Enhancement of Nitric Oxide Production Is Responsible for Minimal Intimal Hyperplasia of Autogenous Rabbit Arterial Grafts

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Background: Vascular endothelium induces smooth muscle cell (SMC) relaxation mainly mediated by endothelium-derived nitric oxide (EDNO) and endothelium-derived hyperpolarizing factor (EDHF). It has previously been reported that functions of these endothelium factors have been greatly impaired in vein grafts. The present study was undertaken to determine whether the functions of EDNO and EDHF might be altered in artery graft.

Methods and Results: In rabbits, the right carotid artery was excised and implanted in its original position as an autogenous graft (“artery graft”) and the non-operated left carotid artery served as the “control artery”. Histochemical changes, acetylcholine (ACh)-induced effects on the intracellular concentration of Ca^{2+} ($[\text{Ca}^{2+}]_i$) in endothelial cells, endothelium-dependent SMC hyperpolarization and relaxation, and tissue cGMP content were examined on post-operative day 28. “Artery graft” displayed a minimal amount of intimal hyperplasia. When compared with the “control artery”, it exhibited greater ACh-induced, endothelium-dependent relaxation, but the reverse was true when EDNO production was blocked. In the “artery graft” (vs. the “control artery”), basal cGMP content was greater, whereas the $[\text{Ca}^{2+}]_i$ increase in endothelial cells and the endothelium-dependent SMC-hyperpolarization induced by ACh were less.

Conclusions: It is suggested that the $[\text{Ca}^{2+}]_i$ -independent EDNO production covers the loss of function of endothelium-dependent SMC hyperpolarization and minimizes intimal hyperplasia caused by surgical operation in autogenous carotid artery graft.

Key Words: Acetylcholine; Calcium-activated K^+ channels; Endothelial cell Ca^{2+} concentration; Endothelium-dependent smooth muscle cell hyperpolarization; Nitric oxide

Vascular endothelium induces relaxation in smooth muscle cells (SMCs) mainly through actions mediated by endothelium-derived nitric oxide (EDNO)^{1,2} and endothelium-derived hyperpolarizing factor (EDHF).^{3,4} The former induces an increased cGMP content in SMCs through activation of soluble guanylyl cyclase (sGC), thus inducing SMC relaxation.^{2,5} Endothelial cell receptor agonists, such as acetylcholine (ACh), increase the intracellular concentration of Ca^{2+} ($[\text{Ca}^{2+}]_i$) and induce endothelial cell hyperpolarization (ECH) through activation of endothelial calcium-activated K^+ channels, with either intermediate conductance ($\text{K}_{\text{Ca}3.1}$, IK_{Ca}) or small conductance ($\text{K}_{\text{Ca}2.3}$, SK_{Ca}), and thereby lead to SMC hyperpolarization and relaxation.^{6–8} It has recently been suggested that ECH may play a central role in EDHF-mediated SMC relaxation in arteries and arterioles.⁴ However, it remains unclear what roles are played by EDNO and EDHF in vascular remodeling in a bypass graft.

Bypass grafting of the artery and vein is an effective and durable treatment for many patients with atherosclerotic occlusive diseases of the coronary or peripheral circulations.^{9–12} In such grafts, certain aspects of the surgical operation itself, such as skeletonization (loss of vasa vasorum, innervation, and lymphatic drainage) and anastomosis-construction, are thought to induce inflammation in the adventitia and intima layers, leading to intimal hyperplasia.^{11,13,14} We recently found that the walls of grafted veins displayed a massive increase in the number of SMCs in the intimal layer, and subsequently an accelerated atherogenesis, factors that have been suggested to be responsible for graft occlusion.^{15,16} We also found that the functions of EDNO and endothelium-dependent SMC hyperpolarization activated by endothelium receptor agonists were both impaired in rabbit vein grafts.^{17–19} However, it is unknown how the surgical operation itself affects the functions of EDNO and EDHF in such grafted arteries and veins.

