Cardiac Preservation Is Enhanced in a Heterotopic Rat Transplant Model by Supplementing the Nitric Oxide Pathway

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Abstract

Nitric oxide (NO) is a novel biologic messenger with diverse effects but its role in organ transplantation remains poorly understood. Using a porphyrinic microsensor, the first direct measurements of coronary vascular and endocardial NO production were made. NO was measured directly in the effluent of preserved, heterotopically transplanted rat hearts stimulated with L-arginine and bradykinin; NO concentrations fell from 2.1±0.4 μM for freshly explanted hearts to 0.7±0.2 and 0.2±0.08 μM for hearts preserved for 19 and 38 h, respectively. NO levels were increased by SOD, suggesting a role for superoxide-mediated destruction of NO. Consistent with these data, addition of the NO donor nitroglycerin (NTG) to a balanced salt preservation solution enhanced graft survival in a time- and dose-dependent manner, with 92% of hearts supplemented with NTG surviving 12 h of preservation versus only 17% in its absence. NTG similarly enhanced preservation of hearts stored in University of Wisconsin solution, the clinical standard for preservation. Other stimulators of the NO pathway, including nitroprusside, L-arginine, or 8-bromoguanosine 3′,5′ monophosphate, also enhanced graft survival, whereas the competitive NO synthase antagonist N^G-monomethyl-L-arginine was associated with poor preservation. Likely mechanisms whereby supplementation of the NO pathway enhanced preservation included increased blood flow to the reperfused graft and decreased graft leukostasis. NO was also measured in endothelial cells subjected to hypoxia/reoxygenation and detected based on its ability to inhibit thrombin-mediated platelet aggregation and serotonin release. NO became undetectable in endothelial cells exposed to hypoxia followed by reoxygenation and was restored to normoxic levels on addition of SOD. These studies suggest that the NO pathway fails during preservation/transplantation because of formation of oxygen free radicals during reperfusion, which quench available NO. Augmentation of NO/cGMP-dependent mechanisms enhances vascular function after ischemia and reperfusion and provides a new strategy for transplantation of vascular organs. (*J. Clin. Invest.* 1994. 93:2291–2297.) Key words: nitric oxide • cyclic GMP • cardiac • preservation • transplantation

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Introduction

Nitric oxide (NO)[1] has been ascribed a central role in processes as diverse as neurotransmission and destruction of invading bacteria (1, 2). First identified as an endothelium-derived relaxing factor (3), NO plays a critical role in the maintenance of vascular homeostasis through its interaction with neutrophils, platelets, and cellular components of the vessel wall (4–7). Because vascular dysfunction leading to organ failure is an important limitation to successful transplantation, we explored the role of the NO pathway in this setting. This report demonstrates direct measurements of NO production by the coronary vasculature and endocardium and shows that NO levels are diminished in the cardiac vasculature and endocardium after preservation and transplantation, as well as in endothelial cells (ECs) subjected to hypoxia and reoxygenation. Augmentation of the NO pathway significantly enhances preservation in a heterotopic rat model of cardiac transplantation. Application of these principles could have a major impact on the current strategy and success of human organ transplantation.

Methods

Heterotopic rat cardiac transplant model

Donor cardiectomy, preservation, and heterotopic transplant. Male Sprague-Dawley rats (350 g) (Camm Research Lab Animals, Wayne, NJ) were used, and harvest, preservation, and transplantation procedures were performed as described (8). Preservation solutions included lactated Ringer's (LR) solution (Baxter, Edison, NJ) or LR supplemented with nitroglycerin (NTG) (Du Pont Co., Wilmington, DE), sodium nitroprusside (Roche Laboratories, Nutley, NJ), N^G-monomethyl-L-arginine (L-NMMA) (Chem Biochem Research, Inc., Salt Lake City, UT), L-arginine (L-arg) (Sigma Chemical Co., St. Louis, MO), or 8-bromoguanosine 3′,5′ monophosphate (8-Br-cGMP) (Sigma Chemical Co.). Components were combined within 4 h of donor cardiectomy, and preservation solutions were kept at 4°C. All of these solutions were at the pH of LR (6.6). University of Wisconsin solution (UW) was purchased from Du Pont Co. Blood flow was restored to the graft exactly 1 h after removal from the cold preservation solution, and pulsation of the donor aortic stump was observed to assure patency of the anastomosis. 10 min after removal of the cross-clamp, an electrocardiogram (ECG) was taken (polygraph from Grass Instrument Co., Quincy, MA), and the heart was judged by the same

1. *Abbreviations used in this paper:* 8-Br-cGMP, 8-bromoguanosine 3′,5′ monophosphate; EC, endothelial cell; HUVEC, human umbilical vein endothelial cell; L-arg, L-arginine; L-NMMA, N^G-monomethyl-L-arginine; LR, lactated Ringer's solution; NO, nitric oxide; NOS, nitric oxide synthase; NTG, nitroglycerin; UW, University of Wisconsin solution.

blinded investigators throughout based on the presence/absence of regular contractions and a transplant score (0–5, worst–best, respectively) using the following criteria: contraction (poor = 0, average = 1, vigorous = 2), tissue turgor (hard = 0, average = 1, soft = 2), and color (dusky = 0, pink = 1). Overall, a graft was considered to have survived if regular contractions were observed with confirmation of corresponding depolarizations by ECG, with a transplant score ≥ 2.

