Title: Reduced function of endothelial nitric oxide and hyperpolarization in artery grafts with poor runoff

Short title: NO and EDHF Function in Artery Grafts

Authors: Masashi Sakakibara, MD[1]; Akio Kodama, MD, PhD[1]; *Kimihiro Komori, MD, PhD[1]; *Takeo Itoh, PhD[2]

Affiliations:

[1]Division of Vascular Surgery, Department of Surgery, Nagoya University Graduate School of Medicine, Nagoya, Japan

[2]Department of Rehabilitation, Faculty of Health Sciences, Nihon Fukushi University, 26-2 Higashihaemi-cho, Handa, Japan

***Corresponding authors:**

Kimihiro Komori, MD, PhD, Division of Vascular Surgery, Department of Surgery, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan. Phone: +81-52-744-2224. Fax: +81-52-744-2226. E-mail: komori@med.nagoya-u.ac.jp

Takeo Itoh, PhD, Department of Rehabilitation, Faculty of Health Sciences, Nihon Fukushi University, 26-2 Higashihaemi-cho, Handa 477-0031, Japan. Phone: +81-569-20-0118. E-mail: titoh@n-fukushi.ac.jp

Author Contributions: M.S. and T.I. performed the research. M.S. and T.I. analyzed the data. A.K. assessed vascular walls as a blinded pathologist. K.K. and T.I. designed

the research study. M.S and T.I. wrote the manuscript. All authors provided comments on the initial and final drafts of the manuscript.

Abstract

Background: The endothelium regulates vascular tonus by releasing nitric oxide (endothelium-derived nitric oxide, EDNO) and hyperpolarizing factor (endothelium-derived hyperpolarizing factor, EDHF). In vein grafts with poor runoff, lack of function of these factors causes severe intimal hyperplasia. This study evaluated how the functions of EDNO and EDHF are altered in artery grafts under poor runoff conditions.

Materials and Methods: The right common carotid arteries of rabbits were excised and implanted in their original positions as autogenous grafts under normal runoff conditions ("nonoccluded grafts") or poor runoff conditions ("poor runoff grafts"). Histochemical changes, acetylcholine (ACh)-induced effects on endothelium-dependent relaxation and smooth muscle cell (SMC) hyperpolarization were examined.

Results: Both artery graft types displayed negligible intimal hyperplasia. In the absence and presence of an EDNO synthase inhibitor, ACh-induced relaxation was lower in grafts with poor runoff than in nonoccluded grafts. Furthermore, ACh-induced but not nonreceptor agonist A23187-induced SMC hyperpolarization was lower in the poor runoff graft group than in the nonoccluded graft group.

Conclusions: Unlike in those in vein grafts, the functions of EDNO and EDHF in autogenous carotid artery grafts under poor runoff conditions were reduced but partly maintained. In such artery grafts, intimal hyperplasia caused by surgical operation was

not present. These results may explain some of the mechanisms underlying the improved patency of artery grafts compared with vein grafts.

Keywords: Artery graft; Endothelium-dependent smooth muscle cell hyperpolarization; Intimal hyperplasia; Nitric oxide

Introduction

Endothelial cells induce vascular smooth muscle cell (SMC) relaxation mainly through actions mediated by endothelium-derived nitric oxide (EDNO)[1,2] and endothelium-derived hyperpolarizing factor (EDHF)[3-9]. The former is released spontaneously and by receptor activation or shear stress[10-14]. Endothelial cell receptor agonists such as acetylcholine (ACh) and substance P increase the intracellular concentration of Ca^{2+} ($[Ca^{2+}]_i$) and then induce endothelial cell hyperpolarization (ECH) through activation of endothelial calcium-activated K^+ channels, with either intermediate conductance ($K_{Ca}3.1$, IK_{Ca}) or small conductance ($K_{Ca}2.3$, SK_{Ca}), thereby leading to SMC hyperpolarization and relaxation[7-11]. It was recently suggested that ECH plays a central role in EDHF-mediated SMC relaxation in arteries and veins[4,7,10,11,14]. However, it remains unclear what roles EDNO and EDHF play in vascular remodeling in a bypass with artery grafts under poor runoff conditions.

Bypass grafting with arterial and venous conduits is an effective and durable treatment for many patients with atherosclerotic occlusive disease, such as coronary artery disease and peripheral artery disease[15-18]. We have found that in rabbit venous grafts under poor runoff conditions, the functions of both EDNO and EDHF are completely lost, and the vascular wall displays a massive increase in the number of SMCs, factors that are responsible for graft occlusion[11-13,19-21]. It is well known that in terms of use 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[15,16,22,23]. However, 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 arterial grafts under poor runoff conditions, we developed a

simple autogenous common carotid artery graft model in rabbits. We studied whether, and if so how, the functions of EDNO and endothelium-dependent SMC hyperpolarization are modulated in such grafts. The changes were examined in endothelium-dependent SMC membrane potentials and relaxation induced by an endothelium receptor agonist (ACh or substance P) or by a nonreceptor stimulant (A23187)[24]. These effects were compared between a "poor runoff graft" and a "nonoccluded graft" that had undergone grafting in one common carotid artery or the contralateral "nongrafted control artery".

Materials and 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 they were approved by the Committee on the Ethics of Animal Experiments of that institution.

Male Japanese albino rabbits (2.5–3.0 kg; Nippon SLC, Hamamatsu, Japan) were randomized to the following 2 groups: rabbits that had an artery graft under normal distal conditions ("nonoccluded graft"; n=23) or poor runoff conditions ("poor runoff graft"; n=23). Males were chosen to minimize the effects of estrogen and other female hormones. The procedure used to create the arterial graft was as follows. Anesthesia was induced intramuscularly with ketamine hydrochloride (50 mg/kg) and xylazine (10 mg/kg) and then maintained with an intravenous administration of ketamine hydrochloride (10 mg/kg) and xylazine (10 mg/kg), given as and when required[12-14,19]. After a longitudinal neck incision, the right common carotid artery was exposed and

then clamped distally and proximally. An approximately 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 ("nonoccluded graft"). To create a "poor runoff graft", the internal carotid artery and two of the three branches of the external carotid artery were ligated so that the most inferior branch of the external carotid artery served as the only outflow[12,13,19]. The wound was closed in a layer-to-layer fashion.

On postoperative day 28, common carotid artery-grafted rabbits (n=18) were used to supply tissues for histological examination (n=7), tension measurements (n=5), and electrophysiological study (n=6).

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

On postoperative day 28, under anesthesia with intravenous ketamine (10 mg/kg) and xylazine (10 mg/kg), mean blood pressure, heart rate, and common carotid artery blood flow were measured in rabbits with "nonoccluded grafts" (n=5) or with "poor runoff grafts" (n=5). Blood pressure was measured invasively from the femoral artery (using Life Scope VS; Nihon Kohden, Tokyo, Japan), and blood flow was measured from the common carotid arteries (using a TS420 transit time perivascular flowmeter; Transonic System, Inc., Ithaca, NY, USA).

Histochemical Examination

On postoperative day 28, the "artery graft" was harvested under anesthesia with intravenous 10 mg/kg ketamine and 10 mg/kg xylazine, and the rabbit was then sacrificed with an overdose of pentobarbital (50 mg/kg intravenously). The harvested artery graft was fixed with 4% formaldehyde at a pressure of 100 mmHg for 30 min and then incubated overnight at room temperature in the same fixative.

A middle portion of the harvested graft was used for the morphometric analysis. Each paraffin-embedded sample was cut into 5-μm sections and stained by the Van Gieson staining method and hematoxylin and eosin method[12,13,19,25].

The vascular wall thickness was taken as the average of measurements made at 8 randomly selected places per section. Six sections were examined in the same way, and their values were averaged to represent the wall thickness of the arteries, as reported previously[12,13,21]. The lumen area in each section was examined, and the average value of 6 sections was taken as the value of the lumen area[12,13,21]. Vascular wall thickness and lumen area were measured with ImageJ software (National Institutes of Health; http://rsb.info.nih.gov/ij/).