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Table 1. Concentrations of Phenylephrine Used and the Tension They Induced in the Presence and Absence of Endothelium and of the Nitric Oxide-Synthase Inhibitor, L-NNA

	Endothelium (+)					Endothelium (–)		
	Concentration ($\mu\text{mol/L}$)		Tension (mN/mm)		n	Concentration ($\mu\text{mol/L}$)	Tension (mN/mm)	n
	L-NNA (–)	L-NNA (+)	L-NNA (–)	L-NNA (+)		L-NNA (–)	L-NNA (–)	
ACh-induced relaxation								
Control	0.92±0.12	0.06±0.01*	1.12±0.18	1.43±0.05	5	0.11±0.02	1.12±0.14	5
Graft	0.38±0.06†	0.04±0.01*†	0.96±0.01	1.41±0.09*	5	0.05±0.02†	1.01±0.13	5
NOC-7-induced relaxation								
Control	ND	0.09±0.03	ND	1.25±0.16	5	ND	ND	
Graft	ND	0.03±0.01	ND	1.14±0.13	5	ND	ND	
A23187-induced relaxation								
Control	ND	0.06±0.00	ND	1.46±0.20	5	ND	ND	
Graft	ND	0.03±0.01†	ND	1.29±0.27	5	ND	ND	

Endothelium (+), endothelium-intact preparations; Endothelium (-), endothelium-denuded preparations; L-NNA (+), in the presence of L-NNA; L-NNA (-), in the absence of L-NNA; ACh, acetylcholine; Control, control artery; Graft, artery graft; ND, not determined; L-NNA, N^G-nitro-L-arginine. Data are shown as mean $\pm$ SEM. *P<0.05 vs. 'L-NNA (-)'. †P<0.05 vs. 'Control'.

Furthermore, although it is known that when used for coronary artery bypass, the patency of artery grafts, such as internal mammary and radial artery grafts, is superior to that of a saphenous vein graft,^{9,10,20–23} the mechanism underlying this superiority in patency remains to be clarified.

Here, to examine the effects on endothelial function caused by the surgical operation required for “artery graft”, we developed a simple autogenous common carotid artery graft model in the rabbit. In such artery-grafted rabbits, both blood pressure and the blood flow through the grafted artery were normal when compared with those in non-operated rabbits. Using this model, we studied whether, and if so how, the functions of EDNO and endothelium-dependent SMC hyperpolarization in the “artery graft” are modulated by the surgical operation. The changes in endothelial cell [Ca²⁺]_i, SMC membrane potential, and endothelium-dependent relaxation induced by an endothelium receptor agonist (ACh) or by a non-receptor stimulant (A23187) were examined. The effects were compared between “artery graft” and the contralateral “control artery” in rabbits that had undergone grafting in one common carotid artery.

Methods

Animals and Artery Graft Implantation

All experiments conformed to the 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.

Male Japanese albino rabbits (2.5–3.0 kg; Nippon SLC, Hamamatsu, Japan) were randomized to the following 2 groups: non-operated rabbits (n=5) and carotid artery-grafted rabbits (n=25). The procedure used to create the artery graft was as follows. Anesthesia was induced intramuscularly with ketamine hydrochloride (50 mg/kg) and xylazine (10 mg/kg), then maintained with an intravenous administration of ketamine hydrochloride (10 mg/kg) and xylazine (10 mg/kg), given as and when required.^{17–19} After a longitudinal neck incision, the right common carotid

artery was exposed, then clamped distally and proximally. An approximate 2.5-cm segment of the carotid artery was taken with meticulous care (to avoid injuring the graft wall), and kept moist in heparinized saline (5 IU/mL) at room temperature. The blood inside the carotid artery was flushed out with heparinized saline. The segment was then returned to its original position, and anastomosed in an end-to-end fashion into the divided artery with interrupted 8-0 polypropylene sutures under a surgical microscope (“artery graft”). The wound was closed in a layer-to-layer fashion.

On post-operative day 28, carotid artery-grafted rabbits (n=20) were used to supply tissues for histochemical examination (n=5), tension and endothelial [Ca²⁺]_i measurements (n=5), cGMP measurement (n=5), and electrophysiological study (n=5).

Measurement of Mean Blood Pressure, Heart Rate, and Common Carotid Artery Blood Flow Under Anesthesia

Under anesthesia induced by intravenous administration of ketamine (10 mg/kg) and xylazine (10 mg/kg), mean blood pressure, heart rate, and common carotid artery blood flow were measured in age-matched, non-operated rabbits (n=5) and in rabbits that had undergone right carotid artery grafting (n=5). The mean blood pressure was measured invasively from the femoral artery (using Life Scope VS; Nihon Kohden, Tokyo, Japan) and blood flow from the right common carotid artery (TS420 transit time perivascular flowmeter; Transonic System Inc., Ithaca, NY, USA).

