Profound Inhibition of Myogenic Tone in Rat Cardiac Allografts Is Due to eNOS- and iNOS-Based Nitric Oxide and an Intrinsic Defect in Vascular Smooth Muscle Contraction

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Background—The physiological consequences of inducible NO synthase (iNOS) expression were studied in allograft coronary arteries by pressure myography.

Methods and Results—Septal coronary arteries (diameter, 200.6±3.3 μm) were harvested from allograft and isograft hearts, and their myogenic properties were measured before and after iNOS and nonselective NOS inhibition with aminoguanidine (AG, 100 μmol/L) and N^G-nitro-L-arginine methyl ester (L-NAME) (200 μmol/L). Fura 2 fluorescence microscopy was used to measure $[Ca^{2+}]_i$ in isolated endothelial cells. Monoclonal anti-iNOS immunostains demonstrated iNOS protein in day 2, 7, 14, and 28 allograft vessels, but only in day 2 isograft vessels. Myogenic tone was profoundly inhibited in allograft vessels from day 4 onward. In day 4 allograft vessels, these differences were abolished by L-NAME but not AG, suggesting greater basal release of eNOS-based NO from allograft endothelium. Fluorescence measurements confirmed elevation of $[Ca^{2+}]_i$ in day 4 allograft endothelium, providing a mechanism for enhanced eNOS activity. For days 7 to 28, AG potentiated myogenic tone in allograft but not isograft vessels, indicating that vasoactive iNOS-based NO was present. In mature vessels, constriction via agonist- and depolarization-mediated mechanisms showed parallel inhibition, suggesting an intrinsic defect in vascular smooth muscle cell contraction.

Conclusions—Our data indicate that the profound inhibition of myogenic tone in allograft arteries involves direct vasodilation by eNOS- and iNOS-based NO, as well as an intrinsic defect in vascular smooth muscle contraction. The hemodynamic profile resulting from these changes in allograft resistance vessel function would favor movement of extracellular fluid from the intravascular space into the myocardial interstitium, resulting in edema, increased ventricular stiffness, and poor ventricular performance. **(*Circulation*. 2000;101:1303-1310.)**

Key Words: nitric oxide ■ nitric oxide synthase ■ transplantation ■ hemodynamics

Nitric oxide (NO) has recently been proposed to play an important role in transplant physiology, with several investigators having identified the inducible isoform of NO synthase (iNOS) in experimental and human cardiac allografts but not isografts.[1–3] Unlike the constitutively expressed endothelial NOS (eNOS), which normally produces low basal levels of NO in a manner very tightly regulated by endothelial intracellular Ca^{2+}, iNOS activity is Ca^{2+}-independent and is regulated largely by the amount of enzyme present; thus, iNOS is capable of producing large quantities of NO for sustained periods of time.[4] The coronary vasculature could be a target of iNOS-based NO, leading to sustained vasodilation of resistance vessels and increased coronary flow. During biopsy-proven cellular rejection in human cardiac allografts, basal work-corrected coronary flow is elevated, with a corresponding decrease in flow reserve, indicating enhanced dilation of the coronary circulation under immune assault.[5] On the basis of this evidence, we hypothesized that the expression of iNOS in cardiac allografts would result in the production of NO and enhanced vasodilation. Our results indicate that the basal myogenic tone of isolated allograft arteries is profoundly inhibited, occurring through NO-dependent and NO-independent mechanisms.

Results

iNOS and eNOS expression

iNOS expression was clearly evident in day 2, 7, 14, and 28 allograft arteries (Figure 1). In contrast, isograft arteries

