vein graft vascular wall, expression of MMP-2 as well as that of collagen was lower in the vildagliptin group than in the control group. Furthermore, in endothelium-denuded preparations, the rate of relaxation after removal of either 5-HT or high $K^+$ was greater in the vildagliptin group than in the controls. Moreover, it has been suggested that a relative deficiency of NO leads to increases in intimal hyperplasia[32] and collagen production,[33-35] thereby stiffening vein grafts.[3] Thus, we speculate that long-term administration of vildagliptin may enhance endothelial NO release and downregulate MMP-2 and collagen expressions, thus accelerating VSMC relaxation. It was recently found that sitagliptin inhibits atherosclerosis development through activation of the GLP-1/cyclic adenosine

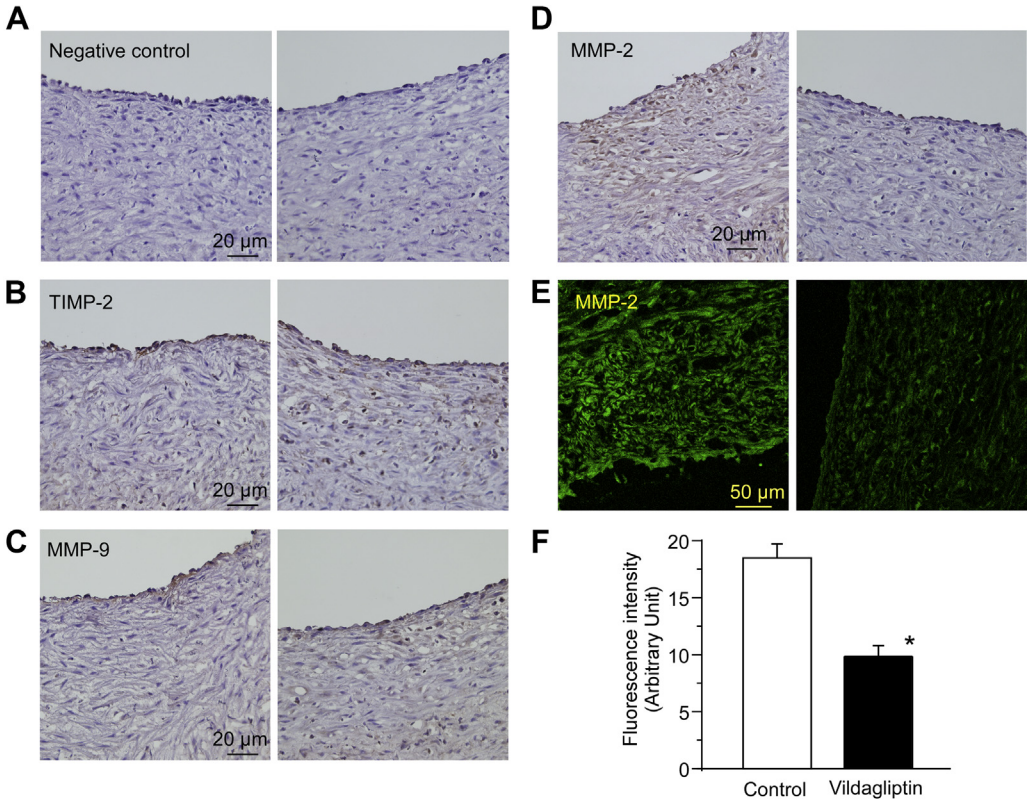


**Fig 8.** Immunohistochemical staining of matrix metalloproteinase 2 (*MMP-2*), matrix metalloproteinase 9 (*MMP-9*), and tissue inhibitor of metalloproteinase 2 (*TIMP-2*) in vein grafts from control group (*left panel*) and vildagliptin group (*right panel*). **A-D,** DAB staining: **(A)** negative control staining without primary antibody; **(B)** TIMP-2 staining; **(C)** MMP-9 staining; **(D)** MMP-2 staining. Expressions of TIMP-2 and MMP-9 were not apparently different between the two groups, but that of MMP-2 was apparently less in the vildagliptin group than in the control group. Similar observations were made in three other preparations. **E,** Immunofluorescence intensities for MMP-2 were detected by confocal laser scanning microscopy. **F,** Summary of the immunofluorescence results. The measurement was made at eight randomly selected different sites per section, and these values were averaged. The four sections were each examined in the same way, and the values were averaged for that graft. Data are shown as mean ± standard error of the mean for four samples from each group. **P* < .05 vs "Control." Measurements were made by blinded researchers who were not otherwise involved in the present study.

monophosphate/protein kinase A pathway in the aorta of apoE-deficient mice fed a high-fat diet.[7] It remains to be clarified whether vildagliptin reduces intimal hyperplasia in vein grafts by activating this pathway.

## CONCLUSIONS

Long-term administration of the DPP-4 inhibitor vildagliptin to rabbits may increase endothelial NO release and decrease MMP-2 and collagen expressions in autologous vein grafts, effects of vildagliptin that may be associated with reductions in both intimal hyperplasia and vascular stiffening. We therefore suggest that vildagliptin may have vascular-protective actions in vein grafts.

We thank Dr R. J. Timms for a critical reading of the manuscript.

## AUTHOR CONTRIBUTIONS

Conception and design: KK, TI
Analysis and interpretation: AK, RO, JK, TI
Data collection: AK, RO, JK, TI
Writing the article: KK, TI
Critical revision of the article: JK, KK, TI
Final approval of the article: KK, TI
Statistical analysis: RO, JK, TI
Obtained funding: KK, TI
Overall responsibility: TI

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Submitted Oct 1, 2014; accepted Dec 17, 2014.