Isometric Tension Measurement

After the rabbits had been sacrificed with an overdose of pentobarbital (50 mg/kg intravenously), both the "arterial graft" and the "control artery" (nonoperated left common carotid artery) were immediately excised, placed in Krebs solution, and cleaned by connective tissue removal.

A ring preparation (~1 mm wide) from the middle portion of the excised artery containing intact endothelium was suspended for measurement of isometric tension

(calculated per millimeter length of the ring) in an organ chamber containing Krebs solution at 37°C and gassed with 95% oxygen and 5% carbon dioxide[12-14,19]. The resting tension was adjusted to obtain maximum contraction induced by a 128 mmol/L-K^+ solution.

To obtain concentration-dependent responses, ACh (10^{-8}-$3x10^{-5}$ mol/L) was cumulatively applied during the contraction induced by phenylephrine in endothelium-intact preparations. To study the influence of EDNO, the effects of ACh 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 90 min and was present thereafter. The concentration of phenylephrine was adjusted in each preparation to obtain matched amplitudes of contraction between the "nonoccluded graft" and "poor runoff graft" (**Table 1**).

Electrophysiological Study

The SMC membrane potential measurements were made using a conventional microelectrode technique, as previously described[10-14]. To observe the concentration-dependent responses, ACh at each concentration was applied for 90 s at 20-30 min intervals. When the effects on the membrane were compared, ACh (10 μmol/L for 90 s), substance P (0.2 μmol/L for 90 s) and A23187 (1 μmol/L for 60 s)[24] were applied at 30 min intervals in the same preparations. In such experiments, substance P was applied only once to avoid tachyphylaxis of the response.

Solutions

The composition of the Krebs solution was as follows (mmol/L): Na^+, 137.4; K^+,

178 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
179 solutions were bubbled with 95% oxygen and 5% carbon dioxide (pH, 7.3–7.4).
180 Diclofenac sodium (3 μmol/L, to inhibit the production of cyclooxygenase products)
181 was present throughout the experiments.
182
183 **Drugs**
184 The drugs used were ACh hydrochloride (Daiichi Pharmaceutical, Tokyo, Japan),
185 L-phenylephrine hydrochloride and diclofenac sodium (Sigma Chemical Co, St Louis,
186 MO, USA), L-NNA, apamin, charybdotoxin, substance P (Peptide Institute, Inc., Osaka,
187 Japan), and A23187 (Merck Chemicals GmbH, Darmstadt, Germany).
188
189 **Statistical Analysis**
190 All results are expressed as the mean ± standard deviation (SD), with n values
191 representing the number of rabbits used (each rabbit provided one "artery graft"
192 segment for a given experiment). The normality of the distribution of continuous data
193 was assessed with the Kolmogorov-Smirnov test, and all variables were found to be
194 normally distributed. One-way or 2-way repeated-measures ANOVA, with post hoc
195 comparisons made using the Scheffé procedure or Student's unpaired t-test, was used
196 for the statistical analysis. The level of significance was set at $P<.05$.
197
198 ***Results***
199 **Mean Blood Pressure, Heart Rate and Common Carotid Artery Blood Flow**
200 Neither the mean blood pressure nor the heart rate measured under anesthesia was
201 significantly different between rabbits regardless of whether some of the distal artery

branches were ("poor runoff graft") or were not ("nonoccluded graft") occluded (n=5, in each case; P>.05). The mean blood pressure (mmHg) in rabbits with "nonoccluded grafts" (n=5, 74.6 ± 5.1) was similar to that in rabbits with "poor runoff grafts" (n=5, 76.8 ± 11.7; P=.71). The heart rate (bpm) in rabbits with "nonoccluded grafts" (130 ± 14) was the same as that in rabbits with "poor runoff grafts" (148 ± 39, n=5 for each case; P=.355).

The blood flow of the nonoperated left common carotid artery (noted as the "control artery") was 34.7 ± 6.5 mL/min in rabbits with "poor runoff grafts" and 33.5 ± 4.1 mL/min in rabbits with "nonoccluded grafts" (**Figure 1**). These values were not significantly different (n=5, in each case; P=.743). When compared with the "control artery", the blood flow was reduced to a minor extent in the "nonoccluded graft", while it was greatly reduced in the "poor runoff grafts". Blood flow was significantly lower in the "poor runoff graft" (n=5, 2.8 ± 1.8 mL/min) than in the "nonoccluded graft" (n=5, 24.2 ± 3.0 mL/min; P<.001; **Figure 1**).

Intimal Hyperplasia and Lumen Area in Artery Grafts

Both the "nonoccluded graft" and "poor runoff graft" displayed minimal amounts of intimal hyperplasia (**Figure 2A**). The media thickness (**Figure 2B-a**), number of nuclei across the media (**Figure 2B-b**) and intimal thickness (**Figure 2B-c**) were not significantly different between the "nonoccluded graft" and "poor runoff graft". Lumen area was significantly smaller in the "poor runoff grafts" (n=7, 0.29 ± 0.09 mm^2) than in the "nonoccluded grafts" (n=7, 0.56 ± 0.28 mm^2; P<.05; **Figure 2B-d**).

Effects of EDNO on High K^+-induced Tension

Before application of L-NNA, 128 mmol/L-K^+ induced a large phasic and subsequently generated tonic contraction in endothelium-intact rings from both the "poor runoff graft" and "nonoccluded graft". The maximum tension (E_{max}) induced by 128 mmol/L-K^+ was significantly larger in the "poor runoff graft" (n=5, 5.98 ± 1.34 mN/mm) than in the "nonoccluded graft" (n=5, 3.60 ± 1.18 mN/mm; P<.05; **Figure 3**).

A 90-min application of L-NNA enhanced the high K^+-induced tension in both grafts (n=5, P<.05), with the maximum tension being similar between the two grafts. These values were 7.14 ± 1.06 mN/mm for the "poor runoff graft" and 5.71 ± 1.00 mN/mm for the "nonoccluded graft" (n=5 in each case; P>.05; **Figure 3**).

Effects of EDNO on Phenylephrine-Induced Tension

In endothelium-intact rings, phenylephrine (10^{-7}-$3x10^{-5}$ mol/L) induced a concentration-dependent contraction in both grafts. Their EC_{50} values were 2.48 ± 0.47 μmol/L in the "poor runoff graft" and 3.13 ± 1.67 μmol/L in the "nonoccluded graft", which were not significantly different (n=5, in each case; P=.428). The E_{max} values of phenylephrine-induced tension were 5.67 ± 1.24 mN/mm in the "poor runoff graft" and 4.51 ± 1.19 mN/mm in the "nonoccluded graft", which were not significantly different (n=5, in each case; P=.171).

L-NNA shifted the concentration-response curve for phenylephrine to the left and enhanced the tension induced by phenylephrine at any given concentration. The EC_{50} values of phenylephrine in the presence of L-NNA were 0.33 ± 0.35 μmol/L for the "poor runoff graft" and 0.39 ± 0.26 μmol/L for the "nonoccluded graft", which were not significantly different (n=5, in each case; P=.794). The E_{max} of phenylephrine-induced tension in the presence of L-NNA was 7.43 ± 1.46 mN/mm for

the “poor runoff grafts” and 6.84 ± 1.37 mN/mm for the “nonoccluded grafts”, but this difference was not significant (n=5, in each case; P=.527).

ACh-Induced, Endothelium-Dependent Relaxation

ACh (10^{-8}-$3x10^{-5}$ mol/L) induced a concentration-dependent relaxation in both “control artery” and “artery grafts” during the contraction induced by phenylephrine: the concentrations of phenylephrine used to induce contraction were adjusted to obtain matched amplitudes of contraction before and after the application of L-NNA (**Table 1**).

Before the application of L-NNA, when compared with the “control artery”, ACh-induced relaxation was greater in the “nonoccluded graft” (n=5; P<.05) but lower in the "poor runoff graft" (n=5; P<.001; **Figure 4**). ACh-induced relaxation was significantly lower in the “poor runoff graft” group than in the “nonoccluded graft” group (n=5; P<.05).

