Comparison of Nitric Oxide Release and Endothelium-Derived Hyperpolarizing Factor–Mediated Hyperpolarization Between Human Radial and Internal Mammary Arteries

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Background—Arterial grafts for CABG have been used increasingly, and the radial artery (RA) has become a preferable graft, secondary to the internal mammary artery (IMA). In the present study, we investigated and compared NO release and endothelium-derived hyperpolarizing factor (EDHF)-mediated hyperpolarization for IMA and RA.

Methods and Results—IMA and RA segments taken from CABG patients were placed in an organ chamber. An NO-sensitive electrode (to directly measure NO release) or intracellular glass microelectrode (to measure membrane potential) was used to study NO or EDHF in response to acetylcholine (ACh) and bradykinin (BK) before and after incubation with indomethacin (a cyclooxygenase inhibitor), N\textsuperscript{-}G-nitro-L-arginine (an NO synthase inhibitor), and oxyhemoglobin (an NO scavenger). The resting membrane potential of the smooth muscle cells of IMA and RA was $-58 \pm 0.84$ (n=61) and $-61 \pm 1.3$ (n=46) mV, respectively ($P=0.03$). BK-induced EDHF-mediated hyperpolarization in the IMA was significantly greater than that in RA (BK $10^{-7}$ mol/L: $-10.9 \pm 1.5$ [n=7] versus $-5.8 \pm 0.9$ [n=6] mV, $P=0.04$). The basal (16.8±1.9 versus 11.1±1.0 nmol/L, n=12, $P=0.02$) and stimulated releases of NO in IMA were significantly greater for BK (44.3±4.0 versus 25.8±3.6 nmol/L, n=8, $P=0.004$) and lasting longer for ACh (9.5±2.0 versus 6.6±3.6 minutes, n=12, $P=0.03$) than those in RA.

Conclusions—The basal and stimulated releases of NO and EDHF-mediated hyperpolarization in the IMA are significantly greater than that in the RA. The lower capacity of NO release may contribute to the susceptibility of RA to the perioperative vasospasm and may have an impact on the long-term graft patency. (Circulation. 2001;104[Suppl I]: I-344-I-349.)

Key Words: nitric oxide □ endothelium-derived factors □ coronary disease □ bypass □ grafting □ electrophysiology

Due to the superior long-term patency and survival of the internal mammary artery (IMA) graft,\(^1\) arterial grafts for CABG are increasingly used. The radial artery (RA) has become the second choice of arterial graft, after the IMA.\(^2\) However, the long-term patency rate of this graft remains unknown, although a recent report shows that the 5-year patency rate of the RA graft is 83%,\(^3\) which is higher than that of the saphenous vein (SV) graft (55%)\(^4\) but slightly lower than that of the IMA graft.\(^4\)

Pathological studies have revealed that the main reason for prolonged IMA graft patency is freedom from atherosclerosis of the conduit, which may be attributed to the unique histological feature of the IMA in contrast to other arterial and venous grafts,\(^5\) and the endothelium-dependent relaxation.\(^6\) Most recently, we reported differences in NO release through the direct measurement of NO and hyperpolarization between the human IMA and the SV.\(^7\) However, many fundamental physiological properties of the RA that may contribute to the graft spasm and patency remain unclear.

Endothelial function of the coronary bypass grafts is believed to be important in the long-term graft patency.\(^8\) In response to variety of agonists, endothelial cells generate 3 major autacoids that regulate vascular relaxation and other endothelium-dependent vascular functions.\(^9\) These autacoids are NO, prostacyclin (PGI\(_2\)), and endothelium-derived hyperpolarizing factor (EDHF).\(^9,10\)