Isometric Tension Measurement

After the rabbits had been sacrificed with an overdose of pentobarbital (50 mg/kg intravenously), both the “artery graft” and the “control artery” (non-operated left common carotid artery) were immediately excised, placed in Krebs solution, and cleaned by removal of connective tissues.

A ring preparation (~1 mm wide) from the middle portion of the excised artery was suspended for measurement of isometric tension (calculated per millimeter length of ring) in an organ chamber containing Krebs solution at 37°C and gassed with 95% oxygen and 5% carbon dioxide.^{17,18,24} The inner diameter of the control artery and

artery graft was 0.44 ± 0.02 mm and 0.47 ± 0.03 mm respectively ($P=0.367$). Resting tension was adjusted to obtain maximum contraction induced by 128 mmol/L- K^+ solution. Guanethidine ($5 \mu\text{mol/L}$, to prevent effects due to release of sympathetic transmitters) and diclofenac sodium ($3 \mu\text{mol/L}$, to inhibit the production of cyclooxygenase products) were present throughout the experiments.

To obtain concentration-dependent responses, ACh was cumulatively applied during the contraction induced by phenylephrine in endothelium-intact or endothelium-denuded preparations. To study the influence of EDNO, the effects of ACh were examined in the presence and absence of the nitric oxide (NO)-synthase inhibitor, N^{ω} -nitro-L-arginine (L-NNA, 0.1 mmol/L), which was applied as pre-treatment for 60 min and was present thereafter. The concentration of phenylephrine was adjusted in each preparation so as to obtain matched amplitudes of contraction between "control artery" and "artery graft" (Table 1). In some preparations, the endothelium was removed, as described elsewhere.¹⁷⁻¹⁹

Concentration-response data for the calcium ionophore, A23187,²⁵ and the NO donor, NOC-7,¹⁸ were obtained by their cumulative application during the contraction induced by phenylephrine in endothelium-intact strips pre-treated for 60 min with 0.1 mmol/L L-NNA.

Measurement of $[Ca^{2+}]_i$

The $[Ca^{2+}]_i$ in endothelial cells was estimated using the ratiometric fluorescence Ca^{2+} -indicator, Fura 2, as previously described.^{17-19,24} 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-s intervals, as previously described.^{18,19} ACh was applied for 90 s in ascending order with a 10-min interval, then A23187 was applied for 90 s. The mean values of F_{340}/F_{380} obtained from 6 endothelial cells in each

preparation were averaged, and one value per preparation was used for later analysis.

Electrophysiological Study

The SMC membrane potential measurements were made using a conventional microelectrode technique, as previously described.^{17,19,24} ACh was applied for 90 s at 20–30 min intervals. Then, A23187 was applied for 60 s in the same preparations.

Immunohistochemical Staining

The harvested "artery graft" and "control artery" were immersion-fixed in 4% paraformaldehyde, embedded in optical-cutting temperature compound (Tissue-Tek; SAKURA Finetechnical, Tokyo, Japan), and then frozen. The sections ($5\text{-}\mu\text{m}$ thickness) were incubated overnight at 4°C with the appropriate primary antibody. The primary antibodies used were mouse monoclonal antibodies to rabbit smooth muscle myosin heavy chain (MHC) (SM1; 1:1,000 dilution; Yamasa, Tokyo, Japan), non-muscle MHC (SMemb; 1:750 dilution; Yamasa), endothelial cells (CD31; 1:30 dilution; Dako, Glostrup, Denmark), and rabbit macrophages (RAM11; 1:100 dilution; Dako). A 3,3'-diaminobenzidine tetrahydrochloride (DAB) was used for chromogenic reaction and hematoxylin for counter-stain.^{19,26}

Assessment of Vascular Wall Thickness

Vascular wall thickness was taken as the average of measurements made at 8 randomly selected places per section. Five sections were examined in the same way and the values obtained from them were averaged to represent the wall thickness of the arteries.^{17,19}

Determination of cGMP Content

The levels of cGMP were assayed using an enzyme-

immunoassay kit according to the manufacturer’s instruction (Cayman Chemical, Ann Arbor, MI, USA), as previously described.²⁶

