Myocardial Contractile Effects of L-Arginine in the Human Allograft

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Objectives. In the present study, we investigated, in transplant recipients, whether L-arginine (L-arg) potentiates the myocardial contractile effects of receptor-mediated coronary endothelial stimulation. Moreover, because inducible nitric oxide synthase (iNOS) is frequently expressed in transplanted myocardium, we also performed intracoronary infusion of L-arg in the absence of receptor-mediated coronary endothelial stimulation to investigate whether similar left ventricular (LV) contractile effects could be induced by providing more substrate for iNOS.

Background. Nitric oxide (NO), released from coronary endothelium after receptor-mediated stimulation by substance P (SP), affects vascular smooth muscle tone and modulates LV contractile performance. L-arg augments receptor-mediated endothelium-dependent coronary vasodilation in transplant recipients by increasing substrate availability for endothelial NO production.

Methods. Sixteen transplant recipients were studied at the time of annual coronary angiography. In eight transplant recipients, microtip LV pressures were recorded before and during intracoronary (IC) SP (20 pmol/min) and after the addition of IC L-arg (160 µmol/min) to IC SP. In eight transplant recipients, microtip LV pressures were recorded before and during IC L-arg (160 µmol/min) alone, and in six of these patients, endomyocardial biopsy samples were obtained to detect the expression of iNOS gene by reverse transcription–polymerase chain reaction.

Results. Addition of IC L-arg to IC SP induced a fall (mean ± SEM) in LV peak systolic pressure (−16 ± 4 mm Hg), which was larger (p < 0.01) than that observed during IC SP (−7 ± 2 mm Hg). During IC L-arg alone, there was no change in LV peak systolic pressure despite the presence of iNOS mRNA in five of the six biopsy samples.

Conclusions. In transplant recipients, L-arg potentiates the paracrine myocardial contractile effects of receptor-mediated coronary endothelial stimulation, probably by providing more substrate for endothelial NO production. Despite the myocardial expression of iNOS gene, L-arg alone fails to elicit similar contractile effects.

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Nitric oxide (NO), derived from NO donors or produced by endothelial cells, has recently been demonstrated to influence myocardial contractile performance of the human heart, as evident from the development of lower left ventricular (LV) systolic pressure and increased LV diastolic distensibility observed during bicornary infusion of sodium nitroprusside (1) or substance P (SP) (2), which releases NO from coronary endothelium. These hemodynamic changes were attributed to a direct, probably 3',5'-cyclic guanosine monophosphate (cGMP)–mediated, myocardial action of NO. They were unrelated to peripheral vasodilation because right atrial infusion failed to reproduce these effects and also appeared unrelated to autonomic reflexes because similar results were observed in transplant recipients who have deficient afferent cardiac innervation (3).

In transplant recipients (4), L-arginine (L-arg) administration improves agonist-induced endothelium-dependent coronary vasodilation, probably by increasing substrate availability for coronary endothelial NO production. In the present study, we investigated, in transplant recipients, whether the LV myocardial contractile effects of NO, released from the coronary endothelium during intracoronary (IC) infusion of SP, could be potentiated by IC co-infusion of L-arg. Moreover, because of the frequent myocardial expression of inducible NO synthase (iNOS) in the human cardiac allograft (5) as a result of long-term exposure to rejection-related cytokines, we also infused IC L-arg without IC infusion of SP to investigate whether similar LV myocardial contractile effects could be induced by providing more substrate for myocardial iNOS, which causes a sustained, large, L-arg transport–dependent production of NO (6–8). These observations may also be relevant to congestive cardiomyopathy because of the recent demonstration of iNOS gene expression in patients with nonischemic congestive cardiomyopathy (9,10) and the recently reported effects of intravenous (11) and oral (12,13) L-arg in heart failure.

