ISCHEMIC PRECONDITIONING ATTENUATES POSTISCHEMIC CORONARY ARTERY ENDOTHELIAL DYSFUNCTION IN A MODEL OF MINIMALLY INVASIVE DIRECT CORONARY ARTERY BYPASS GRAFTING

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Objective: Unmodified reperfusion without cardioplegia in minimally invasive direct coronary artery bypass grafting procedures causes endothelial dysfunction that may predispose to polymorphonuclear neutrophil-mediated myocardial injury. This study tested the hypothesis that ischemic preconditioning in a minimally invasive direct coronary artery bypass grafting model attenuates postischemic endothelial dysfunction in coronary vessels. **Methods:** In anesthetized dogs, the left anterior descending coronary artery was occluded for 30 minutes and reperfused for 3 hours without ischemic preconditioning (no-ischemic preconditioning; $n = 7$); in 7 dogs, the left anterior descending occlusion was preceded by 5 minutes occlusion followed by 5 minutes of reperfusion. Relaxation responses to stimulators of nitric oxide synthase were used to evaluate endothelial function in arteries from the ischemic-reperfused (left anterior descending) and nonischemic (left circumflex coronary artery) zones. **Results:** Stimulated endothelial-dependent relaxation of epicardial left anterior descending artery to incremental concentrations of acetylcholine in the no-ischemic preconditioning animals was shifted to the right, and maximal relaxation was attenuated compared with the nonischemic left circumflex coronary artery ($117\% \pm 4\%$ vs $138\% \pm 5\%$). In contrast, acetylcholine-induced maximal relaxation was comparable in the left anterior descending artery versus left circumflex coronary artery in the ischemic preconditioning group ($130\% \pm 6\%$ vs $135\% \pm 5\%$). In 150- to 200- μm left anterior descending microvessels, 50% relaxation occurred with a lower concentration ($\log[M]$) of acetylcholine in ischemic preconditioning versus no-ischemic preconditioning (-8.0 ± 0.4 vs -7.0 ± 0.1) with no group differences in smooth muscle relaxation to sodium nitroprusside, suggesting endothelial-specific damage. Adherence of fluorescent labeled polymorphonuclear neutrophils to epicardial coronary artery endothelium, used as an index of basal (unstimulated) anti-polymorphonuclear neutrophil function, was significantly attenuated by ischemic preconditioning versus no-ischemic preconditioning (293 ± 25 polymorphonuclear neutrophils/ mm^2 vs 528 ± 29 polymorphonuclear neutrophils/ mm^2). **Conclusion:** In this minimally invasive direct coronary artery bypass grafting model, both agonist-stimulated and basal postischemic endothelial dysfunction were attenuated by ischemic preconditioning. (J Thorac Cardiovasc Surg 1999;117:383-9)

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Presented at the Seventieth Scientific Sessions, American Heart Association, Orlando, Florida, Nov 9-12, 1997.

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0022-5223/99 \$8.00 + 0 12/1/94978

In 1986, Murry and colleagues¹ reported a reduction in myocardial necrosis after repeat periods of ischemia and reperfusion in an in vivo canine model of transient coronary occlusion. Since this report, a plethora of studies have observed and characterized the “ischemic preconditioning” phenomenon. Ischemic preconditioning (IP) has been defined as an endogenous adaptation of the heart to a prolonged ischemic

insult by exposing the myocardium to a brief period of ischemia before the prolonged event. IP has been found to ameliorate regional contractile dysfunction in some models,² ischemic contracture,² infarct size,^{1,3} and reperfusion arrhythmias.⁴ Although the mechanisms of IP have not been clearly defined, the exogenous infusion of adenosine,⁵ noradrenaline,⁶ bradykinin,⁷ nitric oxide,⁸ and endothelin⁹ before the occlusive event have been shown to mimic IP. Furthermore, protein kinase C and adenosine triphosphate-sensitive potassium channel-mediated mechanisms have been suggested as likely end-effectors that lead to myocardial protection.^{10,11}