A 90-min application of L-NNA significantly attenuated the ACh-induced relaxation in both the “nonoccluded graft” and the “poor runoff graft” (comparison of the data between ‘□’ in **Figure 4** and ‘Δ’ in **Figure 5**, n=5 in each case; P<.001). The ACh-induced relaxation in the presence of L-NNA was lower in the “poor runoff graft” than in the “nonoccluded graft” (n=5; P<.05; **Figure 5A, 5B**). In the presence of L-NNA, charybdotoxin (nonselective $K_{Ca}3.1$ inhibitor) together with apamin ($K_{Ca}2.3$ inhibitor) completely blocked ACh-induced relaxation in both grafts (n=5, in each case; **Figure 5**).

SMC Hyperpolarization Induced by ACh, Substance P and A23187

The resting membrane potential of SMCs in the "nonoccluded graft" was -52.4 ± 1.7 mV, and ACh (0.3-10 μmol/L) induced concentration-dependent hyperpolarization (**Figure 6**). The maximum hyperpolarization was obtained at 10 μmol/L ACh (17.6 ± 1.9 mV, n=6; **Figure 6C**). Charybdotoxin together with apamin depolarized SMCs (to -48.2 ± 4.8 mV, n=4; P<.05) and completely blocked ACh (10 μmol/L)-induced hyperpolarization in the "nonoccluded graft" (1.5 ± 4.4 mV depolarization, n=4; P<.05; **Figure 6A**).

The resting membrane potential of SMCs in the "poor runoff graft" group was -51.0 ± 4.2 mV (n=6), and the values in this group were not significantly different from those in the "nonoccluded graft" group (n=6; P>.1). In the "poor runoff graft" group, ACh-induced hyperpolarization was lower than in that in the "nonoccluded graft" group (P<.001; **Figure 6C**), but the EC_{50} value was not significantly different between these groups (0.8 ± 0.9 μmol/L in the "nonoccluded graft" and 1.6 ± 0.8 μmol/L in the "poor runoff graft", n=6 in each case; P>.1).

The SMC hyperpolarization induced by substance P (0.2 μmol/L) was 12.2 ± 2.8 mV (n=3) in the "nonoccluded graft" and 5.7 ± 2.8 mV (n=3) in the "poor runoff graft". These values were significantly different (P<.05). In contrast, the SMC hyperpolarization induced by the nonreceptor agonist A23187[24] (1 μmol/L) was 12.4 ± 0.9 mV (n=3) and 12.9 ± 5.1 mV (n=3) in the "nonoccluded graft" and "poor runoff graft", respectively, and these values were not significantly different (P=.86, **Figure 7**).

Discussion

In the present "poor runoff graft" (vs. "control artery"), the receptor-activated functions of both EDNO and EDHF were reduced by half. However, apparent intimal

hyperplasia was not seen in the graft. These results suggest that a part of the remaining function of EDNO and EDHF is enough to inhibit intimal hyperplasia formation in rabbit "poor runoff artery grafts".

In 1980, Furchgott et al demonstrated that the relaxation of isolated preparations of rabbit thoracic aorta and other blood vessels by ACh required the presence of endothelial cells and that ACh, which acts on the muscarinic receptors of these cells, stimulated the release of a substance(s) that caused the relaxation of the vascular smooth muscle[1]. Various studies have also shown that endothelium receptor agonists such as ACh and substance P increase $[Ca^{2+}]_i$ in endothelial cells and induce the endothelium-dependent vascular relaxation through actions mediated by EDNO[1,2], prostacyclin[29], and EDHF[4,6,10,11]. We previously found that in both "control" and "nonoccluded" rabbit common carotid artery grafts, ACh-induced relaxation is lost in endothelium-denuded preparations, indicating that such ACh-mediated responses are completely dependent of the presence of endothelium[14]. We also found that in "nonoccluded artery grafts" compared to "control artery grafts", (1) spontaneous release of EDNO is increased in a $[Ca^{2+}]_i$-independent manner and (2) ACh-induced EDNO-mediated relaxation is enhanced through an increase in the sensitivity of NO production to $[Ca^{2+}]_i$ (possibly due to endothelial NO-synthase phosphorylation and/or increased expression of NO synthases)[30-33] but that (3) ACh-induced EDHF-mediated relaxation is reduced through downregulation of ACh-induced endothelial cell $[Ca^{2+}]_i$ increase[14]. Here, we found that in the "poor runoff grafts" compared to "nonoccluded grafts", (4) the absolute tension induced by high K^+ was greater before the application of L-NNA but similar after the application of L-NNA (suggesting that less NO was spontaneously released; **Figure 3**), (5) ACh-induced relaxation was lower whether or

not L-NNA was applied (suggesting less release of receptor-activated EDNO; **Figure 4 and Figure 5**), and (6) the SMC hyperpolarization induced by ACh and substance P was reduced, while hyperpolarization induced by the nonreceptor agonist A23187 was similar (suggesting that the increase in receptor-activated endothelial cell $[Ca^{2+}]_i$ was selectively decreased; **Figure 7**). These results clearly indicate that some parts of the function of EDNO and EDHF remain to induce endothelium-dependent vascular relaxation in rabbit "poor runoff grafts".

It has recently been suggested that endothelial hyperpolarization (ECH), rather than EDHFs, plays an essential role in agonist-induced EDHF-mediated relaxation[4,6,7]. Receptor agonists activate both $K_{Ca}3.1$ and $K_{Ca}2.3$ via increased $[Ca^{2+}]_i$ in endothelial cells and then produce ECH, which induces SMC hyperpolarization through direct electrical coupling via myoendothelial gap junctions, thus inducing SMC relaxation[4,6,7,14]. In the rabbit carotid "control artery" and "nonoccluded graft", ACh induced SMC hyperpolarization that was blocked by either charybdotoxin plus apamin (present experiments) or a myoendothelial gap junction inhibitor[14], suggesting that ECH, acting via myoendothelial gap junctions, plays an essential role in ACh-induced SMC relaxation in the rabbit common carotid artery.

We previously found that in rabbit carotid "nonoccluded grafts", only small amounts of the smooth muscle myosin heavy chain (MHC) isoforms SM1 and the nonmuscle MHC SMemb[26,27] but not the macrophage marker were present within a limited amount of intimal hyperplasia[14]. The result is in part consistent with the findings seen in the present "poor runoff graft" (**Figure 2**) and in the canine femoral artery[28]. These results are in contrast with the findings in poor runoff vein grafts in which pronounced intimal thickening (containing massive amounts of SM1 and

346 SMemb)[26,27] was associated with complete loss of function of both EDNO and
347 EDHF[11-13,19]. We also found that long-term *in vivo* administration of drugs for
348 atherosclerotic occlusive diseases (such as the serotonin 2A receptor antagonist
349 sarpogrelate, the cholesterol uptake inhibitor ezetimibe and the dipeptidyl peptidase 4
350 inhibitor vildagliptin) not only restored EDNO function but also reduced intimal
351 hyperplasia formation in vein grafts[11-13,19]. These results suggest that the expression of
352 proliferative SMCs by surgical operation-induced vascular inflammation could be
353 minimized by the action mediated by the remaining function of EDNO and EDHF in
354 "poor runoff artery grafts".

355 It is known that, when used for coronary artery bypass grafting, an artery graft
356 (internal mammary artery) is superior in patency to a vein graft (saphenous vein)[15,16].
357 Low blood flow in arterial grafts anastomosed to chronic totally occluded lesions has
358 also been reported to be a predictor of early postoperative graft failure[34]. The present
359 study indicates that the functions of both EDNO and EDHF are well retained, and no
360 intimal hyperplasia is observed in rabbit carotid artery grafts even under low blood
361 flow conditions. Thus, it is suggested that the artery graft is beneficial to maintain the
362 function of EDNO and EDHF, which may be responsible for the superiority in graft
363 patency.

364 We previously found that electron-microscopic examination revealed mild
365 endothelial cell damage in canine autologous arterial grafts on the 1st or 3rd day after
366 grafting, but endothelial cells appeared to be normal on days 7-14 after grafting[35].
367 Therefore, in the present study, functional changes in endothelial factors in poor runoff
368 artery grafts were examined on postoperative day 28. However, the vessel wall may
369 change later than 4 weeks after the transplantation[36,37]. Thus, future study should

clarify whether the function of vascular endothelium is modified in artery grafts later than 4 weeks (e.g., 3 months or 1 year) after transplantation.