The roles of endothelium in vascular tone modulation and vascular homeostasis are well investigated in blood vessels. A few recent studies also reported the role of endothelium in commonly used bypass grafts such as the IMA, SV, and RA.\(^6,11–17\) Usually, the role of endothelium in these grafts is determined through an indirect method: the endothelium-dependent relaxation by NO agonists such as bradykinin (BK) and acetylcholine (ACh). We recently reported the
results of a newly developed method: the direct electrochemical measurement of the concentration of NO released from the SV in comparison with the IMA. However, such direct measurement has never been reported in the RA. In addition, direct measurement of the cellular membrane potential to detect the EDHF-mediated hyperpolarization in the RA has never been reported. Therefore, the present study was designed to directly measure the amount of NO released from the endothelium of the RA and the cellular membrane potential of the smooth muscle cell to determine the role of NO and EDHF-mediated hyperpolarization of the RA in comparison with the IMA.

**Methods**

**Vessel Preparation**

The discarded IMA from 18 patients and RA segments from 8 patients undergoing CABG were immediately collected and placed into a container with oxygenated physiological solution (Kreb's) maintained at 4°C and then transferred to the laboratory. The time delay between the collection of the specimen and the receipt in the laboratory was normally ≤10 minutes. Approval to use the discarded human vessel tissue was given by the Ethics Committee of Grantham Hospital, Hong Kong SAR, China. The vessels were immediately placed in a glass dish filled with Krebs' solution and dissected free from surrounding fat and connective tissue. All vessels were cut into 5-mm-long rings, opened longitudinally, and fixed on the bottom of an organ chamber (volume 3 mL) with the endothelium side up. The organ chamber was continuously superfused with Krebs' solution bubbled with 95% O₂ and 5% CO₂ at a constant rate of 3 mL/min, and the temperature was maintained at 37°C. The composition of Krebs' solution (in mmol/L) is NaCl 144, KCl 5.9, CaCl₂ 2.5, MgCl₂ 1.2, Cl - 128.7, HCO₃⁻ 25.4, SO₄²⁻ 1.2, H₂PO₄⁻ 1.2, and glucose 11. After 60 minutes of incubation, the following procedures were performed.⁷,⁸

**Electrophysiological Study**

A smooth muscle cell was impaled from the intimal side using a conventional intracellular glass microelectrode filled with 3 mol/L KCl (tip resistance 40 to 80 MΩ). Electrical signals were continuously monitored on an oscilloscope (model 2020B; BK Precision, and Electro 705; WPI), and the membrane potential was recorded with a computer (data logging software: PICOLOG; Pico Technology Limited).⁷,¹⁸,¹⁹ The following criteria were used to assess the validity of successful impalement: a sudden negative shift in voltage followed by (1) a stable negative voltage for ≥2 minutes and (2) an instantaneous return to the previous voltage level on dislodgement of microelectrode. At least 4 impalements of the same artery were made to assess the variability of the electrophysiological parameters, and the average was taken as 1 measurement. After a stable membrane potential for ≥2 minutes, the response to ACh or BK was studied.

**Direct Measurement of NO**

The membrane-type NO-sensitive electrode (ISO-NOP; WPI) and isolated NO meter (ISO-NO Mark II; WPI) were used to measure the isolated NO generated by vascular endothelium.¹⁸,¹⁹ The detection of NO is an electrochemical method in which a potential is applied to the measuring electrode relative to the reference electrode, and the resulting current due to the electrochemical oxidation of NO is monitored. The membrane-type NO-sensitive electrode consists of a working electrode covered by a gas-permeable polymeric membrane. NO diffuses through the selective membrane or coatings and is oxidized on the surface of the prepolarized electrode, resulting in an electrical current. The magnitude of the redox current is in direct proportion to the concentration of NO in the sample and is amplified by the NO meter and registered by a computer (DuoDip 18 data recording system; WPI). The ISO-NOP has an inherently high selectivity due to the fact that the electrodes are separated from the sample in which measurements are being made by gas-permeable hydrophobic membranes. This rules out any interference from solution or dissolved species other than gas.¹⁹

The selectivity of the NO-sensitive electrode was tested in connection with calibration, where a lack of response to strong saline solution (3 mol/L) or sodium nitrite (NaNO₂) up to 100 μmol/L was taken as an evidence for an intact coating of the electrode. The electrodes did not respond to ACh (10 μmol/L), BK (1 μmol/L), indomethacin (7 μmol/L), N⁶-nitro-L-arginine (L-NNA, 300 μmol/L), or oxyhemoglobin (Hb, 20 μmol/L) added to the calibration glass vial.