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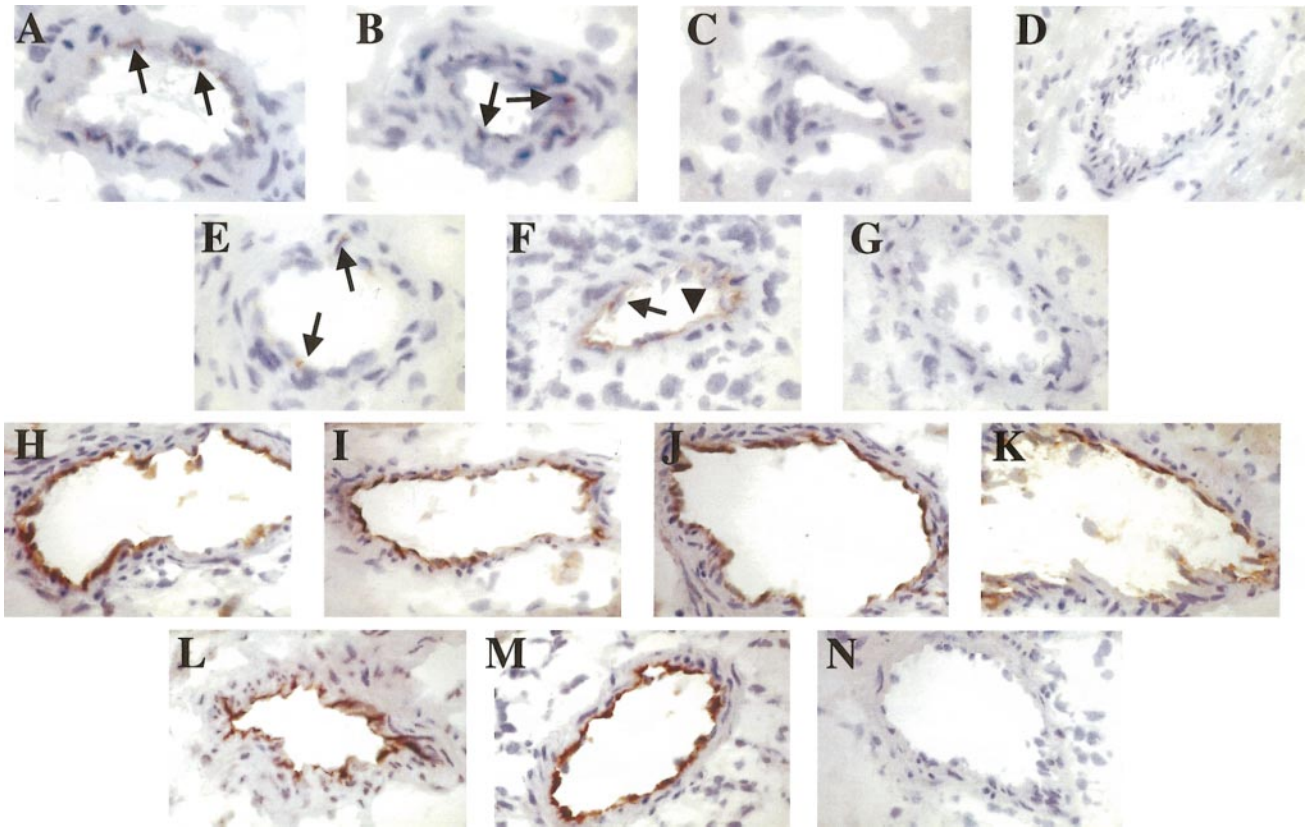

Figure 1. iNOS and eNOS expression in allograft and isograft coronary arteries. A and B, Day 2 isograft and allograft arteries; in most cases, immunostaining appears in close proximity to nuclei (arrows), consistent with iNOS positivity in adherent or infiltrating macrophages. C and D, Day 4 isograft and allograft vessels. E and F, Day 14 vessels, showing minimal iNOS expression in isograft intima (E, arrow) but impressive expression of iNOS antigen in allograft intima (F); these day 14 examples are representative of matched grafts for days 7 to 28. G, Day 14 allograft negative control. eNOS positivity is evident in allograft and isograft arteries at all time points. H and I, Day 2 isograft and allograft. J and K, Day 4 isograft and allograft. L and M, Day 14 isograft and allograft. N, eNOS-negative control.

showed clear iNOS expression only at day 2 posttransplantation, with infrequent staining for subsequent time points. Notably, day 4 lacked iNOS expression in both groups. Thus, allospecific iNOS expression is first identified at day 7 and persists at day 28, whereas transient nonspecific iNOS expression (allografts and isografts) occurs at day 2. eNOS protein was identified in allograft and isograft arteries at all time points.

Vascular Functional Studies

Vessel Size and Structure

The average diameter for all vessels was 200.6±3.3 μm at 10 mm Hg in Ca^{2+}-free physiological salt solution (PSS) (n=85). For all graft and time-point groups, pressure-diameter curves in Ca^{2+}-free PSS were similar, indicating identical passive characteristics.

Myogenic Tone

Day 2 Posttransplantation. Isograft and allograft vessels showed similar myogenic profiles at day 2; myogenic tone developed in a graded fashion as transmural pressure was increased in the physiological range. In both groups, there was a nonsignificant trend toward greater tone after aminoguanidine (AG), indicating the presence of iNOS-based vasoactive NO (Figure 2A). N^G-Nitro-L-arginine methyl ester (L-NAME) resulted in similar potentiation in both groups, indicating equal underlying myogenic tone (Figure 2B).