At present, the mechanism underlying the significant decrease in lumen area in the aortae from the poor runoff group *in vivo* remains unclear. In the present experiments, without L-NNA, high K^+ induces a larger maximum tension in the poor runoff graft than in the nonoccluded graft; with L-NNA, identical maximum tension was induced by high K^+ in the poor runoff and nonoccluded grafts. Thus, the function of EDNO is reduced in the poor runoff graft. Such conditions may induce vascular smooth muscle contraction, which reduces the lumen diameter. This hypothesis should be clarified in future experiments.

The present study used only male rabbits. The use of only one sex could be a limitation to understanding the potential impact of the condition being studied. Future studies, including studies focusing on sex differences, are clearly required to validate the functions of NO and EDHF in arterial and venous grafts.

Conclusions

We developed a rabbit model of common carotid artery grafts under poor runoff conditions to examine the effects of surgical operation on the function of EDNO and EDHF in relaxation and vascular remodeling. In such grafts, receptor-activated endothelium-dependent relaxation was downregulated but partly remained. No apparent intimal hyperplasia was present in the graft. These results are in contrast with those of rabbit vein grafts in which the functions of EDNO and EDHF are completely lost and display severe intimal hyperplasia. It is suggested that the preserved function of EDNO and EDHF in arterial grafts may be responsible for minimizing intimal

hyperplasia in the vascular wall. Thus, EDNO and EDHF are suggested to have important roles in improving the patency of autogenous grafts.

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510 ***Figure Legends***

511 **Figure 1.** Blood flow in the "control artery" and grafted artery ("nonoccluded graft"
512 and "poor runoff graft"). The nonoperated left common carotid artery served as the
513 "control artery" in all rabbits. The blood flow of the grafted right common carotid
514 artery was modified by no ligation ("nonoccluded graft") or by ligation of the distal
515 branch of 3 arteries ("poor runoff graft") (see Method). The data are plotted as a box
516 plot (n=5). *P<0.05 vs. the "control artery"; †P<0.05 vs. "nonoccluded graft".
517

518 **Figure 2.** Morphometric changes in the vascular wall. (**A**) Van Gieson staining of the
519 "nonoccluded graft" (**A-a**, **A-b**) and the "poor runoff graft" (**A-c**, **A-d**). **Panels A-b**
520 and **A-d** show magnifications of the boxed regions shown in **A-a** and **A-c**, respectively.
521 (**B**) A morphometric analysis of medial thickness (**B-a**), calculated the number of
522 nuclei across the media (**B-b**), the intimal thickness (**B-c**), and the lumen area (**B-d**) in
523 the "nonoccluded graft" and "poor runoff graft". The data are plotted as a box plot
524 (n=7). *P<0.05 vs. the "nonoccluded graft".
525

526 **Figure 3.** Effects of N^{ω}-nitro-L-arginine (L-NNA) on high K^{+}-induced maximum
527 tension in endothelium-intact rings. ["L-NNA (+)"], in the presence of L-NNA;
528 ["L-NNA (-)"], in the absence of L-NNA. The data are plotted as a box plot (n=5).
529 *P<0.05 vs. ["L-NNA (-)"]. †P<0.05 vs. ["L-NNA (-)"] in the "nonoccluded graft".
530

531 **Figure 4.** Acetylcholine (ACh)-induced relaxation in endothelium-intact rings obtained
532 from the "control artery" and grafted artery ("nonoccluded graft" or "poor runoff
533 graft") without L-NNA. ACh (10^{-8}-$3x10^{-5}$ mol/L) was cumulatively applied at low to

high concentrations. The ring was obtained from rabbits with a “nonoccluded graft” (“black symbols”, n=5) or a “poor runoff graft” (“red symbols”, n=5). The tension immediately before the first ACh concentration was applied was normalized to a relative tension of 1.0. The data are shown as the mean ± SD. *P<0.05 vs. the “control artery”; †P<0.05 vs. the “nonoccluded graft”.

Figure 5. Effects of L-NNA with and without charybdotoxin (CTX) + apamin on the acetylcholine (ACh)-induced relaxation during the phenylephrine-induced contraction in endothelium-intact rings from the “nonoccluded graft” or “poor runoff graft”. After the ACh-induced responses had been recorded (shown in **Figure 4**), L-NNA (0.1 mmol/L) was applied for 90 min, and the ACh-induced responses were again obtained in the presence of L-NNA [“L-NNA (+)”]. Then, CTX + apamin was applied for 30 min in the presence of L-NNA. Finally, ACh-induced responses were observed in the presence of L-NNA + CTX + apamin [“L-NNA (+)+CTX+apamin”]. The tension immediately before the first ACh concentration was applied was normalized to a relative tension of 1.0. The data are shown as the mean ± SD. (**A**) “Nonoccluded graft” (n=5). *P<0.05 vs. “L-NNA (-)” (“black □ symbol” data in **Figure 4**); †P<0.05 vs. “L-NNA (+)” (“Δ” data in **Figure 5A**). (**B**) “Poor runoff graft” (n=5). *P<0.05 vs. L-NNA (-) (“red □ symbol” data in **Figure 4**); †P<0.05 vs. “L-NNA (+)” (“Δ” data in **Figure 5B**). ‡P<0.05 vs. “nonoccluded graft, L-NNA (+)” (“Δ” data in **Figure 5A**).

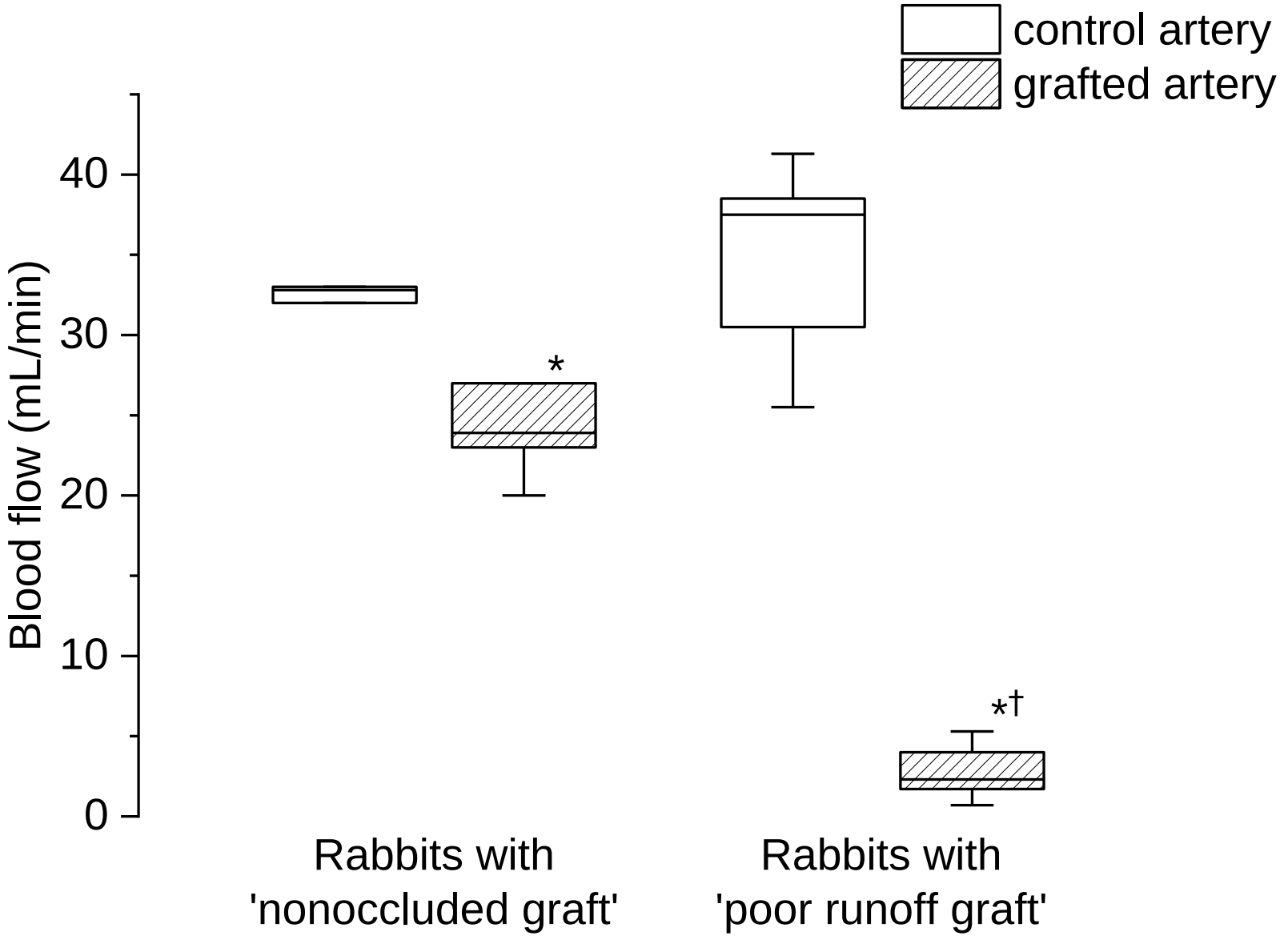
Figure 6. Effects of acetylcholine (ACh) on the smooth muscle cell membrane potential in the “nonoccluded graft” and “poor runoff graft”. (**A**) Membrane potential changes induced by ACh (10 μmol/L) before and after application of CTX (0.1

