Difference in Endothelium-Derived Hyperpolarizing Factor–Mediated Hyperpolarization and Nitric Oxide Release Between Human Internal Mammary Artery and Saphenous Vein

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Background—The greater nitric oxide (NO) release that occurs in the internal mammary artery (IMA) when compared with the saphenous vein (SV) has been suggested by more endothelium-dependent relaxation in the IMA or measured by bioassay; however, no direct measurement of NO- or endothelium-derived hyperpolarizing factor (EDHF)–mediated hyperpolarization has been reported. The present study measured such hyperpolarization, as well as NO release, in these vessels.

Methods and Results—IMA (n=46) and SV (n=61) segments taken from patients undergoing coronary surgery were studied in the organ chamber. Hyperpolarization (by intracellular glass microelectrode) and NO release (by NO-sensitive electrode) in response to acetylcholine and bradykinin, with and without incubation with Nω-nitro-L-arginine, indomethacin, and oxyhemoglobin, were measured. The resting membrane potential of the smooth muscle cells from the IMA (58±0.8 mV; n=15) was higher than that in those from the SV (52±0.9 mV; n=23; P=0.0001). The EDHF-mediated hyperpolarization induced by acetylcholine (10⁻⁵ mol/L: −9.4±1.5 mV in IMA, n=10, versus −4.5±1.0 mV in SV, n=17; P<0.01) and bradykinin (10⁻⁷ mol/L: −10.9±1.5 mV in IMA, n=8, versus −5.1±0.5 mV in SV, n=8; P<0.01) and the basal release of NO (16.8±1.6 nmol/L in IMA, n=13, versus 9.9±2.8 nmol/L in SV, n=13; P<0.001) were significantly greater in the IMA than in the SV. The duration of acetylcholine- and bradykinin-induced NO release was longer in the IMA than in the SV.

Conclusions—The basal release of NO and EDHF-mediated hyperpolarization were significantly greater in the IMA than in the SV. In addition, the duration of the stimulated release of NO was longer in the IMA than in the SV. These differences may contribute to the superior long-term patency of IMA grafts. (Circulation. 2000;102[suppl III]:III-296-III-301.)

Key Words: endothelium-derived factors | nitric oxide | arteries | veins | electrophysiology

The long-term benefit of myocardial revascularization depends largely on the long-term patency of bypass grafts. It is widely accepted that the long-term patency of an internal mammary artery (IMA) graft is superior to that of a saphenous vein (SV) graft.¹² Clinical data show that at 10 years postoperatively, left IMA grafts placed in the left anterior descending branch of the coronary artery have a patency rate of 85% to 95%.³⁴ In contrast, only 38% to 45% of aortocoronary SV grafts remain open.² Most importantly, 10-year survival in patients who have received a left IMA to left anterior descending coronary artery graft is ≈85%; it is only 75% in those who receive SV grafts.³⁴ Although pathological studies revealed that the main reason for prolonged IMA graft patency is freedom from atherosclerosis in the conduit, which may be attributed to histological features of the IMA,¹³ the primary causes contributing to the different patency rates between arterial and venous grafts are not fully understood.

The endothelium plays a pivotal role in the regulation of vascular tone and homeostasis.⁵⁻⁷ Apparently, the endothelial function of the coronary bypass grafts is crucial to long-term graft patency. In response to a variety of stimuli, the endothelial cells generate the following 3 major endothelium-derived relaxing factors: nitric oxide (NO), prostacyclin (PGI₂), and endothelium-derived hyperpolarizing factor (EDHF).⁷ Of these factors, NO and PGI₂ have attracted major attention. This is partially related to the fact that the chemical identification of EDHF has not been finalized, despite recent studies suggesting epoxyeicosatrienoic acids, K⁺, or other substances as candidates.⁸

A great amount of information concerning the physiological effects and biosynthesis of NO and PGI₂ has been

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acquired. Experimental studies have demonstrated that the IMA has greater basal and stimulated production of PGI₂ than does the SV\(^{10}\) and that endothelium-dependent relaxation is significantly greater in the IMA than in the SV.\(^{10}\) Further, NO release from the IMA and SV was measured by a group from the Mayo Clinic.\(^{11,12}\) However, direct quantitative measurement of the NO released from the IMA and SV has never been reported. In addition, the role of EDHF in human coronary bypass grafts has not been well studied, although we have reported the effect of EDHF in human SVs.\(^{13}\)

Therefore, the present study was designed to measure NO release from the endothelium of the IMA and SV directly and to examine the role of EDHF-mediated hyperpolarization, with an emphasis on the different regulating effects of NO and EDHF in the arterial and venous coronary bypass grafts.