Methods

Patients. Sixteen patients were studied at the time of annual cardiac catheterization and coronary angiography 1
Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>l-arg</td>
<td>L-arginine</td>
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<tr>
<td>cNOS</td>
<td>Constitutive nitric oxide synthase</td>
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<tr>
<td>IC</td>
<td>Intracoronary</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
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<tr>
<td>LV</td>
<td>Left ventricular</td>
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<tr>
<td>LV dp/dt</td>
<td>Rate of change of LV pressure</td>
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<tr>
<td>LV dp/dt max</td>
<td>Maximal rate of rise of LV pressure</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>SP</td>
<td>Substance P</td>
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year (n = 1), 2 years (n = 3), 3 years (n = 7), 4 years (n = 3), 5 years (n = 1) and 6 years (n = 1) after orthotopic heart transplantation (2 women, 14 men; mean age 52 years, range 26 to 65). All patients were receiving immunosuppressive therapy, consisting of various combinations of ciclosporine, prednisone and azathioprine. For ethical reasons, immunosuppressive therapy and antihypertensive medications (angiotensin-converting enzyme inhibitors [n = 7], calcium channel blockers [n = 7] and beta-adrenergic blocking agents [n = 1]) were continued at the time of study. No patient was taking digitalis at the time of study.

Coronary angiography, which preceded the study protocol, revealed angiographically normal coronary arteries without evidence of accelerated graft atherosclerosis (14). LV angiography, which was performed after the study protocol, showed normal LV end-diastolic volume index (mean [±SEM] 59 ± 9 ml/m²) and normal LV ejection fraction (77 ± 7%) in all patients. No patient had biopsy evidence requiring adjustment of therapy at the time of study. Informed consent was obtained from all patients. The study protocol was approved by the local ethical committee, and there were no complications related to procedure or study protocol.

Study protocol. As described previously (1.2), left and right heart catheterization was performed from the right and left femoral arteries and the right femoral vein. LV pressure was measured with a high fidelity tip micromanometer catheter calibrated externally against a mercury reference and matched against lumen pressure. To achieve homogenous delivery of SP or L-arg, or both, to all portions of the left ventricle, a bicoronary infusion technique was used (1,2), except in four patients with a left dominant coronary system and three with a right dominant coronary system in whom simultaneous stable coronary catheter positions in both coronary ostia could not be achieved. In the latter two groups of patients, only the left coronary artery was infused. When a bicoronary infusion technique was used, the dose of SP or L-arg, or both, was divided between the two coronary arteries, in accordance with coronary anatomy (1,2). When only left coronary artery infusion was used, the entire dose of SP or L-arg, or both, was infused in the left coronary artery. As described in our previous study (2), the total dose of SP was progressively increased from 2.5 to 20 pmol/min, which was then kept constant throughout the entire protocol. A total dose of L-arg of 160 µmol/min was used throughout the entire study protocol, in accordance with previous investigations (15,16) studying the effects of IC L-arg on endothelium-dependent coronary vasomotion.

LV pressure, rate of change of LV pressure (LV dp/dt), right atrial pressure and three leads of the electrocardiogram were continuously recorded on a Gould ES 1000 multichannel recorder (Fig. 1) before, during and for 5 minutes following the IC infusions. Fast paper speed recordings (250 mm/s) covering several respiratory cycles were obtained at 1-min intervals. After baseline recordings, the first series of eight transplant recipients received IC infusion of SP for 5 min, followed by IC co-infusion of SP and L-arg for 8 min. The second series of eight transplant recipients received IC infusion of L-arg for 8 min after baseline recordings, which were obtained either in control conditions (n = 3) or during intravenous administration of dobutamine (n = 5). Intravenous dobutamine was administered to enhance the eventual myocardial contractile effects of L-arg-induced NO production (17), individually titrated to raise rest heart rate by 20 beats/min and kept constant (4 ± 2 µg/kg body weight per min) throughout the IC L-arg infusion and for 5 min after cessation of the IC L-arg infusion.

Right ventricular endomyocardial biopsy samples were obtained at the end of the study in all patients. In six patients, who underwent single IC L-arg infusion, additional biopsy samples were immediately frozen in liquid nitrogen and stored at −80°C for subsequent detection of iNOS mRNA by reverse transcription–polymerase chain reaction (PCR).

Reverse transcription–PCR. Quantification of iNOS mRNA was performed by reverse transcription followed by PCR.