Solutions

The composition of the Krebs solution was as follows (mmol/L): Na⁺, 137.4; K⁺, 5.9; Mg²⁺, 1.2; Ca²⁺, 2.5; HCO⁻₃, 15.5; H₂PO⁻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 ACh hydrochloride (Daiichi Pharmaceutical, Tokyo, Japan), L-phenylephrine hydrochloride, 1-[(2-chlorophenyl)diphenylmethyl]-1H-pyrazole (TRAM-34), 18-β-glycyrrhetic acid, diclofenac sodium, and DAB (Sigma Chemical Co, St Louis, MO, USA), apamin, charybdotoxin, and L-NNA (Peptide Institute Inc., Osaka, Japan), A23187 (Merck Chemicals GmbH, Darmstadt, Germany), NOC-7 (Dojindo Laboratories, Kumamoto, Japan), guanethidine (Tokyo Kasei, Tokyo, Japan), and Fura 2-acetoxymethyl ester (Molecular Probes, Eugene, OR, USA).

Statistical Analysis

All results are expressed as mean±SEM, with n values

Table 2. Effects of the Presence of Endothelium and of the Nitric Oxide-Synthase Inhibitor, L-NNA, on the Contraction Induced by 128 mmol/L K ⁺		
	Control artery	Artery graft
Endothelium (+) (mN/mm)		
L-NNA (-)	5.15±0.32	3.75±0.10†
n	5	5
L-NNA (+)	6.60±0.32*	5.90±0.20*
n	5	5
Endothelium (-) (mN/mm)		
L-NNA (-)	5.75±0.45	5.40±0.43
n	5	5

Endothelium (+), endothelium-intact preparations; Endothelium (-), endothelium-denuded preparations; L-NNA (+), in the presence of L-NNA; L-NNA (-), in the absence of L-NNA. Data are shown as mean±SEM. L-NNA, N^ω-nitro-L-arginine. *P<0.05 vs. ‘L-NNA (-)’. †P<0.05 vs. ‘Control artery’.

representing the number of rabbits used (each rabbit provided one “artery graft” segment and one “control artery” segment for a given experiment). A 1-way or 2-way repeated-measure ANOVA, with post-hoc comparisons made using the Scheffé procedure or the Student unpaired t-test, was used for the statistical analysis. The level of

Figure 3. Cyclic guanosine monophosphate (cGMP) production and the action of NOC-7 and A23187 in artery preparations. **(A)** cGMP production in the presence and absence of acetylcholine (ACh) in endothelium-intact strips from “control artery” and “artery graft”. cGMP contents in the absence (“Basal”) and presence of 30 μ mol/L ACh (“ACh”) in “control artery” (“Control”) and “artery graft” (“Graft”). The effects were observed in the absence [“L-NNA (–)”) and presence of N^ω-nitro-L-arginine (L-NNA) [“L-NNA (+)”) (n=5, in each case). Data are shown as mean $\pm$ SEM. *P<0.05 vs. “L-NNA (–)”, [†]P<0.05 vs. “Basal”, #P<0.05 vs. “Control” “L-NNA (–)”. **(B)** Effects of NOC-7 and A23187 on the contraction induced by phenylephrine in endothelium-intact rings treated with L-NNA. Summary of the effects of NOC-7 (**B-a**) and A23187 (**B-b**) in “control artery” (“Control”) and “artery graft” (“Graft”). To obtain matched amplitudes of contraction between “control artery” and “artery graft”, the concentration of phenylephrine was adjusted. The tension attained just before application of the first concentration of ACh was normalized as a relative tension of 1.0. Data are shown as mean $\pm$ SEM (n=5).

significance was set at P<0.05.

Results

Mean Blood Pressure, Heart Rate, and Common Carotid Artery Blood Flow

Neither the mean blood pressure nor heart rate measured under anesthesia was significantly different between non-operated normal rabbits and carotid artery grafted rabbits (n=5, in each case; P>0.05). The mean arterial blood pressures (mmHg) were 69.8 $\pm$ 7.9 for normal rabbits and 69.0 $\pm$ 5.1 for graft-operated rabbits (n=5, in each case; P=0.937). In normal rabbits and graft-operated rabbits, respectively, the values obtained for heart rate (bpm) were 188 $\pm$ 22 and 167 $\pm$ 16 (n=5, in each case; P=0.486) and for blood flow (mL/min) being 20.1 $\pm$ 1.5 and 16.9 $\pm$ 0.5 (n=5, in each case; P=0.092).