### Methods

#### Vessel Preparation

Approval to use discarded tissue was obtained from the Ethics Committee of Grantham Hospital, University of Hong Kong. The discarded IMA (\(n=46\)) and SV (\(n=61\)) segments were obtained from patients undergoing coronary artery bypass grafting; they were immediately placed in a container with oxygenated physiological solution (Krebs) that was maintained at 4°C and transferred to the laboratory. The transportation time was <10 minutes. The vessels were 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 and then 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% \(\text{O}_2\) and 5% \(\text{CO}_2\) at a constant rate of 3 mL/min, and the temperature was maintained at 37°C. The composition of the Krebs solution was as follows (in mmol/L): \(\text{Na}^+\) 144, \(\text{K}^+\) 5.9, \(\text{Ca}^{2+}\) 2.5, \(\text{Mg}^{2+}\) 1.2, \(\text{Cl}^-\) 128.7, \(\text{HCO}_3^-\) 25, \(\text{SO}_4^{2-}\) 1.2, \(\text{H}_2\text{PO}_4^-\) 1.2, and glucose 11. After 60 minutes of incubation, various procedures were performed.\(^{13,14}\)

#### Electrophysiological Study

A conventional intracellular glass microelectrode filled with 3 mol/L KCl (tip resistance, 40 to 80 M\(\Omega\)) was impaled in a smooth muscle cell from the internal side. Electrical signals were continuously monitored on an oscilloscope (BK Precision, Model 2020B and WPI Electro 705), and the membrane potential was recorded by the computer (data logging software: PICOLOG, Pico Technology). The following criteria were used to assess the validity of a 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 the microelectrode. Each artery was impaled ≥4 times to assess the variability of the electrophysiological parameters; the results were then averaged to obtain 1 measurement for membrane potential. After a stable membrane potential for ≥2 minutes, acetylcholine (ACh) or bradykinin (BK) was applied.\(^{13,14}\)

#### Direct Measurement of NO

A membrane-type NO-sensitive electrode (ISO-NOP, World Precision Instruments) and isolated NO meter (ISO-NO Mark II, World Precision Instruments) were used to measure the isolated NO generated by the vascular endothelium. NO was detected using 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.\(^{13,14}\) 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 coating and is oxidized on the surface of the prepolaredized electrode, which results in an electrical current. The magnitude of the redox current is in direct proportion to the concentration of NO in the sample; it is amplified by the NO meter and registered on a computer (Duo · 18 data recording system, World Precision Instruments). The ISO-NOP has an inherently high selectivity because the electrodes are separated from the solution by any dissolved species other than gas.\(^{16}\)

The selectivity of the NO-sensitive electrode was tested in connection with calibration; in this test, a lack of response to strong saline solution (3 mol/L) or sodium nitrite (NaNO\(_2\), up to 100 \(\mu\)mol/L) was taken as evidence of an intact electrode coating. The electrodes did not respond to ACh (10 \(\mu\)mol/L), BK (1 \(\mu\)mol/L), indomethacin (7 \(\mu\)mol/L), N\(^\bullet\)-nitro-L-arginine (L-NNA, 300 \(\mu\)mol/L), or oxyhemoglobin (HbO, 20 \(\mu\)mol/L), which were added to the calibration glass vial.

The membrane-type electrode can be calibrated by chemical titration using the following equation:

\[2\text{KNO}_2+2\text{K}^+2\text{H}_2\text{SO}_4=2\text{NO}^+1+\frac{1}{2}\text{H}_2\text{O}+2\text{K}_2\text{SO}_4\]

where a known amount of KNO\(_2\) is added to produce a known amount of NO. The quantity (and thus the concentration) of the NO generated can be calculated directly using stoichiometry if the concentrations of the reactions are known.\(^{19}\)

The calibration was performed daily before the experiment. 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. NO measurement was then performed.