Reverse transcription. For first-strand cDNA synthesis, 2 µg of total RNA was mixed with 1× reverse transcription buffer (50 mmol/liter Tris-HCl, pH 8.3, 75 mmol/liter KCl, 3 mmol/liter MgCl₂) completed with 0.5 mmol/liter deoxynucleotide triphosphate (Pharmacia Biosystems Ltd., Freiburg, Germany) and 250 pmol of random hexanucleotide primers. These mixtures were heated to 72°C for 3 min. Then, dithiothreitol (10 mmol/liter), RNase inhibitor (2 U/100 ng of total RNA, Amersham Buchler, Ltd., Braunschweig, Germany) and Moloney murine leukemia virus reverse transcriptase (10 U/100 ng of total RNA, Life Technologies Ltd., Eggenstein, Germany) were added to the reverse transcription reaction to a total volume of 25 µl and incubated at 42°C for 60 min, followed by denaturation at 95°C for 5 min.

PCR amplification. Duplicate samples of PCR were performed in a total volume of 50 µl, respectively, each containing 10 µl of reverse transcription reaction, 35 µl of a PCR master mix (16 mmol/liter Tris-HCl pH 8.3, 40 mmol/liter KCl, 0.4 mmol/liter MgCl₂, 20 pmol of sense and antisense primer) and 2.5 U of Taq-DNA polymerase (Pharmacia Biosystems, Ltd.). The mixture was overlaid with mineral oil (Sigma Ltd., Deisenhofen, Germany) and then subjected to 36 cycles of PCR amplification using a DNA thermal cycler (Perkin Elmer Ltd., Ueberlingen, Germany). The cycle profile included denaturation for 1 min at 94°C, annealing for 2 min at 62°C and
extension for 3 min at 72°C. The specificity of the amplified PCR products was confirmed by restriction enzyme analysis and by hybridization with specific internal oligonucleotide probes. The amplification products of 10 μl of each PCR reaction were separated by electrophoresis on a 1.5% agarose gel, stained with ethidium bromide, visualized by ultraviolet irradiation and photographed (Polaroid 665 negative film, Polaroid Ltd., Offenbach, Germany). As a negative control, no amplification product occurred if reverse transcriptase or total RNA was omitted in the first-strand cDNA reaction or when water was used as control.

Selection and synthesis of the PCR primers. Appropriate sense and antisense primer oligonucleotides were selected from the human cDNA sequences of iNOS (sense primer: 1614 to 1633, 5'-GGGAGCATCACCCCCGTGTT-3'; antisense primer: 2012 to 2033, 5'-GAGCGATTTCTTCAGTTTCTCT-3') by computer analysis using the Oligo program (National Biosciences Inc.). To ensure that no genomic DNA contamination was present in the RNA solution, the chosen primer oligonucleotides spanned splice junctions (18).

Data analysis. LV volumes and ejection fraction were derived from single-plane LV angiograms by use of the area-length method and a regression equation (19). All hemodynamic data (Table 1) were averaged over a complete respiratory cycle and derived from the fast paper speed recordings. The duration of LV electromechanical systole (Table 1), which indicates the time to onset of LV relaxation, was measured as the interval from the Q wave on the electrocardiogram to the moment of maximal rate of fall of LV pressure. Data were analyzed using repeated measures analysis of variance followed by a multiple-comparison test (Student-Newman-Keuls). Statistical significance was set at a two-tailed probability level <0.05.

Results
IC co-infusion of SP and l-arg. Figure 1 shows representative electrocardiograms of LV dP/dt, LV pressure (LVP) and right atrial pressure (RAP) during control conditions; after 2 (2'IC-SP) and 5 min (5'IC-SP) of IC infusion of SP, respectively; after 2 (2'IC-SP+L-arg) and 8 min (8'IC-SP+L-arg) of IC co-infusion of SP and l-arginine, respectively; and 5 min after cessation of co-infusion (5'Post-IC).

Figure 1. Representative electrocardiograms (three leads) of LV dP/dt, LV pressure (LVP) and right atrial pressure (RAP) during control conditions; after 2 (2'IC-SP) and 5 min (5'IC-SP) of IC infusion of SP, respectively; after 2 (2'IC-SP+L-arg) and 8 min (8'IC-SP+L-arg) of IC co-infusion of SP and l-arginine, respectively; and 5 min after cessation of co-infusion (5'Post-IC).
Partial recovery of LV pressures was observed at the end of the IC infusion of SP and at the end of the IC co-infusion of SP and L-arg. Recovery was almost complete by 5 min after cessation of the IC co-infusion.