Intimal Hyperplasia in Artery Graft

“Artery graft” displayed a minimal amount of intimal hyperplasia (Figure 1A-c, 1A-d, 1B-c), while “control artery” exhibited no intimal hyperplasia at all (Figure 1A-a, 1A-b, 1A-c). Neither media thickness (Figure 1B-a) nor the number of nuclei across the media (Figure 1B-b) nor the lumen area (Figure 1B-d) was significantly different between the “control artery” and “artery graft”.

Expression of CD31 was detected in both the “control artery” (Figure 1C-a) and the “artery graft” (Figure 1C-e). Expressions of SMemb (Figure 1C-b, 1C-f) and SM1 (Figure 1C-c, 1C-g) were detected within the intimal hyper-

plasia only in the “artery graft”. Similarly, RAM11 was not found in “control artery” (Figure 1C-d), but was expressed in “artery graft” (in very small amounts) just beneath the internal elastic lamina (Figure 1C-h, indicated by arrow-heads).

Effects of Endothelium on High K⁺-Induced Tension

In endothelium-intact preparations, 128 mmol/L-K⁺ induced a phasic and subsequently generated tonic contraction (before application of L-NNA). The maximum absolute tension induced by 128 mmol/L-K⁺ was significantly less in “artery graft” than in “control artery” (Figure 2A; Table 2). The high K⁺-induced tension was enhanced by a 60-min L-NNA application in both arteries (n=5, P<0.05), with the amplitude of contraction in the presence of L-NNA being similar between the two arteries (n=5, P>0.05; Table 2).

In endothelium-denuded preparations, the absolute maximum tension induced by 128 mmol/L-K⁺ in “artery graft” was not significantly different from those in “control artery” (n=5, P=0.633; Table 2).

ACh-Induced, Endothelium-Dependent Relaxation

Actual traces showing the effects of ACh on the tension induced by phenylephrine were obtained for endothelium-intact “control artery” (Figure 2A-a) and “artery graft” (Figure 2A-b). ACh concentration dependently induced a relaxation in both arteries; the concentrations of phenylephrine used to induce contraction having been adjusted to obtain matched amplitudes of contraction (Table 1). In endothelium-intact preparations from “control artery”,

Figure 4. Effects of acetylcholine (ACh) and A23187 on endothelial cell $[Ca^{2+}]_i$ (as estimated from the Fura 2 fluorescence ratio) in “control artery” and “artery graft”. (A) 340nm Fura 2 fluorescence image of endothelial cells in “control artery”. (B) Effects of ACh and A23187 on the Fura 2 fluorescence ratio as a function of time in “control artery”. (C) Basal Fura 2 fluorescence ratio in “control artery” (“Control”) and “artery graft” (“Graft”). The Fura 2 ratio was collected in 6 areas (shown in A). Summary of the effects of ACh (D, $n=5$ in each case) and A23187 (E, $n=5$ in each case) in “control artery” (“Control”) and “artery graft” (“Graft”). Data are shown as mean $\pm$ SEM. * $P < 0.05$ vs. “Control”.

ACh-induced relaxation was inhibited by a 60-min application of L-NNA ($n=5$, $P < 0.05$), and the non-selective $K_{Ca}3.1$ inhibitor, charybdotoxin, together with the $K_{Ca}2.3$ inhibitor, apamin, blocked the ACh-induced relaxation in the presence of L-NNA (Figure 2B-a).

As shown in Figure 2B-b, the ACh-induced relaxation was larger in “artery graft” than in “control artery” in endothelium-intact preparations ($n=5$, $P < 0.05$). A 60-min application of L-NNA attenuated the ACh-induced relaxation in both arteries and, importantly, reversed the potency order for the ACh-induced relaxation (i.e., ACh was more potent in “control artery” than in “artery graft” in the presence of L-NNA).

In endothelium-denuded preparations, ACh did not modify the phenylephrine-induced tension in either “control artery” or “artery graft” (Figure 2B-c; Table 1).

Effects of ACh on cGMP Production

The basal cGMP content was significantly greater in “artery graft” ($n=5$) than in “control artery” ($n=5$, $P < 0.05$; Figure 3A). ACh ($30 \mu\text{mol/L}$) increased the cGMP content in both arteries, with the level attained in the presence of ACh being not significantly different between the two arteries ($n=5$, $P = 0.14$).