Day 4 Posttransplantation. In contrast to day 2, significant differences in myogenic tone were evident between isograft and allograft vessels at day 4. AG did not potentiate tone in either group, indicating an absence of iNOS-based NO (Figure 3A). However, nonselective NOS inhibition with L-NAME abolished the tone differences. This observation suggests greater basal release of eNOS-based NO in allograft vessels (Figure 3B). Because eNOS activity is $[Ca^{2+}]_i$-dependent, we reasoned that enhanced Ca^{2+} availability in allograft endothelial cells might underlie the enhanced eNOS activity in day 4 allografts. We used fura 2 fluorescence imaging to study $[Ca^{2+}]_i$ homeostasis in freshly isolated aortic valvular endothelial cells from day 4 allograft and isograft hearts. A representative tracing, along with cumulative data, is shown in Figure 4. Calculated basal $[Ca^{2+}]_i$ was significantly elevated in allograft endothelium (78.3±4.9 versus 42.8±5.7 nmol/L; 35.6±5.7 nmol/L [control], $P<0.05$ allograft versus isograft and control). Inhibition of the endoplasmic reticulum Ca^{2+}-ATPase with cyclopiazonic acid (CPA) in the presence of extracellular Ca^{2+} caused a large increase in 340/380 ratio in allograft endothelium. In isografts and controls, the increase was small and transient. The elevated 340/380 ratio in allografts could not be rapidly reversed by tetraethylammonium (TEA) (which depolarizes the endothe-

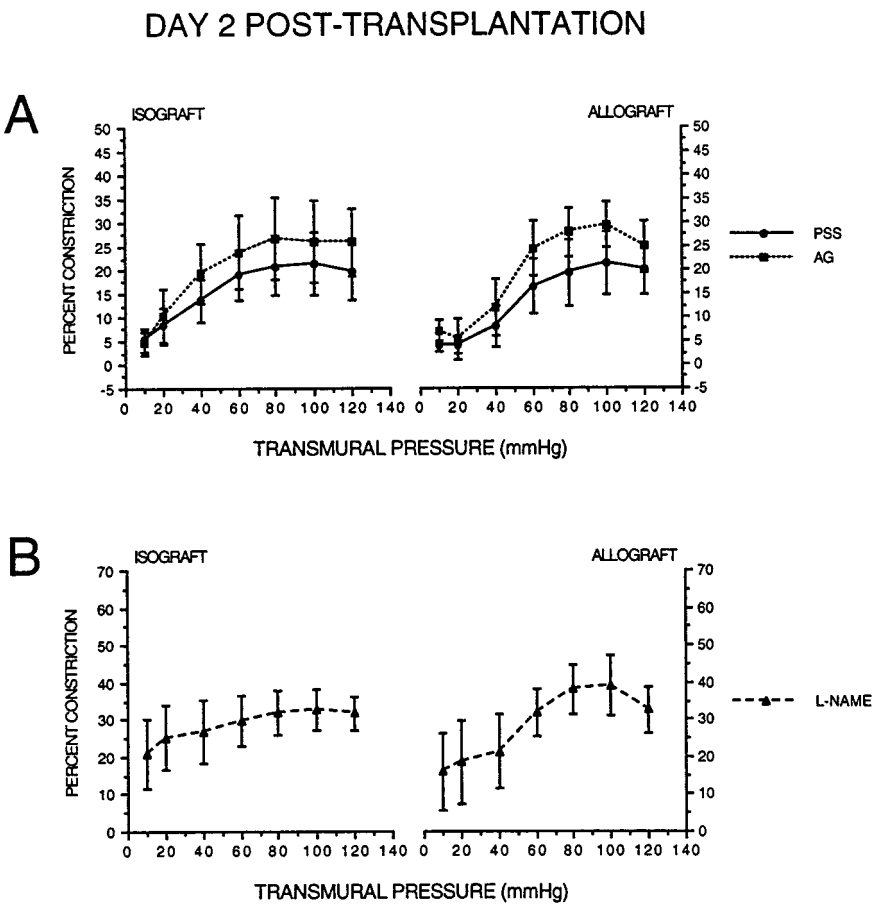

Figure 2. Day 2 posttransplantation. A, Myogenic tone in isograft and allograft vessels before and after iNOS inhibition. B, Potentiation of myogenic tone after nonselective NOS inhibition with L-NAME. Statistical comparison by repeated-measures ANOVA, $P>0.05$.

lial cells, thus decreasing the electrical driving force for Ca^{2+} entry); even removal of CPA caused a very slow decrease in 340/380 ratio. When Mn^{2+} quenching of the 360-nm signal was used as a reflection of Ca^{2+} influx, no differences between allograft and isograft cells were observed, although both were elevated compared with control cells.