**IC infusion of L-arg.** Table 1 (bottom) shows baseline hemodynamic data and the changes observed during IC infusion of L-arg. Baseline recordings were obtained in control conditions (n = 3) or during intravenous infusion of dobutamine (n = 5), which raised (mean ± SEM) heart rate from 83 ± 6 to 120 ± 5 beats/min (p < 0.005) and LV dP/dt max from 1,520 ± 138 to 2,820 ± 132 mm Hg/s (p < 0.0005) and lowered LV end-diastolic pressure from 13 ± 1 to 8 ± 1 mm Hg (p < 0.01) and duration of LV electromechanical systole from 399 ± 10 to 282 ± 10 ms (p < 0.0005). Changes during IC infusion of L-arg are shown after 2 min of infusion at a time corresponding to the moment of maximal effect during IC co-infusion of SP and L-arg, during IC infusion of L-arg after 8 min of infusion (i.e., just before cessation of infusion) and 5 min after cessation of IC infusion of L-arg. IC infusion of L-arg caused no significant changes in LV pressures during or after infusion (Fig. 2). PCR bands indicating expression of iNOS mRNA were present in five of the six patients in whom.

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**Table 1. Myocardial Contractile Effects of Intracoronary L-Arginine**

<table>
<thead>
<tr>
<th></th>
<th>Effects of IC Co-Infusion of SP and L-arg (n = 8)</th>
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<th>Effects of IC Infusion of L-arg (n = 8)</th>
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<tr>
<td><strong>Baseline</strong></td>
<td><strong>Δ2′IC-SP (A)</strong></td>
<td><strong>Δ8′IC-SP (B)</strong></td>
<td><strong>Δ2′IC-L-arg (C)</strong></td>
</tr>
<tr>
<td><strong>HR (beats/min)</strong></td>
<td>107 ± 8</td>
<td>2 ± 1 *#</td>
<td>4 ± 2*</td>
</tr>
<tr>
<td><strong>LVPSP (mm Hg)</strong></td>
<td>128 ± 6</td>
<td>1 ± 2</td>
<td>2 ± 2</td>
</tr>
<tr>
<td><strong>LVESP (mm Hg)</strong></td>
<td>67 ± 4</td>
<td>1 ± 1</td>
<td>4 ± 2</td>
</tr>
<tr>
<td><strong>LVEDP (mm Hg)</strong></td>
<td>10 ± 1</td>
<td>0 ± 0.5</td>
<td>1 ± 0.5</td>
</tr>
<tr>
<td><strong>LV dP/dt max (mm Hg/s)</strong></td>
<td>2,246 ± 286</td>
<td>4 ± 34</td>
<td>54 ± 26</td>
</tr>
<tr>
<td><strong>LVEDP (mm Hg)</strong></td>
<td>1,979 ± 228</td>
<td>39 ± 66</td>
<td>68 ± 70</td>
</tr>
<tr>
<td><strong>LVEST (ms)</strong></td>
<td>329 ± 27</td>
<td>6 ± 21</td>
<td>5 ± 2</td>
</tr>
</tbody>
</table>

*Minimal 95% confidence interval >0. †Maximal 95% confidence interval <0. ‡p < 0.01, A versus C. §p < 0.05, B versus C. |p < 0.05, C versus D. ¶p < 0.05, C versus E. †#p < 0.05, A versus C. Data presented are mean value ± SEM. L-arg = L-arginine; HR = heart rate; IC = intracoronary; LV dP/dt max = maximal rate of rise of LV pressure; LV dP/dt min = maximal rate of fall of LV pressure; LVEDP = left ventricular end-diastolic pressure; LVESP = left ventricular end-systolic pressure; LVEST = left ventricular electromechanical systolic time; LVPSP = left ventricular peak systolic pressure; SP = substance P; Δ2′IC-SP and Δ8′IC-SP = change observed after 2 and 5 min of intracoronary infusion of substance P, respectively; Δ2′IC-SP+L-arg and Δ8′IC-SP+L-arg = change observed after 2 and 8 min of intracoronary substance P and L-arginine co-infusion, respectively; Δ5′Post-IC = change observed 5 min after cessation of intracoronary infusion; Δ2′IC-L-arg and Δ8′IC-L-arg = change observed 2 and 8 min after intracoronary infusion of L-arginine, respectively.