Ischemia-reperfusion has been demonstrated to reduce vascular endothelial-dependent vasoactive properties in both in vitro¹² and in vivo¹³ animal models. Injury to vascular endothelium increases polymorphonuclear neutrophil (PMN) adherence (basal function) and decreases the ability of coronary vessels to vasorelax in response to agonist stimulators of nitric oxide synthase (stimulated function). Although therapeutic strategies (adenosine, nitric oxide) have attenuated PMN attachment and PMN-mediated damage to the endothelium at reperfusion,^{14,15} few studies have examined IP's potential protective effects on the coronary endothelium, and the results of these studies are discrepant.¹⁶⁻²⁰ DeFily and Chilian¹⁷ have reported that IP reduces endothelial dysfunction of coronary arterioles in intact beating canine hearts after reperfusion injury. In contrast, Bauer and colleagues,²⁰ in an in vivo canine model, have shown that IP did not reduce endothelium-dependent coronary vasorelaxation. Therefore the effects of preconditioning on the endothelial cell have not been clearly defined. Furthermore, because coronary epicardial macrovessels and microvessels differ in their normal physiologic features and responses to ischemia and reperfusion,²¹ with the microvascular endothelium being more vulnerable to injury, further studies on the effects of IP on the endothelium in both vascular tissues are warranted.

The present study tests the hypothesis that IP attenuates postischemic endothelial dysfunction in epicardial macrovessels and microvessels in a model of minimally invasive direct coronary artery bypass grafting (MID-CABG) in which short-term occlusions simulate transient intraoperative vessel occlusion during anastomosis.

Methods

General. All dogs were handled in compliance with the "Guide for the Care and Use of Laboratory Animals" published by the National Institutes of Health (NIH Publication #86-23, revised in 1985) and the Institutional Animal Care and Use Committee of Emory University.

Fourteen heartworm-free dogs of either sex were initially anesthetized with pentobarbital (20 mg/kg) and endotracheally intubated. Anesthesia was supplemented with fentanyl citrate (0.3 µg/kg/min) and diazepam (0.03 µg/kg/min) administered intravenously as needed to maintain deep anesthesia. The dogs were ventilated with a volume-cycled respirator with oxygen-enriched room air. Serial arterial blood gases were measured to maintain the arterial oxygen tension above 100 mm Hg and arterial carbon dioxide tension between 30 and 40 mm Hg. A standard lead II electrocardiogram was monitored throughout the experimental protocol. Polyethylene catheters were inserted into the right femoral artery and vein.

A median sternotomy incision was used to expose the heart. Millar catheter-tipped pressure transducers (Millar Instruments, Houston, Texas) were placed in the proximal aorta and left ventricular cavity to measure aortic and left ventricular pressures, respectively. The left anterior descending (LAD) coronary artery distal to the first marginal branch was dissected and loosely encircled with 2-0 silk suture.

The dogs were randomized to a 2-group paradigm comparing preconditioning and no-preconditioning involving 30 minutes of LAD occlusion and 3 hours of reperfusion, as described previously.²² In the group without IP (no-IP), the LAD artery was occluded for 30 minutes and reperfused for 3 hours. In the preconditioning group (IP), animals underwent 5 minutes of LAD occlusion followed by 5 minutes of reperfusion and then 30 minutes of LAD occlusion. After 3 hours of reperfusion, the experiment was terminated with a bolus of intravenous pentobarbital (100 mg/kg). The heart was immediately excised and placed into ice-cold Krebs-Henseleit buffer of the following composition: 118 mmol/L NaCl, 4.7 mmol/L KCl, 1.2 mmol/L KH₂PO₄, 1.2 mmol/L MgSO₄ · 7H₂O, 2.5 mmol/L CaCl₂ · 2H₂O, 12.5 mmol/L NaHCO₃, and 11 mmol/L glucose at pH 7.4. After excision of the coronary artery segments in selected experiments, the hearts were sliced transversely and incubated in 1% triphenyltetrazolium chloride solution (37°C) for 10 minutes to visualize necrotic myocardium within the area at risk. Necrosis was not observed by triphenyltetrazolium chloride staining. Three ex vivo bioassays were used to interrogate the effects of in vivo IP on endothelial function.