L-NNA greatly reduced the cGMP contents of both “control artery” and “artery graft” in the absence and in the presence of ACh ($n=5$, $P < 0.05$).

Relaxations Induced by NOC-7 and A23187 in the Presence of L-NNA

During the contraction induced by phenylephrine in L-NNA-treated preparations (Table 1), NOC-7¹⁷ ($n=5$, Figure 3B-a) or A23187²⁵ ($n=5$, Figure 3B-b) induced a concentration-dependent relaxation that was of similar magnitude between “control artery” and “artery graft” (for NOC-7, 2-way repeated ANOVA, $P = 0.83$; for A23187, $P = 0.71$).

Endothelial Cell $[Ca^{2+}]_i$ Changes Induced by ACh and A23187

Figure 4A shows a 340nm Fura 2 fluorescence image of endothelial cells in “control artery” under basal conditions. Under basal conditions, the Fura 2 fluorescence ratio (340/380nm) in “artery graft” was similar to that in “control artery” ($n=5$, $P = 0.996$; Figure 4B, 4C). ACh concentration dependently increased endothelial cell $[Ca^{2+}]_i$ (estimated from the Fura 2 fluorescence ratio), with the responses being significantly smaller in “artery graft” than in “control artery” ($n=5$, $P < 0.05$; Figure 4D).

The increase in endothelial cells $[Ca^{2+}]_i$ induced by A23187 was greater than that induced by ACh in both “control artery” and “artery graft”. The maximum $[Ca^{2+}]_i$ level attained under A23187 was similar between “control artery” and “artery graft” ($P = 0.389$; Figure 4E).

SMC Hyperpolarization Induced by ACh and A23187

In “control artery”, the resting membrane potential of SMCs was $-50.2 \pm 0.4 \text{ mV}$, and ACh induced hyperpolarization at $19.7 \pm 1.0 \text{ mV}$ ($n=5$). A 60-min application of L-NNA (0.1 mmol/L) modified neither the resting membrane potential of SMCs ($-50.4 \pm 0.6 \text{ mV}$, $n=5$, $P > 0.05$) nor the ACh-induced hyperpolarization ($18.6 \pm 0.7 \text{ mV}$, $n=5$; $P > 0.05$).

In “control artery”, application of the $K_{Ca}3.1$ inhibitor, TRAM-34, together with the $K_{Ca}2.3$ inhibitor, apamin, did not modify the SMC membrane potential ($-49.6 \pm 0.5 \text{ mV}$, $n=5$; $P = 0.704$), but it did inhibit the ACh-induced hyperpolarization ($9.2 \pm 1.8 \text{ mV}$, $n=5$; $P < 0.05$, Figure 5A-a). The non-selective $K_{Ca}3.1$ inhibitor, charybdotoxin, together with apamin depolarized SMCs (to $-45.6 \pm 0.4 \text{ mV}$, $n=5$, $P < 0.05$) and blocked the ACh-induced hyperpolarization ($2.7 \pm 0.6 \text{ mV}$ depolarization, $n=5$; $P < 0.05$; Figure 5A-a). The myoendothelial gap-junction inhibitor, 18- β -glycyrrhetic acid,⁶ depolarized SMCs ($25.2 \pm 2.8 \text{ mV}$, $n=5$) and inhibited the hyperpolarization induced by ACh ($6.3 \pm 1.0 \text{ mV}$, $n=5$;

Figure 5. Effects of acetylcholine (ACh) and A23187 in smooth muscle cell membrane potential in “control artery” and “artery graft”. **(A-a)** Membrane potential (MP) changes induced by ACh before and after application of TRAM-34 (10 μmol/L)+apamin (0.1 μmol/L) or CTX (0.1 μmol/L)+apamin (0.1 μmol/L) in “control artery”. **(A-b)** Membrane potential changes induced by ACh before and after application of 18-β-glycyrrhetic acid (100 μmol/L) in “control artery”. **(B)** Membrane potential changes induced by ACh and A23187 in “control artery” **(B-a)** and “artery graft” **(B-b)**. **(C)** Summary of resting membrane potential (RMP) of smooth muscle cells **(C-a)** and the effects of ACh **(C-b)** and A23187 **(C-c)** on smooth muscle cell membrane potential in “control artery” (“Control”) and “artery graft” (“Graft”). Data are shown as mean±SEM (n=5). *P<0.05 vs. ‘Control’.

Figure 5A-b).