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

#### Experimental Protocol

**Electrophysiological Studies of EDHF-Mediated Hyperpolarization**

To investigate EDHF-mediated hyperpolarization in response to ACh and BK in the IMA and SV, the following substances were added to the organ chamber to completely inhibit the NO and PGI₂ pathway: L-NNA (300 \(\mu\)mol/L), an inhibitor of NO synthase; indomethacin (7 \(\mu\)mol/L), a cyclooxygenase inhibitor; and HbO (20 \(\mu\)mol/L), a NO scavenger.\(^{26}\) After 60 minutes of incubation in the organ chamber, the resting membrane potentials of the smooth muscle cells of the IMA and SV were recorded. ACh (−8 to −5 log M) or BK (−10 to −7 log M) was added to the organ chamber cumulatively, and the change of the membrane potential in the smooth muscle cells from the IMA and the SV was recorded. The organ chamber was then washed with Krebs solution, and L-NNA (300 \(\mu\)mol/L), indomethacin (7 \(\mu\)mol/L), and HbO (20 \(\mu\)mol/L) were added to the organ chamber. After incubation and equilibration for another 30 minutes, the aforementioned steps were repeated, and the change of the membrane potential was recorded.

**Direct Measurement of NO**

To investigate the capacity of NO release from the endothelium of the IMA and SV, ACh- and BK-induced NO release was examined. After 60 minutes of incubation and equilibration for each segment in the organ chamber, ACh (−8 to −5 log M) or BK (−10 to −7 log M) was added to the organ chamber cumulatively, and the NO signals were recorded. The interval between each addition of the different concentrations of ACh or BK was 15 minutes. The organ chamber was then washed with Krebs solution, and L-NNA (300 \(\mu\)mol/L), indomethacin (7 \(\mu\)mol/L), and HbO (20 \(\mu\)mol/L) were added to the organ chamber. After incubation and equilibration for another 30 minutes, the aforementioned steps were repeated.
To evaluate the effect of L-NNA on NO release in the IMA and SV, a subgroup of segments was incubated with L-NNA (300 μmol/L) and indomethacin (7 μmol/L) for 30 minutes, and then the aforementioned steps were repeated. The NO signals were recorded.

### Data Analysis

All results are expressed as means ± SEM. When comparisons were made between the IMA and SV groups, an unpaired Student’s t test (2-tailed) was used. When measurements were performed in the same vessel segment before and after a treatment, a paired t test was used. \( P < 0.05 \) was considered significant.

### Drugs

Acetylcholine HCl, bradykinin, potassium nitrite (KNO₂), potassium iodide (KI), L-NNA, indomethacin, and oxyhemoglobin were purchased from Sigma. The drugs were prepared in distilled water, except for indomethacin, which was dissolved in ethanol.

### Results

#### Electrophysiological Study

The resting membrane potential of the smooth muscle cells of the IMA was higher than that of those from the SV (−58 ± 1 mV, n=15, versus −62 ± 1 mV, n=23; \( P = 0.0001 \)). Mechanical removal of the endothelium produced no significant changes in the resting membrane potential of either the IMA (−56 ± 2 mV, n=5; \( P = 0.7 \) versus control) or the SV (−61 ± 1 mV, n=6; \( P = 0.5 \) versus control). Similarly, after incubation with L-NNA, indomethacin, and HbO for 30 minutes, the resting membrane potential of the smooth muscle cells from the IMA and the SV did not change significantly (IMA: −57 ± 2 mV, n=8; \( P = 0.4 \) versus control; SV: −60 ± 2 mV, n=8; \( P = 0.6 \) versus control).