Neutrophil-endothelial cell adhesion studies

Neutrophil isolation. Neutrophil isolation was performed with techniques previously described.¹⁴ Briefly, peripheral arterial blood was sampled from the femoral artery before the median sternotomy and PMNs were isolated by the Ficol-Pacque (Sigma Chemical, St. Louis, Mo) density gradient technique. In our hands, the isolated cell preparation contains greater than 95% PMNs, and cell viability is greater than 90% (trypan blue exclusion).¹⁴

Neutrophil adherence to coronary artery endothelium. The adherence of unstimulated neutrophils to canine epicardial coronary arteries was assessed with neutrophils labeled with Zynaxis PKH26 vital fluorescent dye (Zynaxis Cell Science, Malvern, Pa). In validation studies performed with the labeling procedure, unlabeled and labeled neutrophils demonstrat-

ed 95% and 97% viability, respectively, using trypan blue excision.¹⁴

After the experiment, LAD (ischemic-reperfused LAD) and left circumflex coronary artery (LCx; nonischemic LCx) segments were isolated after the heart was harvested, cut into 3-mm segments, and carefully opened to expose the endothelium while being submerged in ice-cold Krebs-Henseleit buffer. The segments were then placed in dishes containing Krebs-Henseleit buffer at 37°C. Unstimulated, fluorescent-labeled neutrophils were added to the baths containing the artery segments (final concentration 1×10^6 cells/dish) for 15 minutes. After incubation, coronary segments were washed of nonadherent PMNs by carefully being dipped in Krebs-Henseleit buffer 3 times. The segments were mounted on glass slides, and adherent neutrophils were counted under epifluorescence microscopy (490-nm excitation; 504-nm emission).

Agonist-stimulated vascular relaxation

MACROVESSEL STUDIES. Vasoreactivity in epicardial macrovessels was interrogated as described previously.¹⁴ Briefly, LAD and LCx segments were carefully transected into 2- to 3-mm rings and placed into organ chambers (Radnoti Glass, Monrovia, Calif) containing oxygenated (95% oxygen and 5% carbon dioxide) Krebs-Henseleit solution at 37°C. Indomethacin (10 $\mu\text{mol/L}$) was used to inhibit the release of prostaglandins.¹⁴ The coronary rings were precontracted with an optimal concentration of thromboxane A_2 mimetic agent, U-46619, determined for each experiment (approximately 5 nmol/L). Endothelial function was assessed by comparing the vasorelaxation responses with incremental concentrations of acetylcholine (10 to 685 $\mu\text{mol/L}$) and A23187 (1-91 $\mu\text{mol/L}$), whereas smooth muscle responses were assessed with sodium nitroprusside (1-381 $\mu\text{mol/L}$). The order of dilator agent administration was random, except for A23187, which was always administered last because this causes the acute loss of responsiveness to other endothelial agonists.

MICROVESSEL STUDIES. Coronary microvessels were studied as previously described.²³ Briefly, 1 or 2 myocardial arterial microvessels (150-200 μm in internal diameter) were dissected from the LAD distribution within the ischemic zone. The vessels were placed in a circulating organ bath (Krebs-Henseleit buffer, 37°C), cannulated with dual glass micropipettes, and secured with monofilament suture. The microvessels were pressurized to 20 mm Hg in a no-flow state. With an inverted microscope connected to a video camera (IX50-S8F; Olympus Optical Company, Ltd, Japan), the vessel was projected onto a television monitor. A video dimension analyzer (Halarn; Living Systems Instrumentation, Burlington, Vt), with edge detection algorithms, was used to measure internal lumen diameter.

After 60 minutes of equilibration, the microvessels were precontracted with U-46619 (5 nmol/L) and dilated in a dose-response fashion with acetylcholine (1×10^{-10} - 3×10^{-5} mol/L), A23187 (1×10^{-10} - 3×10^{-5} mol/L), or sodium nitroprusside (1×10^{-10} - 3×10^{-5} mol/L). The order of dilator agent administration was random, except for A23187, which was always administered last.

Fig 1. Adherence of neutrophils to coronary artery endothelium with IP or no-IP. Labeled neutrophils were quantified with epifluorescence microscopy and are represented as the sum of neutrophils adhering to the endothelial surface per square millimeter of endothelium. Values are mean $\pm$ standard error of the mean.