Perfusion of donor graft (9)

10 min after restoration of blood flow, 0.5 ml of 10-μm-diameter microspheres (10^6/ml) (E-Z Trac, Inc., West Los Angeles, CA) was injected into the donor aortic root. Deposition of microspheres in ventricular tissue was assessed after alkaline hydrolysis, washing, and counting of microspheres as described (8), with results expressed as number of spheres per (40×) field per gram of ventricle. A minimum of 10 fields was counted, and the mean±SEM is reported.

Myeloperoxidase assay/histology

10 min after restoration of blood flow, transplanted hearts were excised, and the aortic root was flushed for 5 min (rate of 2 ml/minute controlled by roller pump) with normal saline (Baxter), the atria and great vessels were removed, and intracavitary blood was evacuated. Remaining ventricular pieces were weighed and homogenized, and myeloperoxidase activity was measured as described (10). For histologic study, cardiac graft samples were fixed in an ascending series of aldehydes, embedded, sectioned, and stained with hematoxylin and eosin.

NO measurements

Preparation of platelets. Platelets were obtained from a volunteer who had ingested 650 mg of aspirin 12 h before the experiment, collected, and prepared as described previously (11). Radiolabeled serotonin ([^{14}C]5-hydroxytryptamine) (Amersham Corp., Arlington Heights, IL) was incorporated into the platelets by adding 0.5 μCi during the blood collection process. Inhibition of cyclooxygenase was confirmed by the absence of aggregation to 1 mM sodium arachidonate.

Preparation of human umbilical vein endothelial cells (HUVECs). HUVECs from fresh human umbilical veins were isolated and grown according to previously published methods (12) and were used between the third and fourth passages. HUVEC cyclooxygenase was inhibited with acetylsalicylic acid (1 mmol/liter in M199 was incubated for 30 min at 37°C and then washed twice with M199 to remove residual acetylsalicyclic acid) immediately before collagenase digestion. Collagenase type I (4 ml, 200 U/ml) (Worthington Biochemical Corp., Freehold, NJ) and EDTA mix (2% EDTA in Hepes-buffered saline, pH 7.4, with 1% BSA [Sigma Chemical Co.]) were added to digest ECs from the monolayer, followed by washing twice in an electrolyte buffer (KCl 4.2 mM, $MgSO_4$ 0.5 mM, NaCl 135.5 mM, Na_2HPO_4 6.5 mM, NaH_2PO_4 1.5 mM, and glucose 5.6 mM). A hypoxic environment was administered by placing cells in a specially designed airtight chamber (Coy Laboratory Products, Inc., Ann Arbor, MI) with a normobaric environment comprised of 90% N_2, 5% H_2, 5% CO_2, and 0% O_2. Partial pressures of oxygen of 15 Torr in the culture medium were achieved by the use of a palladium catalyst in the chamber to reduce any residual O_2. All manipulations, including centrifugation and washing of cells (up to the final aggregometry or reoxygenation phases as described), were performed within the hypoxia chamber. Reoxygenation was accomplished by removing an aliquot of washed and counted HUVECs in suspension to room air for the indicated duration.

Aggregometry/serotonin release. A Lumiaggregometer (Chrono-Log Corp., Havertown, PA) was used with a continuously warmed (37°C) siliconized glass cuvette containing 58×10^6 platelets, 3 mM calcium chloride, and 2.5 μM imipramine to prevent the reuptake of released serotonin. Platelets were challenged with graded doses of α-thrombin (Sigma Chemical Co.) to determine the concentration that caused maximal platelet aggregation (0.3 U/ml in all cases), and this dose was used for all further studies. Hypoxic HUVEC aliquots (66 μl of 1×10^6 ml) were removed from the chamber and added immediately to the aggregometry cuvette or exposed to room air for the indicated duration of reoxygenation. Normoxic HUVEC aliquots were prepared identically except that they were maintained in a standard cell culture incubator under ambient conditions. Cell viability between these ECs was comparable by trypan blue exclusion. HUVECs were added just before thrombin in the indicated experiments. Interference with light absorption was measured for 3 min to obtain platelet aggregation curves, after which the cuvette contents were placed on ice, centrifuged at 5,000 rpm for 5 min at 4°C, and 200 μl of supernatant was recovered and counted in a liquid scintillation counter to quantify [^{14}C]serotonin release. To determine the effects of oxygen free radicals, bovine erythrocyte SOD (final concentration 32 U/ml) (Sigma Chemical Co.) was added to the aggregometry cuvette in the indicated experiments just before the addition of the HUVEC aliquot. Results are expressed as both aggregometry curves and as a percentage of maximal serotonin release as defined by thrombin challenge in the absence of ECs.