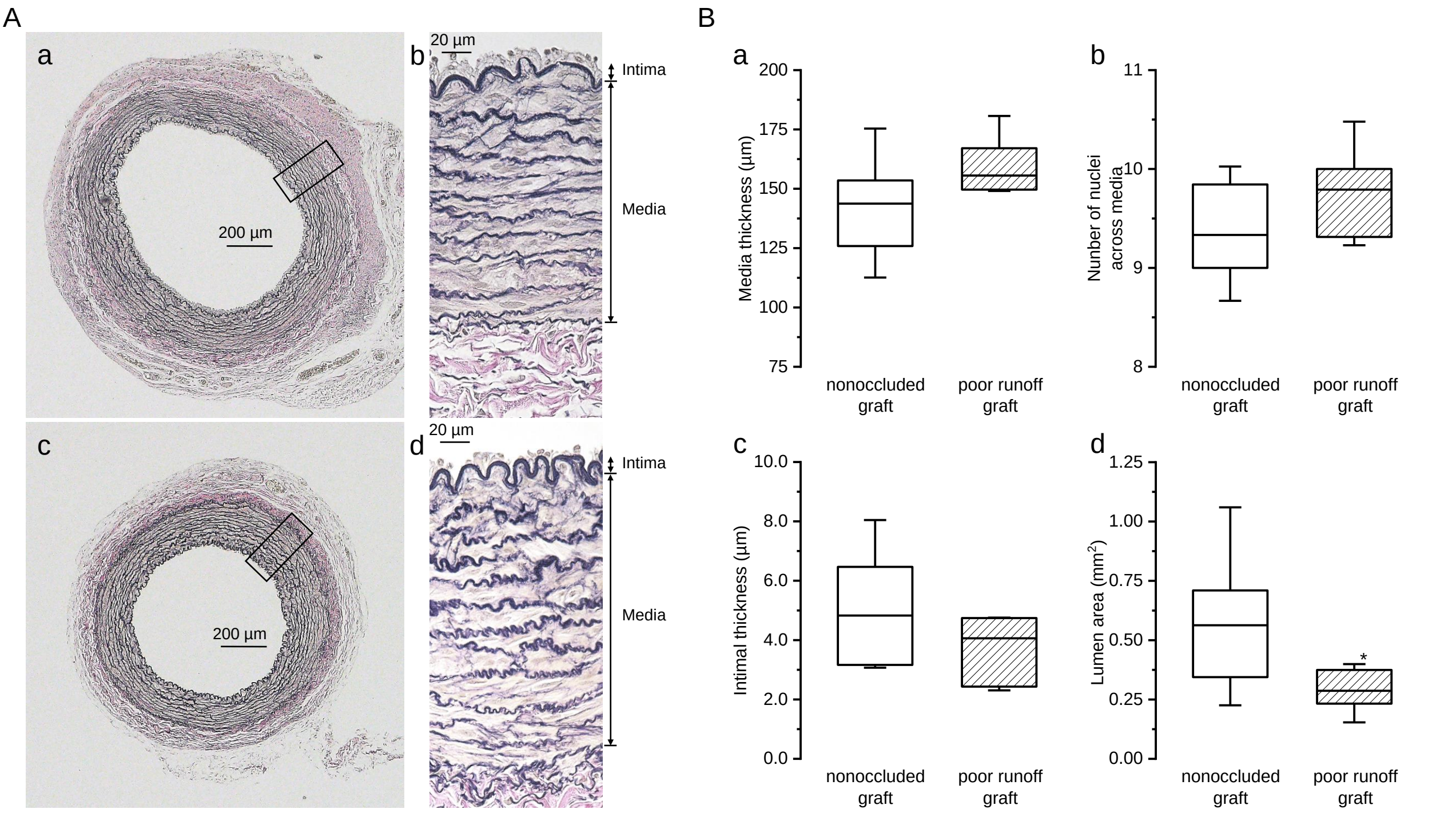
558 μmol/L) + apamin (0.1 μmol/L) in the nonoccluded graft. (**B**) Resting membrane
559 potential of smooth muscle cells in the "nonoccluded graft" (n=6) and "poor runoff
560 graft" (n=6) shown in a box plot. (**C**) Concentration-dependent effects of ACh on the
561 smooth muscle cell membrane potential in the "nonoccluded graft" and "poor runoff
562 graft". The data are shown as the mean ± SD (n=6). *P<0.05 vs. the "poor runoff
563 graft".
564
565 **Figure 7.** Effects of acetylcholine (ACh), substance P and A23187 on the smooth
566 muscle cell membrane potential in the "nonoccluded graft" and "poor runoff graft".
567 (**A**) Membrane potential changes induced by ACh (10 μmol/L), substance P (0.2
568 μmol/L) and A23187 (1 μmol/L) in the "nonoccluded graft". (**B**) Effects of ACh,
569 substance P and A23187 on the smooth muscle cell membrane potential in the
570 "nonoccluded graft" and "poor runoff graft". The data are shown as the mean ± SD
571 (n=3). *P<0.05 vs. the "nonoccluded graft".