Days 7 to 28 posttransplantation. Inhibition of allograft myogenic tone persisted for days 7 to 28. The tracings in Figure 5 demonstrate the profound vasodilation of an allograft artery compared with a matched isograft artery. For days 7 to 28, incubation with AG abolished the statistical differences between isograft and allograft vessels, indicating that iNOS-based NO is present and is vasoactive (Figure 6A through 6C). However, a trend toward less tone in allograft vessels persists after AG, suggesting either incomplete iNOS blockade, greater eNOS activity, or another mechanism of tone inhibition. In these vessels, L-NAME unmasked a time-dependent deterioration of underlying myogenic tone in allograft groups (Figure 7A). Neither indomethacin 1 μmol/L nor endothelium removal could reverse the profound inhibition of tone in L-NAME–treated day 28 allograft arteries (not shown).

Agonist- and Potassium-Induced Tone

Allograft and isograft vessels (with AG) were constricted by agonist and depolarization (potassium) mechanisms for comparison with myogenic tone. As shown in Figure 7B, there is a parallel inhibition of these 3 mechanisms of constriction in mature allograft vessels (day 28), whereas all 3 are preserved in early allografts and matched isografts. This pattern of multimodal inhibition suggests a decrease in the number of viable or functional smooth muscle cells,[6,7] or possibly a signaling defect in the distal common pathway of constriction.

Graft Weight and Wet/Dry Ratios

Dry weight was greater in allografts than in matched isografts from day 4 onward. Wet/dry ratio was significantly elevated in day 4, 7, and 28 allograft hearts, indicating greater myocardial free water content (Table).

Drugs and Concentrations

Previous work has shown inhibition of iNOS in vascular preparations by 100 to 300 μmol/L AG.[8–12] Figures 3A, 4A, and 7A through 7C show potentiation of tone only in vessels expressing iNOS protein. We observed preservation of acetylcholine-induced dilation in native arteries in the presence of 100 μmol/L AG; 200 μmol/L L-NAME abolished acetylcholine-induced dilation, indicating complete blockade of eNOS (not shown).

Discussion

The principal finding of this study is that myogenic tone in allograft coronary arteries is profoundly inhibited compared with site- and size-matched isograft arteries, resulting in a greatly enhanced arterial diameter. We have identified 3 mechanisms: (1) tone inhibition can occur through enhanced basal release of eNOS-based NO due to

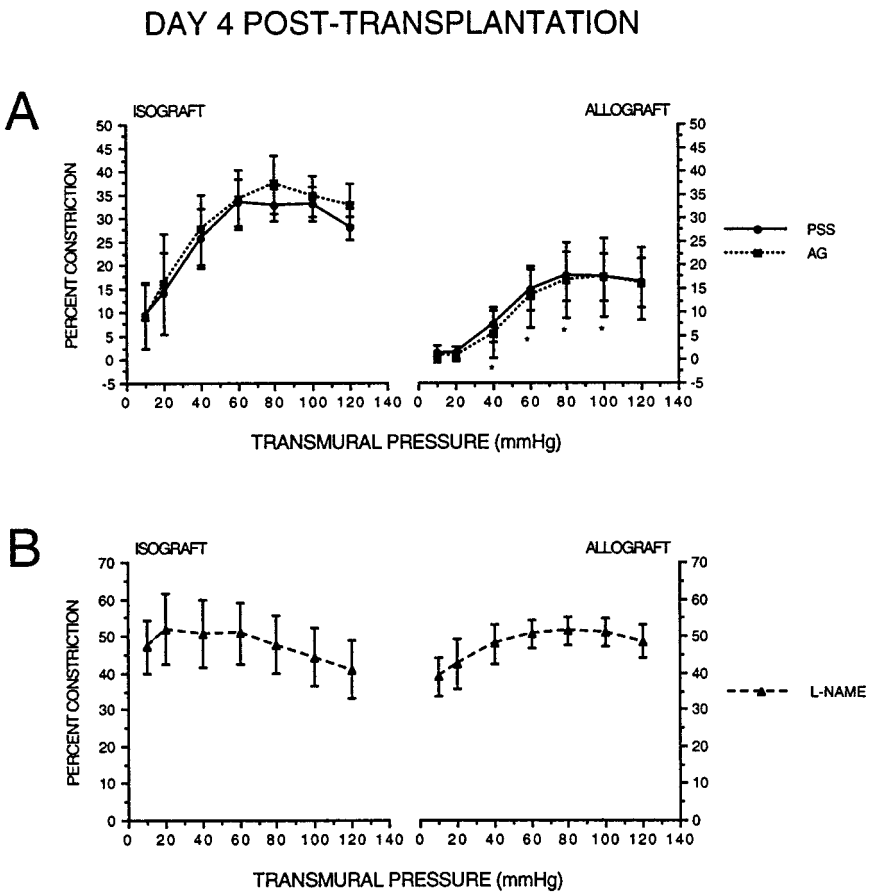

Figure 3. Day 4 posttransplantation. A, Myogenic tone in isograft and allograft vessels before and after iNOS inhibition. B, Nonselective NOS inhibition with L-NAME potentiates tone in both groups and abolishes tone differences, indicating a greater basal release of eNOS-based NO in allograft vessels. *$P<0.05$.