The membrane-type electrode can be calibrated by chemical titration based on the following equation: 2KNO₂ + 2KCl + 2H₂O → 2NO + 1, 2H₂O + 2K₂SO₄, where a known amount of KNO₂ is added to produce a known amount of NO. The quantity (and so the concentration) of NO generated can be calculated directly from the stoichiometry if the concentrations of the reactions are known.⁷,¹⁹

The calibration was performed daily before the experiment. After calibration, the NO-sensitive electrode was inserted into the organ chamber vertically and placed as close to the endothelial surface as possible by means of a micromanipulator (WR-6; Narishige International). The NO electrode was connected to the amplifier, and the signals were recorded. After 60 to 120 minutes of equilibration in the organ chamber, the electrode was stabilized and the baseline of the current became stable. The measurement of NO was then carried out.¹⁹

The NO concentration measured with the NO-sensitive electrode reflects the NO released from the endothelium minus the NO cleared through degradation and diffusion.

**Experimental Protocol**

**Electrophysiological Studies of the EDHF-Mediated Hyperpolarization**

To investigate the EDHF-mediated hyperpolarization in response to ACh and BK in the IMA and RA, L-NNA (300 μmol/L), an inhibitor of NO synthase; indomethacin (7 μmol/L), a cyclooxygenase (COX) inhibitor; and Hb (20 μmol/L), an NO scavenger, were added to the organ chamber to completely inhibit the NO and PG1 pathway.¹⁹

After 60 minutes of equilibration in the organ chamber, the resting membrane potentials of the smooth muscle cells of IMA and RA were recorded. ACh [−8 to −5 log(mol/L)] or BK [−10 to−7 log(mol/L)] was added to the organ chamber cumulatively, and the change of the membrane potential of the smooth muscle cells of IMA and RA was recorded. The organ chamber was then washed with Krebs' solution, and L-NNA (300 μmol/L), indomethacin (7 μmol/L), and Hb (20 μmol/L) were added. After incubation and equilibration for another 30 minutes, the aforementioned steps were repeated, and the change in the membrane potential was recorded.

**Direct Measurement of NO**

To investigate the capacity of NO release from the endothelium of IMA and RA, ACh- and BK-induced NO release was examined. After 60 minutes of incubation and equilibration of each segment in the organ chamber, ACh [−8 to −5 log(mol/L)] or BK [−10 to −7 log(mol/L)] was added to the organ chamber cumulatively, and the NO signals were recorded.⁷,¹⁹,²⁰ The interval between each 2 concentrations of ACh or BK was 15 minutes. The organ chamber was then washed with Krebs’ solution. L-NNA (300 μmol/L), indomethacin (7 μmol/L), and Hb (20 μmol/L) were added into the organ chamber. After incubation and equilibration for an additional 30 minutes, the aforementioned steps were repeated.

**Data Analysis**

All values are expressed as mean±SEM. When the comparison was made between the IMA and RA groups, the unpaired Student's t test was used. When the measurement was performed in the same vessel segment before and after a treatment, paired t test was used. P<0.05 was considered significant.
Results

Electrophysiological Study

The resting membrane potential of the smooth muscle cells of IMA was $-58 \pm 0.8$ mV (n=16), significantly higher than that of RA ($-61 \pm 1.3$ mV, n=14, $P=0.02$). Mechanical removal of the endothelium produced no significant changes in the resting membrane potential of IMA ($-56 \pm 2$ mV, n=6, $P=0.7$ versus control) and RA ($-60 \pm 1.6$ mV, n=5, $P=0.5$ versus control). Similarly, after incubation with L-NNA, indomethacin, and Hb for 30 minutes, there were no significant changes in the resting membrane potential of the smooth muscle cell of either IMA or RA ($-57 \pm 2$ mV, n=8, $P=0.4$ versus control, and $-59 \pm 1.7$ mV, n=7, $P=0.7$ versus control, respectively).