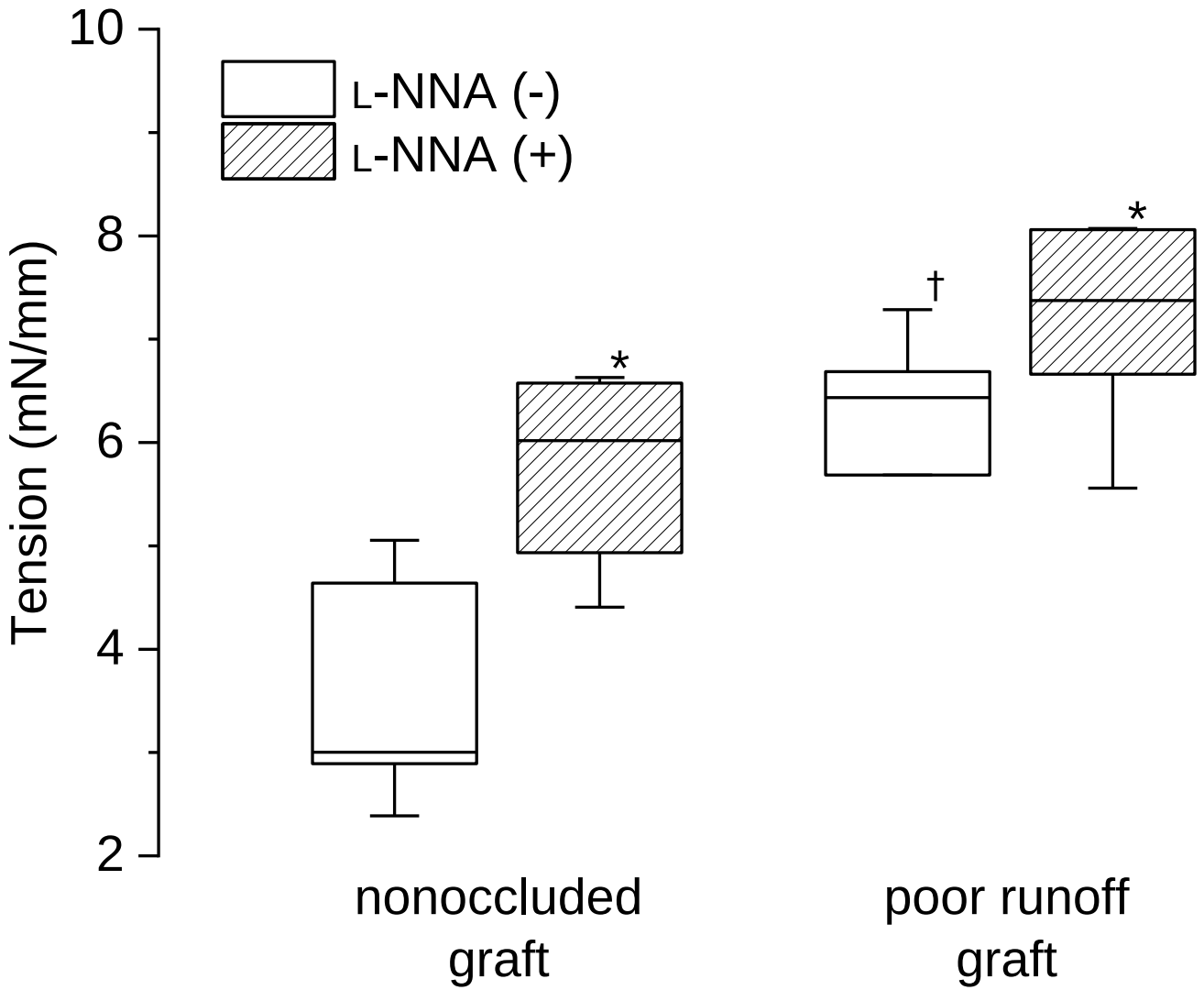
Table 1. Concentrations of phenylephrine used and the tension they induced in the presence and absence of the nitric oxide-synthase inhibitor, L-NNA

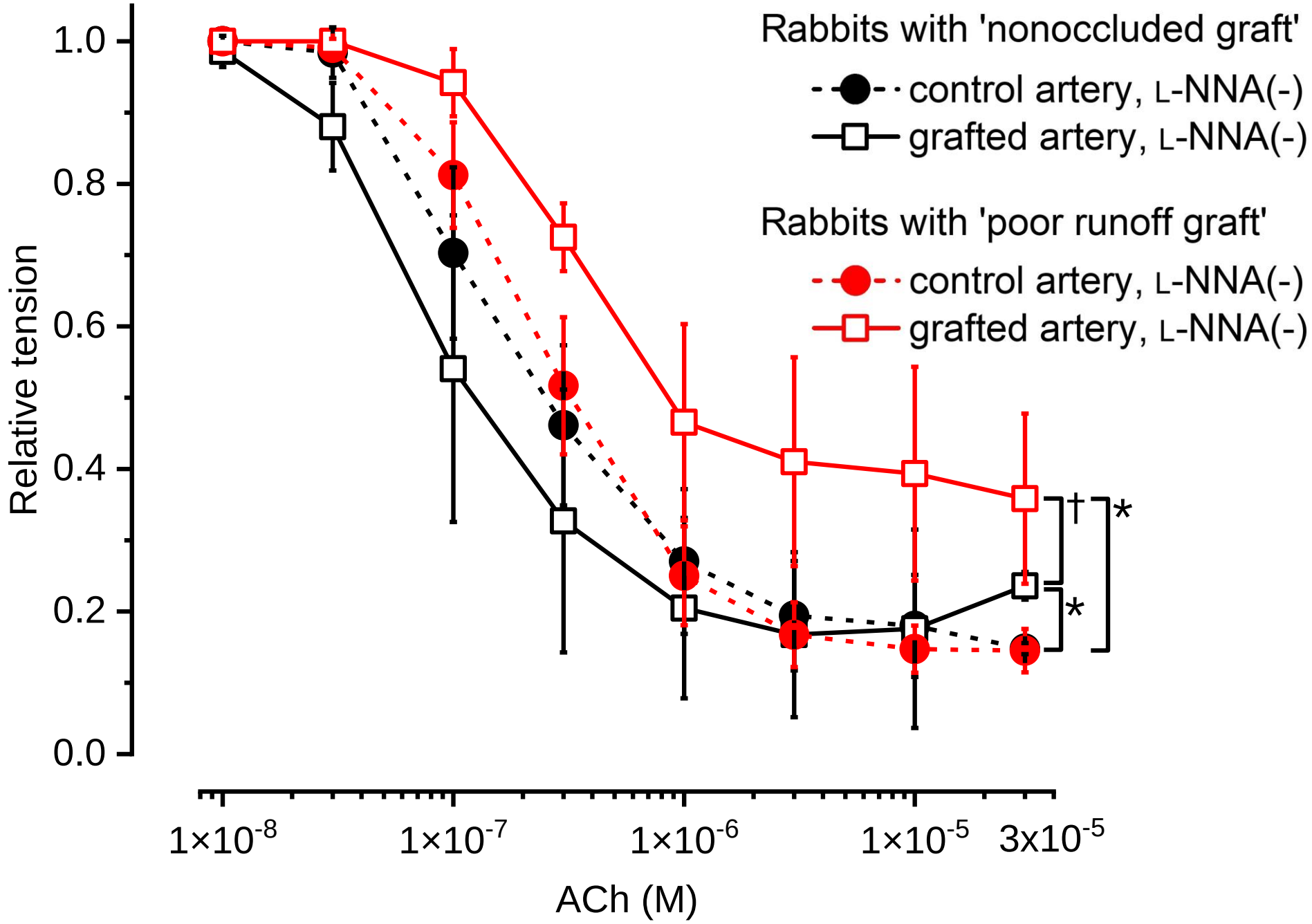
		Concentration (μmol/L)		**Tension (mN/mm)**		**n**
		L-NNA (-)	**L-NNA (+)**	**L-NNA (-)**	**L-NNA (+)**	
Rabbits with the "nonoccluded graft"	"Control artery"	8.60 ± 2.19	0.78 ± 0.19†	1.97 ± 0.38	2.38 ± 0.18	5
	"Nonoccluded graft"	2.10 ± 0.74*	0.16 ± 0.09*†	1.60 ± 0.38	2.26 ± 0.61†	5
Rabbit with the "poor runoff graft"	"Control artery"	8.60 ± 3.13	0.78 ± 0.30†	1.81 ± 0.23	2.23 ± 0.26†	5
	"Poor runoff graft"	1.46 ± 0.56*	0.14 ± 0.10*†	1.73 ± 0.45	2.02 ± 0.67	5

L-NNA (+), in the presence of L-NNA; L-NNA (-), in the absence of L-NNA; ACh, acetylcholine; L-NNA, N^{ω}-nitro-L-arginine. The data are shown as the mean ± SD. *P<0.05 vs. the "control artery" in each case. †P<0.05 vs. "L-NNA (-)" in each case.