enhanced endothelial Ca^{2+} availability; (2) when iNOS is expressed, tone inhibition can occur through release of iNOS-based NO; and (3) in contrast to the NO-dependent vasodilation mechanisms mentioned above, the concerted deterioration of pressure-, agonist-, and depolarization-induced tone seen in mature allograft vessels is consistent with an intrinsic defect in vascular smooth muscle contraction, involving either common signal transduction events or a decrease in the number of viable smooth muscle cells. These findings, which precede the obliterative arteriopathy characteristic of chronic rejection, predict a pattern of coronary hemodynamics that would favor movement of extracellular fluid from the intravascular compartment into the myocardial interstitium, resulting in myocardial edema and ventricular stiffness.

Mechanisms of Myogenic Tone Inhibition

eNOS and Endothelial $[Ca^{2+}]_i$

Arteries from allografts at day 4 manifest less myogenic tone than matched isograft arteries. Selective iNOS inhibition with AG did not potentiate tone in either group, and no iNOS protein was identified immunohistochemically. The tone differences were abolished after nonselective NOS inhibition with L-NAME, indicating a greater basal release of eNOS-based NO from allograft endothelium. Resting 340/380 ratio (and calculated $[Ca^{2+}]_i$) was elevated in isolated allograft endothelial cells; because eNOS is very tightly regulated by $[Ca^{2+}]_i$, this observation provides a mechanism for enhanced basal release of eNOS-based NO.

iNOS-Based Vasoactive NO

From day 7 onward, the inhibition of myogenic tone in allograft arteries paralleled the expression of iNOS protein. In these vessels, selective inhibition of iNOS with AG potentiated tone; thus, iNOS-based NO is vasoactive and is an important mechanism of tone inhibition. To our knowledge, this is the first report to show immunohistochemical evidence of iNOS protein together with vascular hyporesponsiveness and iNOS inhibitor potentiation of resistance vessels. The general theme, however, has been addressed in other models.[10,11]

Smooth Muscle Contractile Defect

A trend toward less tone in day 7 to 28 allografts persists even after iNOS inhibition. These residual differences could be due to alloimmune alteration in endothelial $[Ca^{2+}]_i$ as described above; in mature grafts, however, the residual differences in tone were not abolished by nonselective NOS inhibition. In fact, this approach unmasked a pattern of progressive, time-dependent deterioration of myogenic tone that is not solely due to NO vasodilation, arguing against eNOS as a preeminent factor. Because neither indomethacin nor endothelium removal altered this residual inhibition, prostaglandins and other endothelium-derived vasodilators were eliminated as candidate mechanisms. In these mature (day 28) allograft vessels, profound

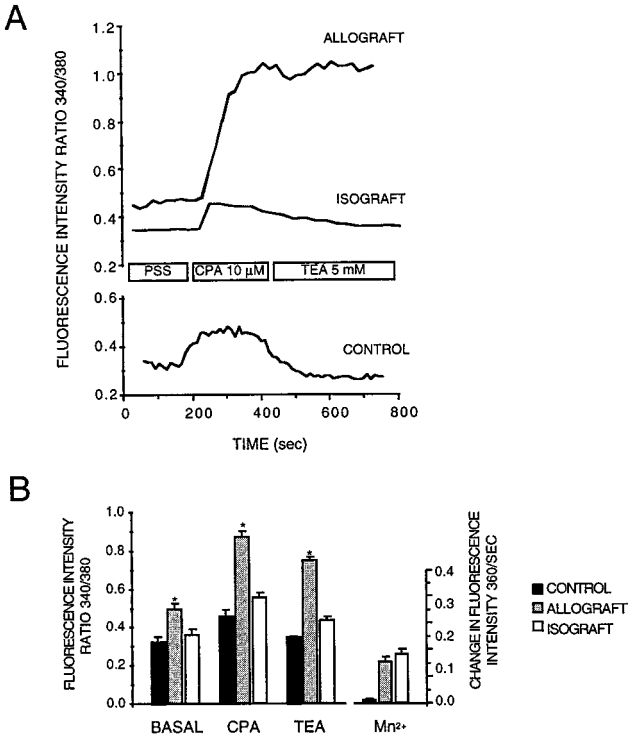

Figure 4. Ca^{2+} signaling in day 4 posttransplantation endothelium. A, Representative tracing of fura 2 340/380 fluorescence ratio in allograft, isograft, and control endothelial cells, showing basal, CPA, and TEA responses (see text). B, Summary of Ca^{2+} signaling experiments in isolated valve leaflet endothelial cells. Control indicates 22 cells from 4 Lewis hearts; allograft, 51 cells from 5 hearts; and isograft, 39 cells from 4 hearts. Statistical comparison by *t* test, $^{*}P<0.05$.

inhibition of myogenic, agonist, and depolarization-induced tone was observed, suggesting a defect in vascular smooth muscle contraction through a common signaling event. Potential events include those of the distal common pathway: Ca^{2+} influx, calmodulin, myosin light chain kinase, contractile filaments, and phosphatases.