Chemical preparation. Acetylcholine chloride, the calcium ionophore A23187, sodium nitroprusside, and indomethacin were obtained from Sigma Chemical Company (St Louis, Mo). The final concentrations of acetylcholine, A23187, sodium nitroprusside, indomethacin, and U-46619 were determined from a previous study.¹⁴ The thromboxane A_2 mimetic, U-46619, was donated from Upjohn Pharmaceuticals, Inc (Kalamazoo, Mich). Ca^{2+} - and Mg^{2+} -free Hanks' balanced salt solution 1X was purchased from Cellgro (Mediatech, Inc, Herndon, Va).

Statistical analysis. An 1-way analysis of variance was used to determine whether group-related differences occurred. If significant interactions were found, Tukey's or Student-Newman-Keuls post hoc multiple comparisons tests were applied to locate the sources of differences. The mean $\pm$ standard error of the mean are reported.

Results

Neutrophil adherence to coronary endothelium.

After the experiment, unstimulated, fluorescent-labeled neutrophils were coincubated with epicardial coronary arterial segments from both the ischemic-reperfused LAD artery and the nonischemic-reperfused LCx (Fig 1). In the no-IP group, neutrophil adherence was significantly greater by 29% in the LAD segments as compared with the LCx segments (528 ± 29 vs 409 ± 25 neutrophils/ mm^2 , respectively; $P = .004$). In contrast, in the ischemic-preconditioned group neutrophil adherence in the ischemic-reperfused LAD coronary seg-

Fig 2. Response curves to incremental concentrations of acetylcholine (ACh) to coronary macrovessels precontracted with U46619, which were treated with no-IP (A) or IP (B). Responses are expressed as percent relaxation from precontracted tension. Values are mean $\pm$ standard error of the mean. The number of coronary rings in each group is given in parentheses. * $P < .05$ versus LCx.

ments was comparable to that observed in the nonischemic-reperfused LCx segments of the same group and was reduced by 44% compared with no-IP LAD segments (293 ± 25 vs 528 ± 29 neutrophils/mm², respectively; $P < .001$).

Macrovascular reactivity studies. To quantify agonist-stimulated endothelial dysfunction in conduit epicardial coronary arteries, we used incremental concentrations of the vasodilators acetylcholine, A23187, and sodium nitroprusside in the vascular ring preparation. Fig 2 shows vasodilator responses to acetylcholine in the isolated coronary rings, expressed as a percentage of U46619-induced precontraction. In the no-IP group (Fig 2, A), there is a general and significant right shift in the concentration-response curve with significantly lower relaxation responses at acetylcholine concentrations greater than 10 nmol/L compared with the nonischemic LCx group. In addition, there is a significant

Fig 3. Response curves to incremental concentrations of acetylcholine (ACh; A), calcium ionophore (A23187; B), and sodium nitroprusside (NP; C) to LAD microvessels (150-200 μ mol/L) precontracted with U46619, which were treated with no-IP or IP. Responses are expressed as percent relaxation from precontracted tension. Values are mean $\pm$ standard error of the mean. The number of microvessels in each group is given in parentheses. * $P < .05$ versus no-IP.

decrease in maximal relaxation in the ischemic-LAD response as compared with the nonischemic LCx response. Conversely, there is no difference in the concentration-response curve and maximal relaxation in

Table I. Macrovascular responses to A23187 and sodium nitroprusside

	A23187		Sodium nitroprusside	
	Maximal relaxation (%)	EC ₅₀ log[M]	Maximal relaxation (%)	EC ₅₀ log[M]
No-IP				
LAD	128 ± 4.0	-7.77 ± 0.04	111 ± 2.3	-7.34 ± 0.03
LCx	134 ± 3.8	-7.76 ± 0.06	120 ± 2.6	-7.40 ± 0.03
IP				
LAD	142 ± 5.0	-7.80 ± 0.03	121 ± 5.1	-7.33 ± 0.03
LCx	141 ± 5.8	-7.84 ± 0.04	125 ± 4.8	-7.39 ± 0.03