Both ACh and BK induced a hyperpolarization of the smooth muscle cell of the IMA and RA in a concentration-dependent manner (Figure 1). When the NO and PGI$_2$ pathways were blocked (in the presence of L-NNA, indomethacin, and Hb), the EDHF-mediated hyperpolarization elicited by BK in the IMA (8 individuals) was significantly greater than that in RA (7 segments from 5 individuals) at the concentration of $-9$ to $-7 \log$(mol/L). At $-9.0 \log$(mol/L), it was $2.1 \pm 0.6$ mV versus $6.5 \pm 1.2$ mV ($P=0.009$). At $-8 \log$(mol/L), it was $4.6 \pm 1.1$ versus $9.4 \pm 1.3$ mV ($P=0.02$). At $-7 \log$(mol/L), it was $5.8 \pm 0.9$ versus $10.9 \pm 1.5$ mV ($P=0.04$) (Figures 1, bottom, and 2). There were no significant differences in the EDHF-mediated hyperpolarization induced by ACh between these 2 vessels (Figures 1, top, and 2).

In endothelium-denuded IMA and RA segments, addition of ACh or BK did not cause significant changes in the membrane potential of the smooth muscle cell (Figure 3).

NO Measurement

Calibrations

The membrane-type NO-sensitive electrodes responded with increases in current to nanomolar concentration of NO. The output current of the probes correlated linearly with the concentration of NO ($r=0.9965 \pm 0.0027$, n=28, $P=0.0002$). Because the sensitivity of the different electrodes varied in a broad range of 0.16 to 0.89 nmol · L$^{-1}$ · pa$^{-1}$ (0.56 ± 0.14 nmol · L$^{-1}$ · pa$^{-1}$), the calibration was carried out daily.

Basal Release of NO

In the resting state, a continuous NO signal (the basal release of NO) was observed in both IMA and RA. In IMA, the basal concentration of NO was $16.8 \pm 1.6$ nmol/L (n=12 different segments from 9 individuals), significantly greater than that in RA ($11.9 \pm 1.8$ nmol/L, n=12 different segments from 8 individuals, $P=0.02$) (Figure 4).

Stimulated Release of NO

Stimulation of the endothelium of IMA and RA with ACh and BK evoked a rapid rise in NO concentration followed by a sustained elevation that lasted for 2 to 14 minutes (Figure 5). There were no significant differences between IMA and RA in the maximum concentration of NO release induced by ACh (Figures 5 and 6, top). However, the duration of ACh-induced NO release in the IMA was significantly longer than that in RA (for ACh at $-5 \log$(mol/L): 9.5 ± 2.0 minutes, $P<0.05$).
n=12, versus 6.6±3.6 minutes, n=12,  P=0.03) (Figure 7), and the time-concentration curves of NO release showed a significant difference between IMA and RA (Figure 7).

The maximum concentration of NO release induced by BK in the IMA (n=8) was significantly greater than that in RA (n=8) at the concentration of −8 to −7 log(mol/L). At −8 log(mol/L), it was 24.7±3.8 nmol/L versus 41±6.5 nmol/L (P=0.04). At −7 log(mol/L), it was 25.8±3.6 nmol/L versus 44.3±4.0 nmol/L (P=0.004) (Figures 5 and 6, bottom).

After incubation with L-NNA (300 μmol/L), indomethacin (7 μmol/L), and Hb (20 μmol/L), NO could not be detected in either IMA or RA (data not shown).

Discussion

In the present study, we have for the first time found (1) that at both basal and stimulated conditions, the concentration of NO released from the IMA is significantly greater and lasts longer than that from the RA and (2) that the EDHF-mediated hyperpolarization exists in the RA and the hyperpolarization induced by BK in the IMA is more significant than that in the RA.