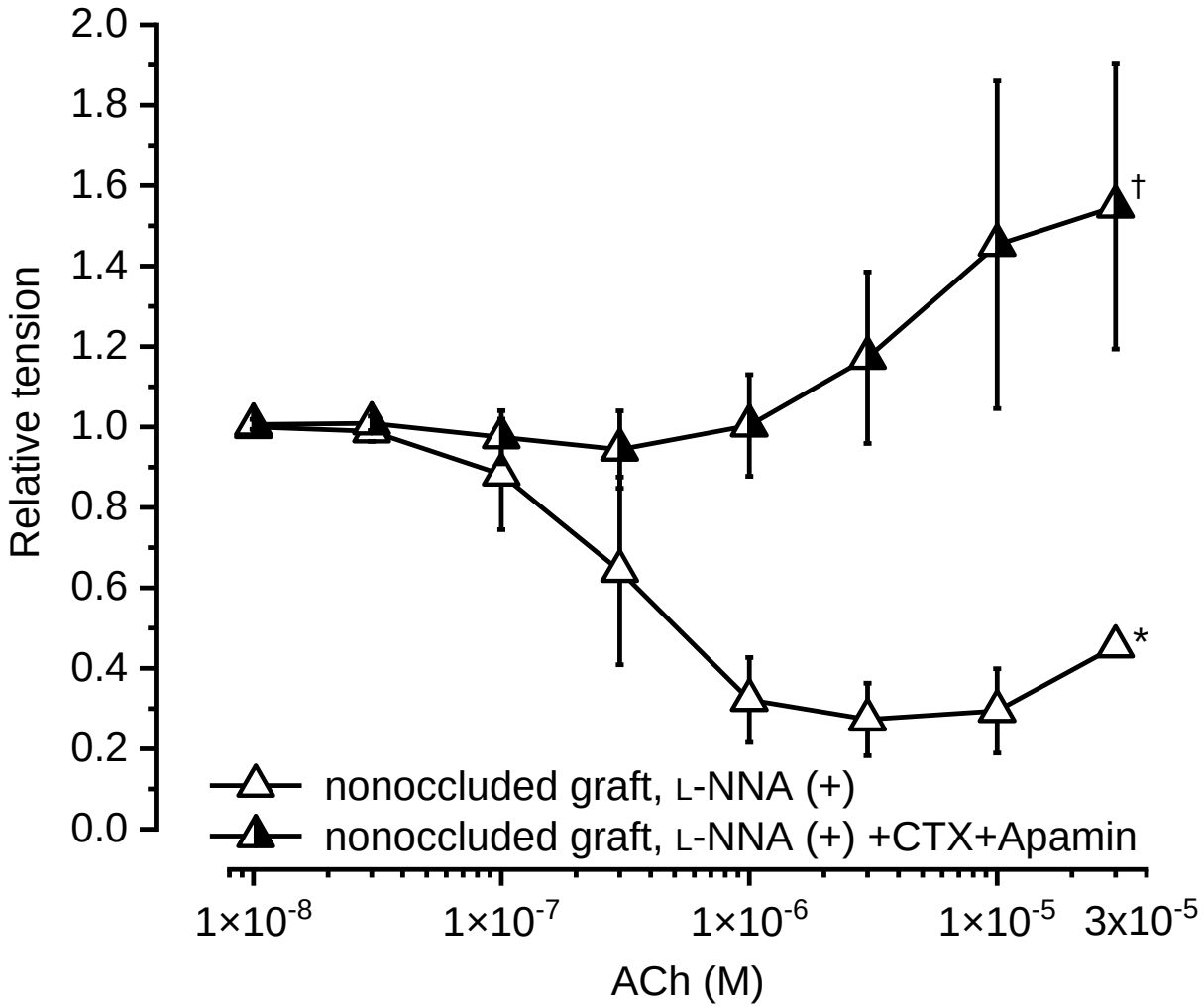

control artery
grafted artery
Blood flow (mL/min)
0
10
20
30
40
*
*†
Rabbits with
'nonoccluded graft'
Rabbits with
'poor runoff graft'


A
a
200 µm
b
20 µm
Intima
Media
c
200 µm
d
20 µm
Intima
Media
B
a
Media thickness (µm)
200
175
150
125
100
75
nonoccluded graft
poor runoff graft
b
Number of nuclei across media
11
10
9
8
nonoccluded graft
poor runoff graft
c
Intimal thickness (µm)
10.0
8.0
6.0
4.0
2.0
0.0
nonoccluded graft
poor runoff graft
d
Lumen area (mm2)
1.25
1.00
0.75
0.50
0.25
0.00
*
nonoccluded graft
poor runoff graft

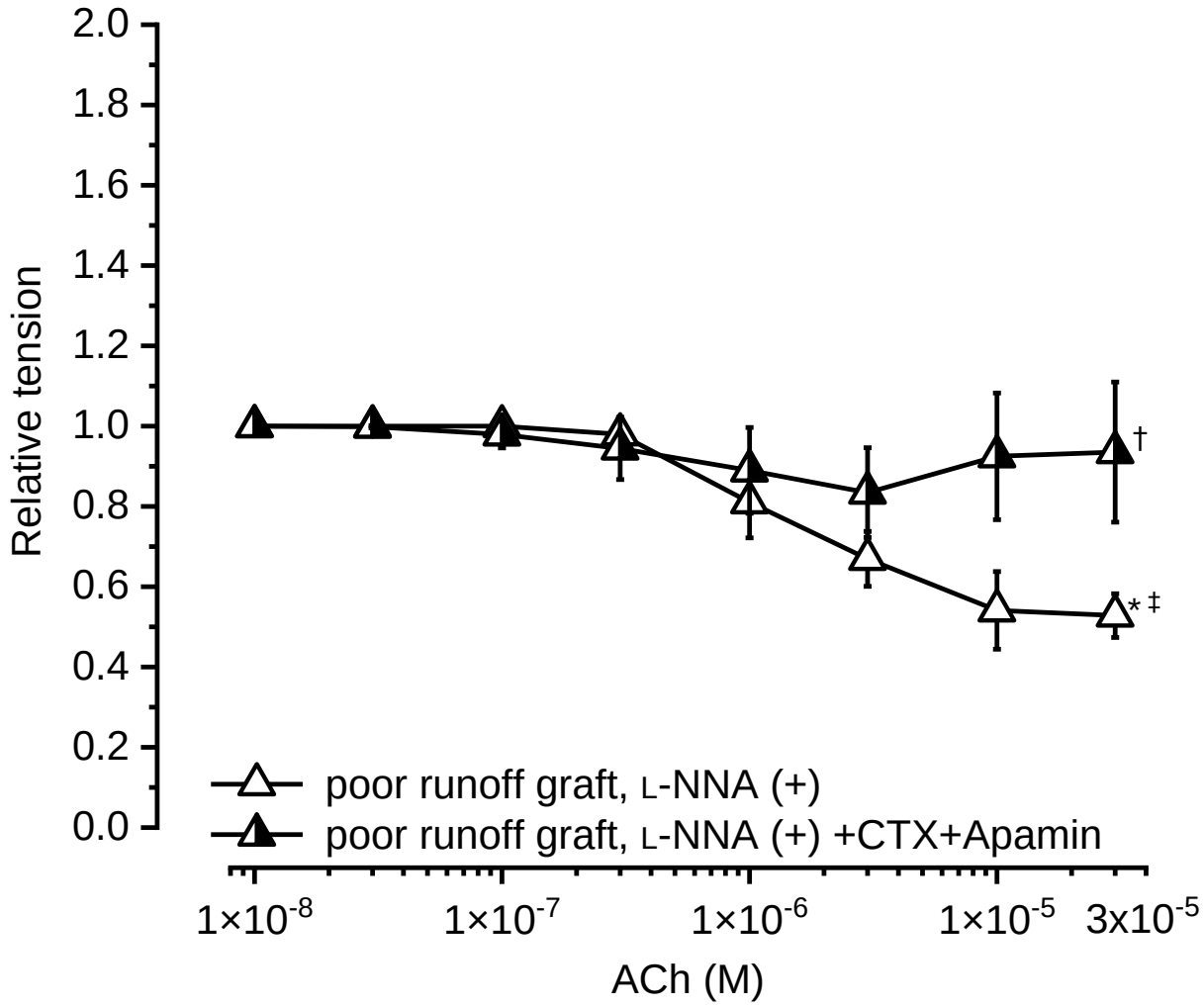

10
8
6
4
2
Tension (mN/mm)
L-NNA (-)
L-NNA (+)
*
†
*
nonoccluded graft
poor runoff graft