ACh and BK induced endothelium-dependent hyperpolarization of the smooth muscle cells of the IMA and the SV in a concentration-dependent manner (Figures 1 through 3). Without inhibitors, no significant difference existed in the maximum hyperpolarization induced by ACh or BK in these 2 vessels (Figures 3a and 4a). However, when the NO and PGI₂ pathways were blocked (in the presence of indomethacin, L-NNA, and HbO), ACh- and BK-induced hyperpolarization was reduced in both the IMA and SV. This reduction (examined in separate experiments in which only 1 concentration of ACh or BK was applied) was more significant in the SV than in the IMA (ACh, −5 log M: 7.2 ± 0.4 mV, n=6, versus 3.2 ± 0.2 mV, n=5; \( P < 0.01 \); BK, −7.0 log M: 8.1 ± 0.6 mV, n=6, versus 4.1 ± 0.1 mV, n=5; \( P < 0.05 \)). Therefore, the EDHF-mediated hyperpolarization (the residual hyperpolarization) in the IMA was significantly greater than that in the SV (ACh, −5.0 log M: 9.4 ± 1.5 mV, n=10, versus 4.5 ± 1.1 mV, n=17; \( P < 0.01 \); BK, −7.0 log M: 10.9 ± 1.5 mV, n=8, versus 5.1 ± 0.5 mV, n=8; \( P < 0.01 \) (Figures 3b and 4b). This hyperpolarization, if expressed as a percentage of the maxi-
The basal concentration of NO—was observed in both the IMA and SV. In the IMA, basal release of NO was significantly greater in the IMA than in the SV (BK -7 log M: 67.9±11.4%, n=8, versus 43.4±21.5%, n=8; P=0.01).

In the presence of indomethacin, the endothelium-dependent hyperpolarization of the smooth muscle cells did not change significantly in either the IMA or SV (data not shown).

In endothelium-denuded IMA and SV segments, the addition of ACh or BK did not cause a significant change in the membrane potential of the smooth muscle cells.

**NO Measurement**

**Calibrations**
The membrane-type NO-sensitive electrodes responded with decreases in current to nanomolar concentrations of NO. The output current of the probes correlated linearly with the concentration of NO (r=0.9965±0.0027, n=28 experiments; P<0.005). Because the sensitivity of the different electrodes varied broadly, from 0.16 to 0.89 nmol·L⁻¹·pA⁻¹ (average, 0.56±0.14 nmol·L⁻¹·pA⁻¹), calibration was performed daily.

**Basal Release of NO**
In the resting state, a continuous NO signal—the basal release of NO—was observed in both the IMA and SV. In the IMA, the basal concentration of NO was 16.8±1.6 nmol/L (n=13 segments from 8 IMAs), which is significantly greater than that in the SV (9.9±2.8 nmol/L, n=13 segments from 9 SVs; P<0.001).

**Stimulated Release of NO**
Stimulation of the endothelium of the IMA and SV with ACh and BK evoked a rapid rise in NO that constituted the initial peak of NO and was followed by a sustained elevation lasting for 3 to 13 minutes (Figures 5 and 6). The ACh- and BK-induced NO release in the IMA and SV occurred in a concentration-dependent manner. Although no significant differences existed between the IMA and the SV in the peak concentration of NO release induced by ACh and BK (Figure 7), the duration of NO release in the IMA was significantly longer than that in the SV (11.6±1.5 minutes, n=8, versus 8.1±1.9 minutes, n=9; P<0.01; Figure 7).

With regard to the effect of inhibitors on NO release, in the presence of L-NNA and indomethacin, the ACh- and BK-induced NO release decreased to ≈26% in the IMA (n=5) and to 29% in the SV (n=5), but it was still detectable. Further addition of HbO (20 μmol/L) abolished NO release (Figure 6).

**Discussion**
In the present study, we found (1) that the EDHF-mediated hyperpolarization in the IMA is significantly greater than that in the SV; (2) that the basal release of NO in the IMA is significantly greater than that in the SV; and (3) that the duration of the stimulated release of NO in the IMA is longer than that in the SV.

To study EDHF-related endothelial function, it is essential to completely inhibit the other 2 endothelium-derived relaxing factors, NO and PGI₂. It was previously demonstrated that the PGI₂ pathway can be blocked by indomethacin,6,7 but none of the NO synthase inhibitors, such as L-NNA and N⁵-nitro-L-arginine methyl ester, completely blocks NO bio-
syntheses and release.17,20 NO has been shown to hyperpolarize the vascular smooth muscle cells19,20 and, therefore, any electrophysiological studies related to EDHF must be conducted under the condition that the residual NO resistant to NO synthase inhibitors is completely eliminated. We added HbO11 in our electrophysiological studies concerning the role of EDHF, and we demonstrated in the present study that in the IMA and SV, NO release is completely inhibited by the combination of L-NNA and HbO. The hyperpolarization of the smooth muscle cells from the IMA and SV in our study is therefore related to EDHF.