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**Figure 2.** Changes (mean ± 95% confidence interval) in LV peak systolic pressure (ΔLVPSP) compared with (left) baseline during single IC infusion of SP after 2 min of infusion at the time of maximal effect (A), at the end of the single IC infusion of SP (B), during IC co-infusion of SP and L-arg after 2 min of infusion at the time of maximal effect (C), at the end of the IC co-infusion of SP and L-arg (D) and 5 min after cessation of all infusions (E); and (right) during single IC infusion of L-arg after 2 min of infusion (C), at the end of single IC infusion of L-arg (D) and 5 min after cessation of infusion (E). ‡p < 0.01, A versus C. §p < 0.05, B versus C. |p < 0.05, C versus D.
additional endomyocardial biopsy samples were obtained for detection of iNOS mRNA by reverse transcription–PCR (Fig. 3).

**Discussion**

**Myocardial contractile response to IC co-infusion of SP and L-arg.** In the present study, addition of IC L-arg potenti- ated the LV contractile effects of coronary endothelial stimulation by SP. These results resemble a previous report (4) in transplant recipients showing attenuation by L-arg of acetylcholine-induced vasoconstriction of epicardial coronary conduit vessels and enhancement by L-arg of acetylcholine-induced vasodilation of coronary resistance vessels. Both these myocardial and vascular effects of L-arg in transplant recipients could be attributed to increased NO production by endothelial cells during receptor-mediated stimulation because of increased provision of substrate for endothelial NO production in the presence of L-arg (20).

Enhancement by L-arg of agonist-induced endothelium-dependent vasodilation has also been observed in the forearm circulation of normal control subjects (16,21), and patients with hypercholesterolemia (22), mild coronary disease (16) and heart failure (23) and in the coronary microcirculation of normal control subjects (16,21), and patients with mild coronary disease (16) and hypercholesterolemia (15). In contrast, intravenous L-arg causes a vasodilator response without agonist-induced endothelial stimulation in systemic resistance vessels of patients with heart failure (11) or with critical limb ischemia (29) and in pulmonary resistance vessels of patients with pulmonary hypertension (30).

In the present study, L-arg infusion provided additional substrate not only for endothelial constitutive nitric oxide synthase (eNOS) but also for myocardial iNOS, which was recently shown (5) to be expressed in myocardial tissue of transplant recipients obtained at the time of surveillance endomyocardial biopsies. Despite myocardial expression of iNOS mRNA in five of the six samples in the present study, a single IC L-arg infusion failed to alter LV function. In view of the recently reported association in transplant recipients between the myocardial expression of iNOS and LV contractile dysfunction (5) and because of the numerous reports from isolated cardiac preparations showing reduced contractile performance after acute cytokine-induced expression of iNOS (31–36), provision of additional substrate for myocardial iNOS by IC L-arg infusion might have been expected to impair LV contractile function. The failure to observe such an impairment of LV function could relate to the absence of substrate dependence of myocardial iNOS, to a relatively low myocardial expression of iNOS in the patients studied or to unequal tissue distribution of endothelial eNOS and myocardial iNOS. Absence of substrate dependence of iNOS seems unlikely because in contrast to endothelial eNOS, iNOS is not regulated by intracellular calcium and generates large amounts of NO (6,7). Moreover, cytokine-induced NO production by iNOS in isolated cardiomyocytes was recently shown (8) to be highly dependent on L-arg transport into the cells, as evident from cytokine-induced co-induction of cationic amino acid transporters. The present investigations were performed at the time of annual cardiac catheterization in the absence of clinical or histologic evidence of rejection. Lewis et al. (5) also used surveillance biopsies to establish the association between myocardial expression of iNOS and LV contractile dysfunction of the allograft. This argues in favor of a similar level of myocardial expression of iNOS mRNA in the present study and in the study by Lewis et al. A more likely cause for the unequal effects on myocardial contractile performance of combined L-arg–SP
infusion and single L-arg infusion seems to be the unequal tissue distribution of endothelial eNOS and of myocardial iNOS, which determines diffusion distance for L-arg, diffusion distance for NO to cardiomyocytes and eventual binding of NO to immune-related reactive oxidants.