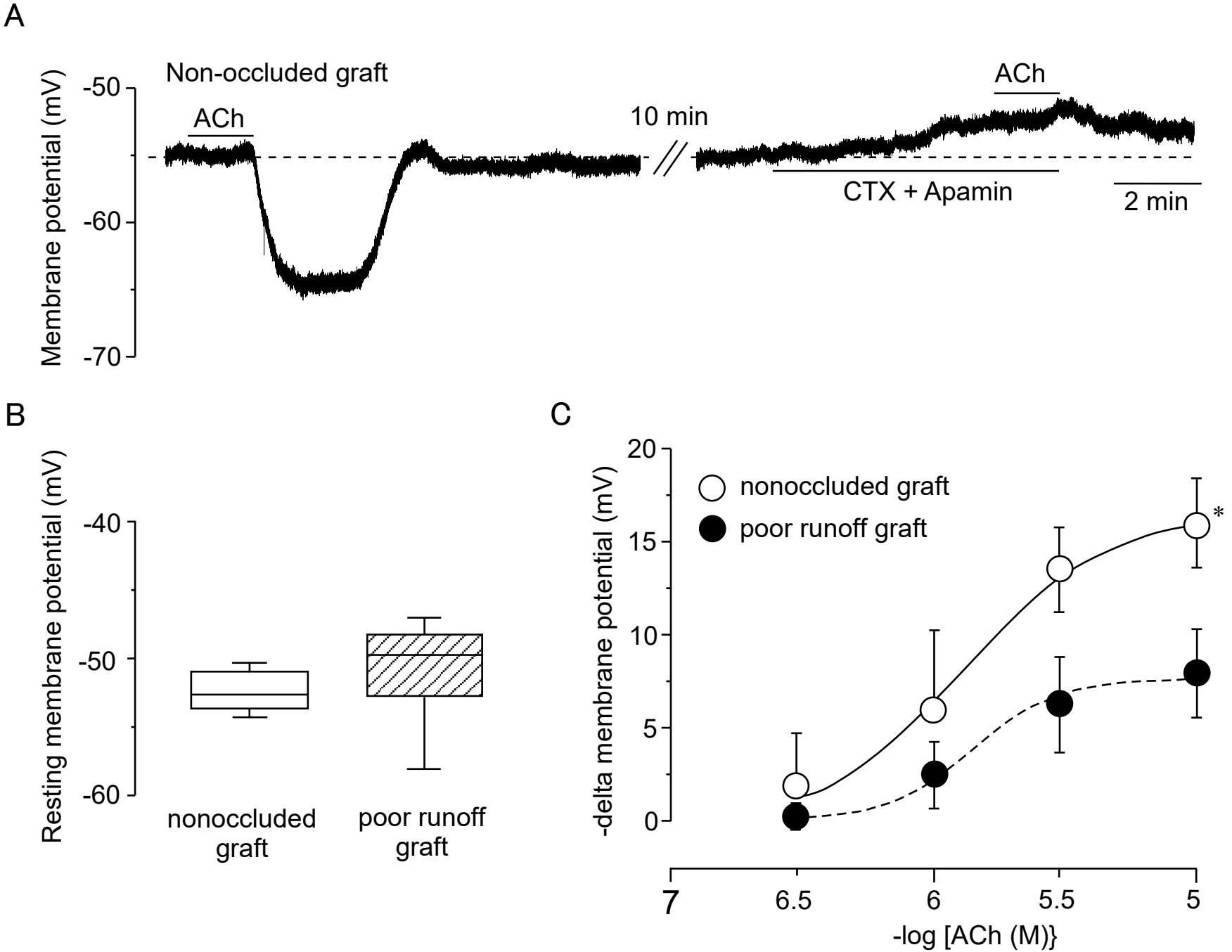

Rabbits with 'nonoccluded graft'
control artery, L-NNA(-)
grafted artery, L-NNA(-)
Rabbits with 'poor runoff graft'
control artery, L-NNA(-)
grafted artery, L-NNA(-)
Relative tension
1.0
0.8
0.6
0.4
0.2
0.0
†
*
*
1×10-8
1×10-7
1×10-6
1×10-5
3x10-5
ACh (M)

A. Rabbits with 'nonoccluded graft'

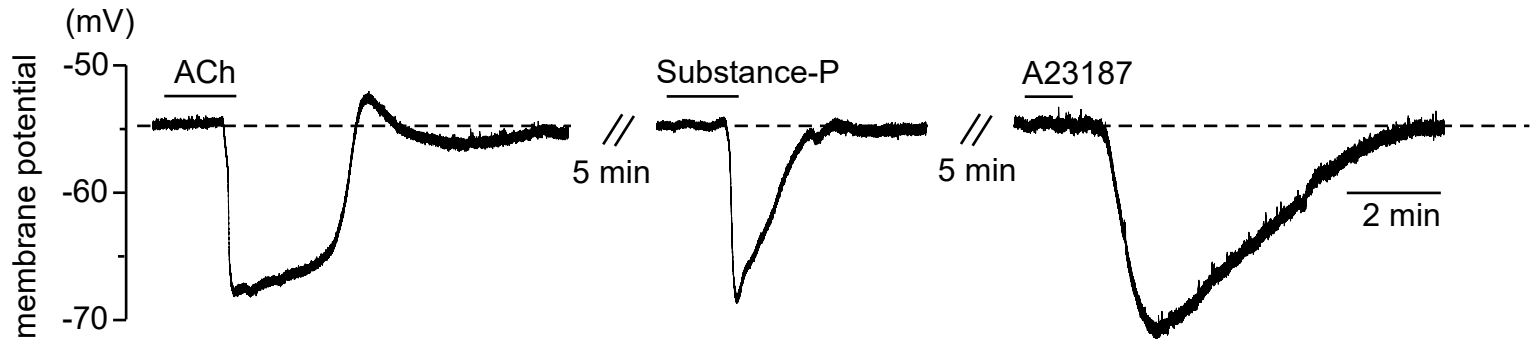


B. Rabbits with 'poor runoff graft'

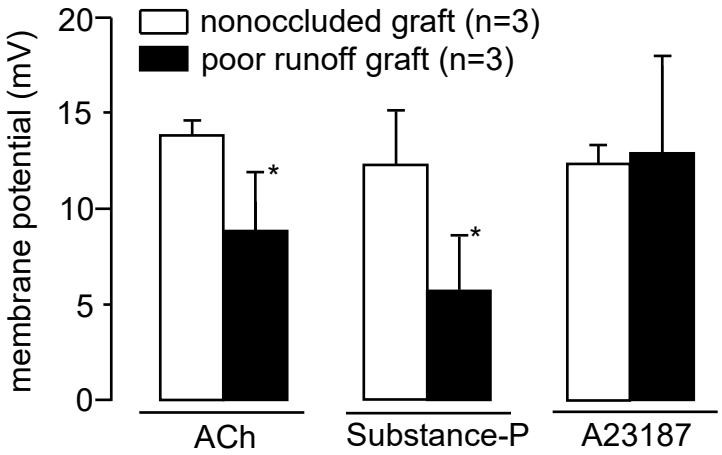



A
Non-occluded graft
ACh
10 min
ACh
CTX + Apamin
2 min
Membrane potential (mV)
-50
-60
-70
B
Resting membrane potential (mV)
-40
-50
-60
nonoccluded graft
poor runoff graft
C
-delta membrane potential (mV)
20
15
10
5
0
nonoccluded graft
poor runoff graft
*
7
6.5
6
5.5
5
-log [ACh (M)}

A


(mV)
-50
-60
-70
membrane potential
ACh
Substance-P
A23187
5 min
5 min
2 min

B


20
15
10
5
0
membrane potential (mV)
nonoccluded graft (n=3)
poor runoff graft (n=3)
*
*
ACh
Substance-P
A23187

Highlights:

- Endothelium regulates vascular tonus by releasing both EDNO and EDHF.
- EDNO/EDHF function is partially maintained in artery grafts with poor runoff.
- Intimal hyperplasia was not apparent in artery grafts with poor runoff.
- EDNO and EDHF likely play important roles in improving artery graft patency.