The superiority of the IMA-to-SV graft for coronary revascularization has been widely accepted,1,4 and other arterial grafts are used increasingly. In some medical centers, the RA has become a preferable choice secondary to IMA.2 The improvement of early and mid-term results of the RA has been attributed to the improved harvesting and preparation technique of the RA graft and the use of antispastic agents.2,21 As a muscular artery, the RA graft is susceptible to vasospasm, which was thought to be the principal cause of early graft failure.2,22 Numerous studies from others11–16 and ourselves12 have revealed that the RA has a higher receptor-mediated contractility compared with the IMA12 and human RA is an α-adrenoceptor dominant artery with weak β-adrenoceptor function.23 In addition, others reported similar results that the RA exhibits greater contraction to potassium chloride, serotonin, thromboxane A2, and norepinephrine than does the IMA.11 These characteristics of the RA may contribute to its vasospastic characteristics.11,14–16 However, the main cause of the perioperative vasospasm of the RA graft and the factors that contribute to the long-term graft patency still remain unknown.8 Apart from our study of a

Figure 7. Time-concentration curves of NO release in IMAs (n=6) and RA (n=6) where ACh −5 log(mol/L) was added. Duration of NO release in IMA was significantly longer than that in RA. At 5 minutes after addition of ACh, concentration of NO in IMA was significantly higher than that in RA. *P<0.05, **P<0.01. Data are mean±SEM.
comparison of the endothelium-dependent relaxation that reported a similar endothelium-dependent relaxation between the RA and IMA.\textsuperscript{12,13} Two other recent studies also investigated the role of the endothelium-dependent relaxation in the RA and IMA, but their results conflicted with each other.\textsuperscript{13,14} The former\textsuperscript{13} reported that the RA has enhanced NO-mediated relaxation in the RA compared with that in the IMA, but the latter gave the opposite result.\textsuperscript{14} This discrepancy promotes further studies on the endothelial function in these 2 arteries.

Nevertheless, the endothelium-dependent relaxation is an indirect index for NO release from the endothelium and may not exactly reflect the content of the NO released from the endothelium. This is due to the following reasons. First, the relaxation is the result of 3 relaxing factors (NO, EDHF, and PGI\textsubscript{2}), and therefore a more prominent relaxation is not necessarily due to the higher production of NO. Other words, the same amount of relaxation is not necessarily due to the same amount of NO release. This may explain the discrepancy between the previously reported “similar endothelial function” in the RA and IMA and the findings in the present study. Second, it also reflects the sensitivity of the smooth muscle to the relaxing factors. Obviously, new technology that may more accurately determine the NO release in these vessels is essential to solve the problem. With the recent technique developed with the use of the NO-sensitive electrode, we are able to directly measure the NO concentration, and this method provides a new approach to compare the endothelial function between the RA and IMA in the present study. The direct measurement of NO in the present study solved the problem of the use of simple indirect methods by different groups that led to different conclusions. In addition, for the first time, we investigated the EDHF-mediated hyperpolarization through direct measurement of the cellular membrane potential in the RA.

EDHF-Mediated Hyperpolarization in the RA and IMA

ACh and BK are 2 endogenous vasodilators. ACh- or BK-induced endothelium-dependent relaxation is widely used in the evaluation of the endothelial function in vivo. In response to ACh or BK, endothelial cells release \( \geq 3 \) vasoactive compounds: NO, PGI\textsubscript{2}, and EDHF.\textsuperscript{9,10} It has been reported that both NO and PGI\textsubscript{2} can cause hyperpolarization of smooth muscle cells.\textsuperscript{24} Therefore, to study the EDHF-related endothelial function, it is essential to completely inhibit the other 2 pathways: NO and PGI\textsubscript{2}. It has been demonstrated that the PGI\textsubscript{2} pathway can be blocked by indomethacin,\textsuperscript{10} but the NO production is not eliminated by the NO synthase inhibitor L-NNA, even at high doses.\textsuperscript{19} We also demonstrated that further addition of the NO scavenger Hb is essential in electrophysiological studies for EDHF.\textsuperscript{19} In the present study, we demonstrated that in the human arteries (IMA and RA), NO release is completely inhibited by the combination of L-NNA and Hb. The hyperpolarization of the smooth muscle cell of the IMA and RA in our study is therefore related to EDHF, and the role of NO in the hyperpolarization is completely excluded.