Interactions between NO and the LV contractile response to beta-agonists have been observed in dogs (37), in patients with left ventricular dysfunction (38) and in transplant recipients (17). Because of the enhancement by beta-agonists of the cardiodepressant effect of NO, we investigated, in five transplant recipients, the myocardial contractile effects of IC L-arg infusion after pretreatment with intravenous dobutamine. In these patients, IC L-arg infusion also failed to alter LV performance.

**Study limitations.** In the present study, IC L-arg was infused at a dose of 160 μmol/min. A similar dose was used in previous studies (15,16) and shown to potentiate endothelium-dependent vasodilation of the coronary microcirculation in hypercholesterolemia (15). In the present study, this dose was also effective in potentiating the myocardial contractile effects of SP-induced coronary endothelial stimulation. An insufficient dosage of L-arg to influence myocardial NO production by iNOS therefore seems unlikely. In the present study, IC L-arg was infused for an 8-min period irrespective of the presence or absence of co-infusion with SP. During IC co-infusion with SP, maximal effects were observed after 2 min, or one fourth of the total infusion time. During single IC infusion of L-arg, no effect was observed during the entire 8-min infusion period. Because of the longer diffusion distance from the coronary lumen to the myocardium than to the endothelium, slower uptake of L-arg by myocardium than by endothelium could explain failure to observe myocardial contractile effects of single IC L-arg infusion despite myocardial iNOS gene expression. A longer IC infusion period could have revealed a direct iNOS-mediated myocardial effect of L-arg. However, spillover into the right atrium and concomitant vasodilator action of L-arg precludes the use of a long IC infusion period because of inability to distinguish a fall in LV pressure related to systemic vasodilation from a fall related to iNOS-induced myocardial contractile depression.

The fall in LV peak systolic, end-systolic and end-diastolic pressures observed in the present study during IC infusion of SP results from altered myocardial contractile performance and not from peripheral vasodilation because of previous studies in which LV contractile performance during IC infusion of SP was assessed from simultaneous LV pressures and angiograms (2,17). In these studies, IC infusion of SP induced a similar fall in LV pressures without change in LV dP/dt max, which was accompanied by a rise in LV end-diastolic volume and no change in LV end-systolic volume. Unaltered LV dP/dt max at larger LV end-diastolic volume and lower LV end-systolic pressure at unaltered LV end-systolic volume were consistent with reduced myocardial contractile performance (39,40). Moreover, both the larger LV end-diastolic volume and the failure of a right atrial infusion to reproduce the hemodynamic changes of the IC infusion argue against vasodilator effects.

The present study was limited to a group of transplant recipients, and extrapolation of the present data to normal control subjects warrants caution because of studies that showed enhancement by L-arg of agonist-induced endothelium dependent vasodilation in transplant recipients (4) but not in normal control subjects (16). In contrast, our previous study (2) on the LV myocardial contractile response to IC SP reported similar hemodynamic changes in normal control subjects and transplant recipients, although the numbers studied were too small to exclude minor differences with certainty.

The presence of iNOS in the myocardium of our study group was derived from demonstration of iNOS mRNA by reverse transcription PCR and not by direct demonstration of iNOS protein. Because of posttranscriptional control of iNOS protein translation, definite proof of myocardial presence of iNOS requires direct demonstration of myocardial iNOS protein, which was not performed in the present study.

**Conclusions.** IC L-arg potentiates the paracrine myocardial contractile effects of receptor-mediated coronary endothelial stimulation, probably by providing more substrate for coronary endothelial eNOS. Despite myocardial expression of iNOS gene, a similar single IC infusion of L-arg failed to induce LV contractile effects.

**References**