The resting SMC membrane potential in “artery graft” (n=5) was not significantly different with that in “control artery” (n=5; $P>0.05$; **Figure 5C-a**). ACh induced SMC hyperpolarization in a concentration-dependent manner in both “control artery” and “artery graft”, with the responses being greater in “control artery” than in “artery graft” ($P<0.05$ for each ACh concentration; **Figure 5B-a,5C-b**). A23187 induced SMC hyperpolarization in both “control artery” and “artery graft”, with the hyperpolarization in “control artery” (n=5) being similar to that in “artery graft” (n=5, in each case; $P=0.08$, **Figure 5B-b,5C-c**).

Discussion

In the autogenous artery graft, agonist-induced EDNO-mediated relaxation was augmented while inhibiting agonist-induced, endothelium-dependent SMC hyperpolarization through downregulation of receptor-activated endothelial $[Ca^{2+}]_i$ mobilization. It is suggested that in such an autogenous artery graft, the increased receptor-mediated EDNO production, being $[Ca^{2+}]_i$ independent, covers for the reduced endothelium-dependent SMC hyperpolarization function and thus minimizes intimal hyperplasia. These results are markedly different from our previous findings with vein grafts in which pronounced intimal thickening was associated with dysfunction of both EDNO and EDHF.¹⁹ Based on these findings, the preserved endothelial function and the minimal intimal thickening may explain the improved patency of autogenous arterial grafts compared to the vein grafts in aortocoronary revascularization.

The “Artery graft” had developed a minimal amount of intimal hyperplasia, while “control artery” displayed no intimal hyperplasia. The smooth muscle MHC isoforms, SM1 and SMemb,^{27,28} but not the macrophage marker, RAM11, were present within the intimal hyperplasia (**Figure 1**), suggesting that proliferative SMCs may be one of the major cells expressed in such hyperplasia in the present artery graft. These results suggest that certain features of the surgical operation for “artery graft” (such as skeletonization and anastomosis-construction) may themselves induce vascular wall inflammation, and thus cause a minimal amount of intimal hyperplasia.

In various arteries and veins, endothelium receptor agonists such as ACh increase $[Ca^{2+}]_i$ in endothelial cells and induce relaxation through actions mediated by EDNO,^{1,2} prostacyclin,²⁹ and EDHF.^{3-5,26} EDNO increases cGMP production in SMCs via activation of sGC, and thereby induces SMC relaxation.^{2,5} In the present “artery graft” (vs. “control artery”), we found that: (1) cGMP content under basal conditions was greater; (2) the endothelial cell $[Ca^{2+}]_i$ under basal conditions was the same (**Figure 4C**); (3) the absolute tension induced by high K^+ in the absence of the NO synthase inhibitor, L-NNA, was less (**Table 2**); and (4) the relaxation induced by the NO-donor, NOC-7, in the presence of L-NNA was not significantly different (**Figure 3B**). These results suggest that in “artery graft”, spontaneous release of EDNO is increased in a $[Ca^{2+}]_i$ -independent manner and then inhibits vascular contraction. We also found in “artery graft” (vs. “control artery”) that: (5) ACh-induced endothelial cell $[Ca^{2+}]_i$ increase was less (**Figure 4D**); (6) ACh-induced L-NNA-sensitive relaxation was greater, while ACh-induced L-NNA-resistant

relaxation was lesser (**Figure 2B-b**); and (7) the resting membrane potential of SMCs was similar, but ACh-induced SMC hyperpolarization was less (**Figure 5C-a, 5C-b**), indicating that ACh-induced EDHF-mediated relaxation was downregulated in “artery graft”.

It is noted that the level of cGMP content in the presence of ACh in “artery graft” was similar to that in “control artery” (**Figure 3A**) under the conditions in which ACh-induced endothelial cell $[Ca^{2+}]_i$ increase was less in “artery graft”. These results suggest that ACh induces greater relaxation in “artery graft” due to not only the enhancement of spontaneous release of EDNO, but also because of the increase in sensitivity of receptor-mediated EDNO production to $[Ca^{2+}]_i$, which possibly caused by $[Ca^{2+}]_i$ -independent endothelial NO synthase (eNOS) activation (such as eNOS phosphorylation)^{32–34} and/or increased expression of NO synthases (nNOS, iNOS and eNOS). We previously found that the enhancement of EDNO production seen in vein grafts in experimental animals could be useful for preventing both intimal hyperplasia and late graft failure.^{15–19,22,30,31} Taken together, these suggest that increased functions of receptor-mediated EDNO may be responsible for inhibition of not only vascular tone but also vascular remodeling in our “artery graft”.