It is also possible that the impairment of constriction in mature allograft vessels is due to a decrease in the number of viable smooth muscle cells. Apparent loss of medial cells has been observed by Dong et al[13] in human coronary arteries, and recent evidence indicates that apoptosis may play a key role in cardiac allograft vasculopathy. Szabolcs et al[14] used

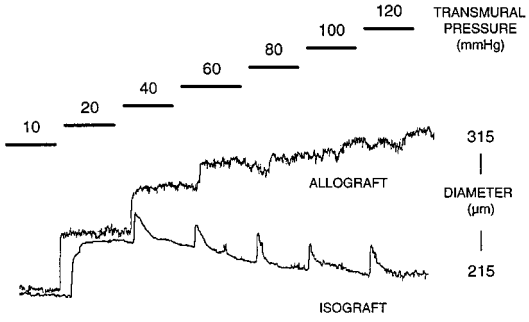

Figure 5. Pressure-diameter tracings from allograft and isograft coronary septal arteries harvested at day 14 posttransplantation.

DNA laddering, terminal dUTP nick end-labeling (TUNEL), and in situ nick translation to identify apoptotic cells in Lewis-to–Wistar-Furth allografts. Apoptotic nuclei were identified in cardiac myocytes, endothelial cells, and infiltrating monocytes; iNOS protein was identified in the same cell types. Importantly, the temporal pattern of apoptosis paralleled that of iNOS expression, NOS activity, and nitrotyrosine staining, suggesting that apoptosis may be triggered by iNOS and peroxynitrite. With this in mind, it is possible that the effect of iNOS expression on allograft arteries is 2-fold and time-dependent: an early phase due to vasodilation by NO itself and a delayed phase due to smooth muscle apoptosis by NO and/or NO adducts.

iNOS Protein Expression

Expression of iNOS mRNA and protein has been demonstrated in cardiac allografts from several heterotopic animal models, and our analysis is similar to these.[1,2] In the Lewis-to-F344 model, reverse transcription–polymerase chain reaction on ventricular homogenate RNA identified iNOS transcript in allografts at days 7, 14, 28, and 75, with a small amount at day 3. Immunohistochemical stains with polyclonal rabbit antisera to macrophage NOS showed iNOS protein predominantly in mononuclear inflammatory cells within the interstitial and perivascular spaces of allograft hearts (days 7, 28, and 75); there was minimal staining in isografts. iNOS protein in smooth muscle and endothelial cells was identified in significant quantity only at later time points (days 75 and 120), although lesser staining was seen in all allografts.[2] Our results with a monoclonal mouse anti-iNOS antibody show that allospecific expression of iNOS in day 7 to 28 allograft arteries occurs mainly in the intimal space. In this location, NO from functional iNOS would be expected to have access to the vascular smooth muscle with resistance vessel sequelae as we have shown. Our structure-function comparison demonstrates that the pattern of iNOS expression parallels the pattern of functional change in allograft vessels and confirms an important physiological consequence of iNOS expression in transplantation.

Clinical Significance

Our experimental observations provide a mechanistic basis for several clinical observations. In the absence of fixed distal disease, a pattern of enhanced arterial diameter due to myogenic tone inhibition would predict a hemodynamic profile of supranormal coronary flow and reduced flow reserve. In nonrejecting human cardiac grafts, resting coronary flow is indeed elevated, with a proportional decrease in flow reserve[15,16]; these findings have been attributed to increased cardiac work in recipients with systolic hypertension and tachycardia, and when corrected for this, coronary flow is appropriate. However, during biopsy-proven acute rejection, corrected coronary flow is significantly elevated (and coronary resistance depressed) compared with flow after successful recovery on immunosuppression,[4] indicating that vasodilation accompanies uncontrolled graft rejection. Interestingly, corrected coronary flow remains significantly elevated after a single