The values are mean ± standard error of the mean.

the IP group between the ischemic-reperfused LAD and nonischemic LCx segments (Fig 2, B). However, the median effective concentration (EC₅₀; log[M]), the concentration of acetylcholine required to effect 50% relaxation, was not significant between the no-IP and IP LAD vessels (-7.21 ± 0.04 vs -7.21 ± 0.06, respectively). Maximal responses and EC₅₀ to the endothelium-dependent receptor-independent calcium ionophore A23187 and to the smooth muscle-dependent vasodilator sodium nitroprusside (Table I) showed no significant difference between LAD and LCx in either the no-IP or IP groups. Therefore endothelial dysfunction in epicardial vessels appears to be endothelial cell-receptor specific.

Microvessel reactivity studies. In the IP group, relaxation responses to acetylcholine were significantly greater at each concentration compared with responses in the no-IP microvessels, with maximal relaxation being significantly greater in IP than no-IP microvessels (Fig 3, A). EC₅₀ (log[M]) in the IP LAD vessels was significantly lower than in no-IP LAD vessels (-8.0 ± 0.36 vs -7.02 ± 0.14, respectively; *P* = .035). Although there is a modest trend toward lower relaxation responses to nonreceptor mediated A23187, there is no significant difference in maximal relaxation between IP and no-IP LAD vessels (Fig 3, B) or EC₅₀ (-8.63 ± 0.39 vs -8.13 ± 0.19, respectively; *P* = .263). Maximal relaxation to the endothelium-independent vasodilator, sodium nitroprusside, was significantly greater in the IP group at selected concentrations (Fig 3, C), and EC₅₀ was significantly lower in the IP-treated LAD vessels as compared with the no-IP treated vessels (-8.42 ± 0.26 vs -7.21 ± 0.29, respectively; *P* = .023).

Discussion

In the present study, we sought to determine whether IP could attenuate postischemic endothelial dysfunction in a canine MIDCABG model in which a period of short-term vessel occlusion is obligatory during coro-

nary artery anastomosis. A recent study by Bufkin and colleagues²² demonstrated contractile dysfunction in the ischemic-reperfused myocardium after the primary occlusion, which was immune to the effects of IP. In contrast, the present study demonstrates that 30 minutes of LAD occlusion followed by reperfusion attenuated the basal antineutrophil and agonist-stimulated components of endothelial function in the ischemic-reperfused epicardial macrovessels, although vasorelaxation responses were also impaired in the microvessels. Injury to the endothelium by myocardial ischemia and reperfusion increased neutrophil adherence (basal response) and decreased the ability of coronary vessels to vasorelax (stimulated response) to agonist stimulation of nitric oxide synthase. IP attenuated endothelial dysfunction in both epicardial coronary macrovessels and microvessels and significantly decreased the adherence of neutrophils to ischemic-reperfused LAD endothelium. In addition, IP reversed moderate smooth muscle dysfunction observed in ischemic-reperfused microvessels. These data suggest that IP may attenuate macrovascular and microvascular endothelial dysfunction and inhibit neutrophil-endothelial cell interactions, although contractile dysfunction in the area at risk may not be attenuated based on a previous study.²²

Since the initial report of IP by Murry and colleagues,¹ a considerable amount of literature regarding this phenomenon has been published focusing primarily on the end points of contractile function and infarct size. Although IP reduces myocyte necrosis resulting from a prolonged ischemia, the effects of preconditioning on the coronary vascular endothelium is less well defined. In 1993, DeFily and Chilian,¹⁷ measuring vasodilator reserve in in vivo canine coronary arterioles, demonstrated that IP preserved endothelial-dependent vasodilation after myocardial ischemia-reperfusion. Similar endothelial protection with IP was reported by Richard and colleagues.¹⁸ Conversely, Bauer and colleagues²⁰ reported that although IP sig-

nificantly reduced myocardial infarct size, the protective effects did not extend to the coronary endothelium because IP did not significantly improve postischemic endothelium-dependent responses to acetylcholine. More recently, Kaeffer and colleagues¹⁶ showed that endothelial dysfunction as the result of ischemia-reperfusion persisted for at least 1 month and that IP attenuated this prolonged endothelial dysfunction. The present study showed that IP preserved acute endothelial function in both epicardial coronary macrovessels and microvessels in agreement with DeFily and Chilian¹⁷ and others^{16,18-20} and extended these observations by demonstrating a reduction in neutrophil adherence with IP. However, it is not known whether endothelial dysfunction in postischemic macrovessels and microvessels is a long-term event in this MIDCABG model, or whether IP offers sustained protection.