It has been suggested that ECH itself, rather than endothelium-derived factors, plays an essential role in ACh-induced EDHF-mediated relaxation.^{4–6} ACh activates both $K_{Ca3.1}$ and $K_{Ca2.3}$ via an increase in endothelial cell $[Ca^{2+}]_i$ and then produces ECH, which induces SMC hyperpolarization through direct electrical coupling via myoendothelial gap junctions, thus inducing SMC relaxation.⁴ This hypothesis is supported by the finding in guinea pig carotid artery in which the gap-junction inhibitor, carbenoxolone (a water-soluble salt of 18- β -glycyrrhetic acid), abolished ACh-induced, endothelium-dependent SMC hyperpolarization, but had no effect on ACh-induced ECH.⁶ Here, we found that in “control artery”, ACh induced a SMC hyperpolarization that was, in part, inhibited by TRAM-34 ($K_{Ca3.1}$ blocker) plus apamin ($K_{Ca2.3}$ blocker) and completely blocked by charybdotoxin (a non-selective $K_{Ca3.1}$ blocker) plus apamin.¹⁹ Furthermore, 18- β -glycyrrhetic acid inhibited the ACh-induced SMC hyperpolarization. Thus, these results suggest that ECH, acting via myoendothelial gap junction, plays an essential role in ACh-induced SMC relaxation in the rabbit common carotid “control artery”.

We found that ACh-induced: (1) $[Ca^{2+}]_i$ increase in endothelial cells; (2) endothelium-dependent SMC hyperpolarization; and (3) vascular relaxation in the presence of L-NNA were all downregulated in “artery graft” vs. “control artery”. In contrast, none of these 3 responses, when induced by A23187, was significantly different between “artery graft” and “control artery”. These results suggest that receptor-mediated signal transduction pathways for increases in $[Ca^{2+}]_i$ in endothelial cells may be dysfunctional in “artery graft”, leading to downregulation of endothelium-dependent SMC hyperpolarization. We previously found that in a rabbit autologous jugular vein graft, ACh induces neither $[Ca^{2+}]_i$ increase in endothelial cells nor endothelium-dependent SMC hyperpolarization nor vascular relaxation.^{15–19} These findings in the “jugular vein graft” are in contrast with those made in the present study on the “carotid artery graft”.

It is known that an artery graft (internal mammary

artery) is superior in patency to a vein graft (saphenous vein) when used for coronary artery bypass grafting.^{9,10} Furthermore, it has also been reported that pronounced intimal thickening is associated with impairment of endothelial responses in the canine venous graft, whereas intact endothelial function and no intimal thickening were observed in the arterial graft.²² Thus, the present study results suggest that endothelial functions are better retained in “artery graft” than in the vein graft, which may contribute to the former’s superiority in patency. However, the detailed mechanism underlying this will need to be clarified in future studies.

In conclusion, we developed a rabbit model of artery grafting to examine the effects of surgical operation on function of endothelium-dependent relaxing factors towards relaxation and vascular remodeling in artery graft. Using this model, we found that receptor-mediated, endothelium-dependent relaxation is enhanced in “artery graft”. We suggest that this enhancement is due to increases in both spontaneous and receptor-activated EDNO release under conditions in which receptor-mediated increases in $[Ca^{2+}]_i$ in endothelial cells are, in part, impaired. Our results also suggest that this upregulation of the EDNO function could be beneficial in reducing intimal hyperplasia in the artery graft. In addition, the minimal intimal thickening may thus explain the improved patency of autogenous arterial grafts compared to the vein grafts in aortocoronary revascularization. This is the first demonstration to show how functions of EDNO and EDHF are modulated by the surgical operation performed for “artery graft”. Ways of restoring the function of EDHF in artery grafts need to be clarified in future studies.

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Author Contributions

K.T., R.O., J.K., and T.I. performed the research. K.T., R.O., J.K., and K.K. analyzed the data. R.O. assessed vascular walls, as a blind pathologist. K.T., K.K., and T.I. designed the research study and T.I., K.K. and J.K. wrote the manuscript. All authors provided comments on initial and final drafts of the manuscript.

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