Through direct measurement of the EDHF-mediated hyperpolarization in the IMA and RA, we have for the first time demonstrated that EDHF exists in the human RA. Further, we have demonstrated that the EDHF-mediated hyperpolarization induced by BK in the IMA is more significant than that in RA.

NO Release From the RA and IMA

In the present study, both the basal and BK-stimulated releases of NO in the IMA are significantly greater than those in the RA. This suggests that the capacity of the endothelium of the IMA to release NO is greater than that of the RA. This is one of the major findings of the present study. This finding again emphasizes that the IMA has superiority not only to the vein grafts but also to other arterial grafts as far as the endothelial function is concerned. As mentioned, there are conflicting results regarding the endothelium-dependent relaxation between the IMA and the RA. These differences may relate to many factors. Through the direct measurement of NO concentration, the present study clearly demonstrates the greater amount of NO released from the endothelium of the IMA than from that of the RA.

In ACh-induced NO release, although the maximum concentration of NO is similar for the RA and the IMA, the duration of the ACh-induced NO release in the IMA is significantly greater than that in the RA (Figures 5 and 7). This result, from another angle, reveals the difference in the pattern of NO release from the IMA and the RA.

It is well known that NO is the major mediator of vascular tone\textsuperscript{25} and homeostasis and that the activity of the media in response to vasoconstrictor agents is reduced by the endothelium through the basal release of NO.\textsuperscript{26} In addition, NO inhibits platelet and neutrophil aggregation and adhesion and arrests smooth muscle cell proliferation.\textsuperscript{26} On the other hand, EDHF is an important mediator of the vascular tone, particularly when the NO pathway is inhibited.\textsuperscript{10,27} Therefore, EDHF may serve as a back-up mechanism of the NO pathway. The greater release of NO and the more significant EDHF-mediated hyperpolarization in the IMA compared with those in the RA predispose the RA to a more spastic characteristic in addition to its higher receptor-mediated\textsuperscript{12} and non–receptor-mediated\textsuperscript{11} contractility than in the IMA. Such differences in NO release and EDHF-mediated hyperpolarization may also have an impact on the long-term patency of the grafts. This forms a basis for future research in this area.

Clinical Implication

The RA produces less NO than that in the IMA at both the basal and the stimulated situations. This may be related to the more spastic characteristics of the RA and may affect the long-term patency rate of this graft. Antispastic therapy in this graft is particularly important during the harvesting and probably also postoperatively for CABG with the RA used to obtain the best clinical results.

In conclusion, our study suggests that the basal and stimulated releases of NO and EDHF-mediated hyperpolarization in the IMA are significantly greater than those in the RA. The lower level of NO basal release, the reduced and shorter period of stimulated NO release, and the less EDHF-mediated hyperpolarization in the RA may account for the predisposition of RA graft to the perioperative vasospasm and
may have an impact on the early and long-term results of the graft patency.

Acknowledgments

This study was fully supported by grants from the Research Grants Council of the Hong Kong Special Administrative Region (project CUHK7246/99M and 4127/01M), China, and the Providence St Vincent Medical Foundation, Portland, Ore. The major part of the experimental work was performed at the Cardiovascular Research Laboratory, Grantham Hospital, headed by Prof He, before it was transferred to the Chinese University of Hong Kong, where Dr Liu was a visiting fellow from Beijing, China.

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Circulation. 2001;104:I-344-I-349
doi: 10.1161/hc37t1.094930
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/104/suppl_1/I-344

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