With the presence of L-NNA and HbO, NO production was not detectable. Theoretically, a possibility exists that a small amount of NO could be diffused into the smooth muscle cell through the endothelium-smooth muscle gap junction. However, it is unlikely that this would affect our results. Martin et al19 demonstrated that with 10 μmol/L HbO, endothelium-dependent relaxation is abolished, which implies that it has minimal or no influence on the direct diffusion of NO. Further, HbO also abolishes increases in cGMP. Therefore, in
our study, the possible influence of the diffusion of NO into the smooth muscle cell is minimal because we used 20 μmol/L HbO.

In the present study, for the first time, we demonstrated the existence of EDHF in the human IMA. In addition, we showed that the magnitude of EDHF-mediated hyperpolarization elicited by ACh and BK in the IMA is significantly greater than that in the SV. Interestingly, without NO and PGI2 inhibitors, endothelium-dependent hyperpolarization is not different between the IMA and SV. Only after the NO and PGI2 pathways were completely blocked did the amplitude of ACh- and BK-induced hyperpolarization, which was mediated by both NO and EDHF, decrease; it decreased by nearly 60% in the SV compared with only 30% in the IMA (Figures 3 and 4). We also observed that when indomethacin was added, the ACh- and BK-induced endothelium-dependent hyperpolarization was almost unchanged in both the IMA and SV. This suggests that the endothelium-dependent hyperpolarization in the IMA and SV is mainly due to EDHF and NO rather than PGI2. In fact, NO may be responsible for nearly 60% of the endothelium-derived NO production in response to ACh and BK in the SV, whereas only 30% of that in the IMA (P<0.01); therefore, NO makes a greater contribution to the ACh- and BK-induced hyperpolarization in the SV than that in the IMA. These data suggest that EDHF might play a more important role in the IMA than in the SV.

Previous studies showed that endothelium-dependent relaxation in the IMA was greater than that in the SV. In addition, Nishioka et al22 recently reported that, by indirect measurement, IMA grafts release more endothelium-derived NO than SV grafts in vivo. These data seem to conflict with our results. One possible reason for this is that the experimental conditions were different. Moreover, none of these studies directly and quantitatively measured NO release. Nishioka et al22 measured NO-related nitrite in coronary artery bypass grafts in vivo, and their SV grafts were prepared during harvest. It has been demonstrated that the surgical preparation of the SV damages the endothelium and abolishes EDHF-related function.13 In the present study, we used nondistended SV for the NO measurement. Therefore, it is understandable that discrepancies exist between our study and those of others.

It has been demonstrated that NO inhibits platelet and neutrophil aggregation and adhesion and arrests smooth muscle cell proliferation.23 These effects are crucial to the long-term patency of coronary artery bypass grafts. For this reason, greater basal release of NO in the IMA may contribute significantly to the superior long-term patency of IMA graft. Further, we recently demonstrated that after surgical preparation, the NO production from the SV is greatly

**Figure 6.** Original tracing of BK-evoked NO release from endothelial cells of IMA and SV (from right to left). Indo indicates indomethacin; Hb, oxyhemoglobin. BK was added at arrows above each recording.

**Figure 7.** Time-course of BK (−7.0 log M)-induced NO release in IMA (n=8) and SV (n=9). *P<0.05 compared with SV group.
reduced, and this may further decrease the patency of the vein graft.

The contribution of EDHF to endothelium-dependent relaxation varies along the vascular tree. In addition, one study recently showed that under physiological conditions, continuous release of EDHF contributes to the adjustment of adequate vascular compliance and tone in the coronary vascular bed. Moreover, some interactions occur between NO and EDHF. EDHF may play a back-up role when NO production in the vascular endothelium is impaired. and NO may regulate EDHF production and effect. A better understanding of the physiological role of EDHF must await its biological characterization and the availability of a specific inhibitor for its release and/or action. In fact, a number of recent studies have focused on the chemical nature of EDHF.

Arterial grafts may provide superior long-term patency than vein grafts. Our study provides an explanation for this superior patency: it may be related to the greater basal release of NO in arterial grafts compared with venous grafts. In addition, the superior patency may also be related to the more significant role of EDHF-mediated endothelial function in arterial grafts. Our study provides a biological basis for the wide use of arterial grafts in coronary bypass surgery.

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References
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