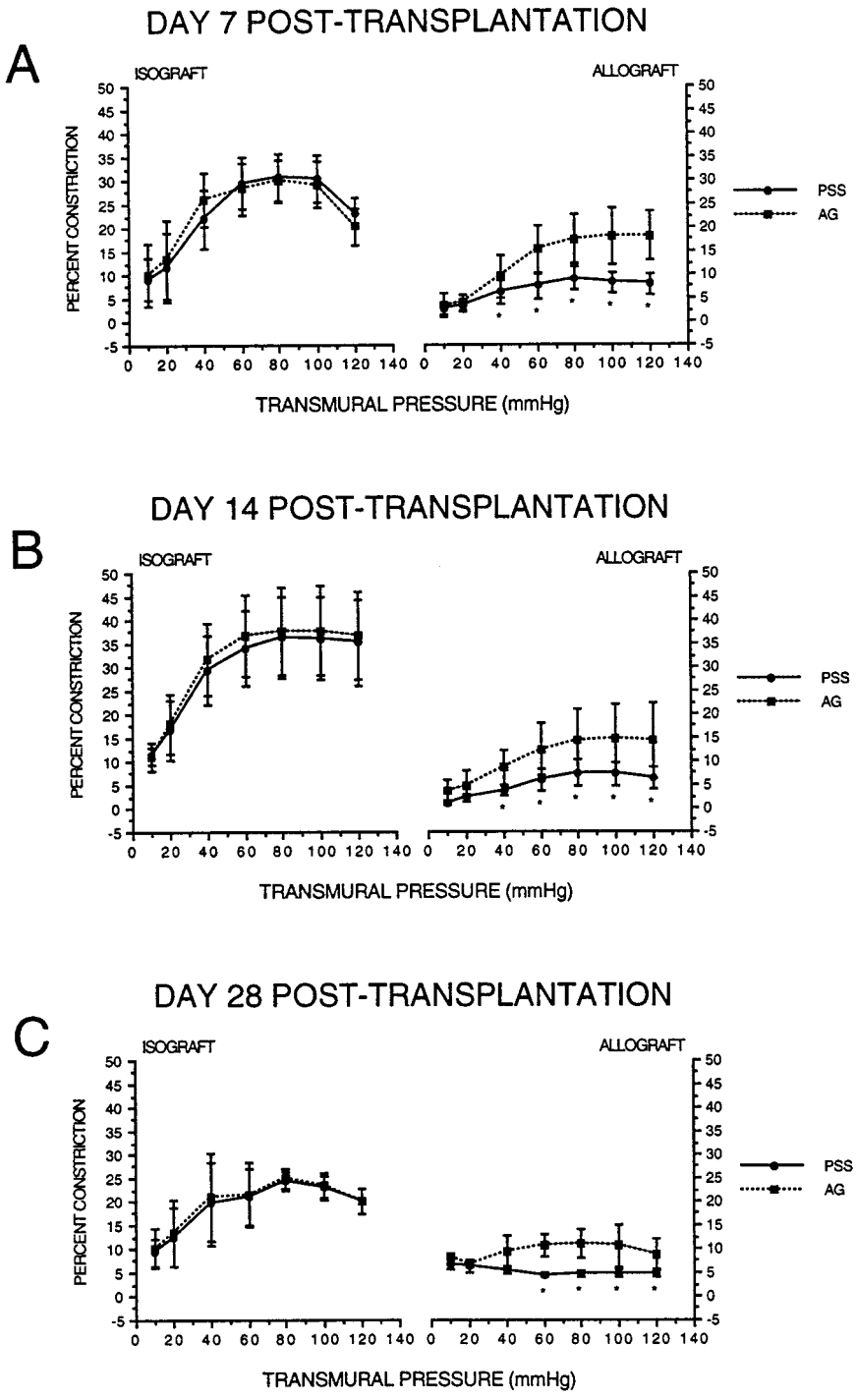

Figure 6. Days 7 to 28 posttransplantation. Myogenic tone in isograft and allograft vessels before and after iNOS inhibition for days 7 (A), 14 (B), and 28 (C). *$P<0.05$.

episode of rejection compared with patients without previous rejection episodes, consistent with a persistent defect in resistance vessel tone due to the rejection event. Because iNOS expression in cardiac allografts is inhibited by immunosuppression,[17] it is possible that the residual elevation in resting corrected coronary flow in transplant patients after rejection is due to an iNOS-independent event such as enhanced endothelial $[Ca^{2+}]_i$ and NO or a smooth muscle contractile defect, as we have shown. If so, these alterations in resistance vessel function may be irreversible.