It is well established that under basal conditions, normal endothelium releases nitric oxide, which acts to tonically retard the adherence of neutrophils to coronary endothelium. In response to myocardial ischemia-reperfusion, the ability to synthesize and release nitric oxide is impaired in relation to the degree of endothelial injury, which leads to increased basal neutrophil adherence and reduced vascular relaxation responses to agonist stimulators of nitric oxide synthase. In our study, we demonstrated that myocardial stunning increases basal neutrophil-coronary endothelial adherence in ischemic LAD when compared with the neutrophil-endothelial adherence in the nonischemic LCx. Although it is well established that the interaction between PMN and coronary vascular endothelium at reperfusion leads to endothelial dysfunction,^{24,25} which can largely be attenuated by anti-PMN therapy (nitric oxide, adenosine, monoclonal antibodies),^{14,15,26} it is not clear from our study whether IP reduced endothelial dysfunction by directly protecting the endothelium or by directly attenuating neutrophil activation and adherence. If the preconditioning response is mediated, in part by adenosine, then both cell types may be effected directly. However, it would be interesting to determine whether endothelial protection by IP involved attenuation of neutrophil events at reperfusion. Nevertheless, the protection afforded to the endothelium is manifested in part by inhibiting PMN adherence.

Study limitations. Although the beneficial effects of IP have not been found in all species, many studies have reported the beneficial effects of IP in both canine and human patients. It is possible that the degree of injury and the effectiveness of IP may be blunted in human patients.²⁷ The effects of IP on human myocardium may depend on whether the ischemia is local

(ie, coronary occlusion) or global. In addition, our studies were performed in normal coronary vessels. However, in instances where underlying pathologic conditions such as atherosclerosis, diabetes mellitus, or hypercholesterolemia may significantly impact the ability of the endothelium to produce endogenous protection with ischemic or chemical preconditioning, the protection afforded by preconditioning may be less than in normal vessels. Further studies interrogating the effects of IP on endothelial function in humans undergoing coronary artery bypass grafting are warranted. Another consideration is the clinical relevance of the duration of LAD occlusion used in the present study. In a large study of MIDCABG procedures, the time to completion of target vessel anastomosis averaged 23 minutes.²⁸ In the present study, the duration of regional ischemia (30 minutes) is only somewhat longer than that encountered during completion of anastomoses in MIDCABG operations. Whether shorter periods of coronary occlusion in patients with underlying disease causes endothelial dysfunction needs to be determined.

In summary, the present study used a canine model of transient coronary occlusion to evaluate the effect of IP on coronary artery endothelium. We conclude that IP attenuated endothelial dysfunction expressed as neutrophil adherence to the ischemic-reperfused LAD and attenuated endothelial dysfunction in both coronary macrovessels and microvessels, which may have implications for the long-term patency of grafts and myocardial viability. Furthermore, we have demonstrated that IP reduces neutrophil adherence to vascular endothelium, which is consistent with an attenuation of neutrophil accumulation in ischemic-reperfused areas at risk observed in a previous study with the same model.²² This study may be clinically important for the practicing cardiac surgeon, because protection of coronary vascular endothelial function by IP may diminish reperfusion injury, reduce vascular permeability and myocardial edema, and possibly reduce end-artery thrombosis and its associated complications.

We thank Ms Gail Nechtman for formatting the manuscript; Mrs Susan Schmarkey, Ms Sara Katzmark, and Ms Jill Robinson from the Cardiothoracic Research Laboratory for technical support; the Carlyle Fraser Heart Center at Emory University School of Medicine for its continued support of the research effort; and Ron Shebusky, PhD, (Pharmacia-Upjohn Pharmaceuticals) for the gift of U46619.

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