Elevated coronary flow in the face of an unrestrained immune assault may at first consideration appear to be an appropriate and beneficial response. However, normal coronary myogenic behavior is necessary not only to regulate myocardial blood flow but also to provide graded vascular resistance. Appropriate vascular resistance protects the microvasculature from central arterial pressures and so preserves the important balance of hydrostatic and oncotic forces at the capillary and venular levels.[18] A massively dilated coronary circulation, predicted by our results, would transmit abnormally high perfusion pres-

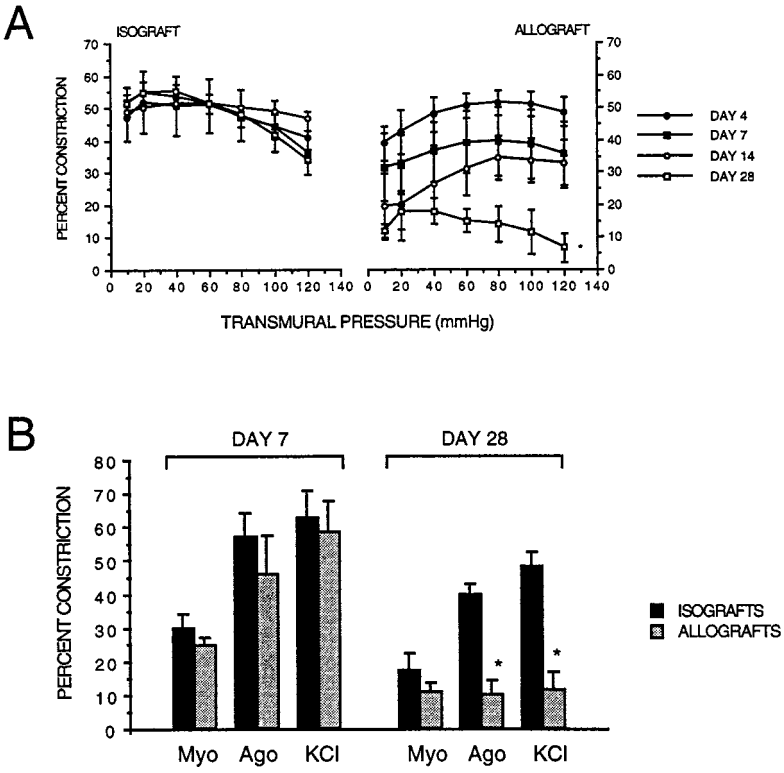

Figure 7. Smooth muscle contractile defect in mature allografts. A, Myogenic tone in isograft and allograft vessels after L-NAME showing graded deterioration in allografts. B, Constrictions of pressurized allograft and isograft vessels by myogenic, agonist-induced, and depolarization-induced mechanisms reveal a time-dependent, parallel deterioration of constriction by all 3 mechanisms. Myo indicates myogenic; Ago, agonist; and KCl, depolarization. Statistical comparison by *t* test, $^{*}P<0.05$.

sures to nutritive vessels, distorting the balance of intravascular oncotic and hydrostatic pressure and favoring a net movement of fluid into the myocardial interstitium. In support of this hypothetical pathogenesis, our results show a greater wet/dry ratio in allograft hearts, reflecting greater myocardial free water content. Because endothelial permeability to serum protein is enhanced in allograft rejection (also an iNOS-dependent event),[19] these 2 mechanisms could act synergistically to cause significant myocardial edema, thereby compromising ventricular compliance and performance.

The association between iNOS expression and ventricular performance in human grafts was recently published. In support of our hypothesis that the altered allograft coronary physiology (in part due to iNOS-based NO) would favor myocardial edema, ventricular stiffness, and poor performance, Lewis et al[3] reported that expression of iNOS correlates with cGMP levels and systolic and diastolic left ventricular contractile dysfunction; importantly, iNOS did not associate with International Society of Heart and Lung Transplantation (ISHLT) rejection grade. Together with our results, this key study indicates that current techniques of rejection assessment (ISHLT grade) may ignore coexistent physiological mechanisms of allograft dysfunction.

Greater Dry Weight and Wet/Dry Ratio in Allograft Hearts

	Allograft	Isograft	*P* (n=3)
Day 4			
D	194±8	151±8	<0.05
W/D	4.86±0.02	4.34±0.06	<0.05
Day 7			
D	204±9	143±11	<0.05
W/D	5.13±0.02	4.45±0.06	<0.05
Day 28			
D	376±9	105±5	<0.05
W/D	5.87±0.15	4.37±0.04	<0.05

D indicates dry weight (mg); W/D, wet/dry ratio.

Summary and Conclusions

We have shown that myogenic tone is profoundly inhibited in cardiac allograft arteries, in part by excess vasoactive NO and in part by a defect in vascular smooth muscle contractility. Excess NO can be derived from eNOS and iNOS isoforms. These findings predict a hemodynamic pattern within the rejecting heart that would favor myocardial edema, ventricular stiffness, and poor myocardial performance.

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