Direct measurement with NO electrode. Direct measurements of coronary vascular and endocardial NO synthesis were made with a porphyrinic microsensor (13) using differential pulse voltammetry or amperometry. Rat hearts were explanted and preserved as described above and studied fresh (no preservation/transplantation) or after hypothermic preservation in LR solution, heterotopic transplantation, and 10 min of reperfusion. The coronary vasculature was flushed free of blood with LR followed by 4 ml of LR supplemented with L-arg (2 mM) and bradykinin (6×10^{-6} M) (Sigma Chemical Co.), which was recycled (1 ml/min) through the coronary vasculature by flushing down the cross-clamped aortic root. Aliquots of perfusate were transferred immediately to an anaerobic environment for measurements and compared with an aqueous NO reference. Differential pulse voltammograms were obtained using a sensor (working electrode), saturated calomel electrode reference, and platinum wire auxiliary electrode, with a pulse amplitude of 40 mV. Endocardial NO was measured by implanting the sensor in the septal endocardium of a freshly explanted or preserved/transplanted heart, and after the baseline stabilization 6×10^{-6} M bradykinin was applied, and NO release was monitored continuously by amperometry at constant potential of 0.63 V.

Measurement of NO synthase (NOS) activity (14). Hearts were either freshly explanted, preserved, or preserved/transplanted (with 10 min of reperfusion), after which they were excised. The coronary arteries were flushed with physiologic saline, the atria and great vessels were excised, and the ventricular cavities were rinsed free of residual intracavitary blood. Samples were flash frozen in liquid nitrogen and stored at −80°C until the time of assay. Extracts were prepared by homogenizing tissue at 4°C in the presence of protease inhibitors (aprotinin, epsilon amino caproic acid, PMSF, pepstatin A, and chymostatin) and centrifuging (20,000 rpm × 20 min) to remove cellular debris. The extract (20 μg of protein by the method of Lowry et al. [15]) was incubated for 60 min in the presence of $CaCl_2$ (10 μM), FAD (8 μM), dithiothreitol (60 μM) (Sigma Chemical Co.), tetrahydrobiopterin (8 μM) (Dr. B. Schirks Laboratories, Jona, Switzerland), and [^{3}H]L-arg (0.072 μM, 5 μCi) (Amersham Corp.). TLC was performed with 5 μl of the reaction mix, using methanol/chloroform/water/ammonium hydroxide (9:2:8:3) as the running solvent. Silica gel TLC plates (Sigma Chemical Co.) were stained with ninhydrin (1 g/500 ml ethanol), and the spots corresponding to L-arg, L-ornithine, and L-citrulline were cut out and counted. The percentage of conversion was defined as the number of counts in the L-citrulline spot divided by the total number of counts (L-arg plus L-ornithine plus L-citrulline). Results are expressed as the mean±SEM of three determinations.

Statistics

Graft survival data were analyzed by contingency analysis (chi-square). Transplant scores, microsphere perfusion, myeloperoxidase, NOS, and platelet serotonin release data were analyzed using ANOVA for unpaired variables with the Tukey test used to discriminate significant differences between group means. Values are expressed as means±SEM, with a $P < 0.05$ considered statistically significant.

Results and Discussion

Experiments with ECs have demonstrated that hypoxia/reoxygenation causes increased leukocyte adhesion and activation, suppression of the cell surface anticoagulant cofactor thrombomodulin along with induction of prothrombotic activity, and increased permeability of the EC monolayer (12, 16, 17). Since NO prevents neutrophil and platelet adherence (5, 6) and enhances endothelial barrier function (18), we used this EC model of hypoxia/reoxygenation to simulate a significant component of ischemia/reperfusion to study the role of NO. Cultured ECs were exposed to a level of hypoxia comparable with that measured within the cardiac vasculature of an explanted heart after overnight storage (partial pressure of oxygen $\approx$ 18–20 Torr), and NO production was evaluated by the ability of ECs to inhibit thrombin-induced platelet aggregation and serotonin release (11) (Fig. 1 *A*, aggregometry curves and bar graph). Inhibition of platelet aggregation by normoxic ECs (Fig. 1 *B*) was blocked by hemoglobin (Fig. 1 *C*), validating that NO was the antiaggregatory agent. Exposure of ECs to hypoxia for 16 h did not significantly alter their capacity to block platelet aggregation (Fig. 1 *D*), indicating NO production was maintained. However, exposure of ECs to hypoxia followed by reoxygenation in room air was associated with undetectable levels of NO (Fig. 1 *E*). Since reoxygenated ECs produce oxygen free radicals which can destroy NO (19, 20), the free radical scavengers SOD (Fig. 1 *F*) or butylated hydroxytoluene (data not shown) were added and found to restore NO levels. This suggested that oxygen free radicals quenched NO during reoxygenation, a process which was maximal by 10–15 min after reoxygenation in the assay system. These results are consistent with observations from experiments with coronary artery rings from feline hearts subjected to ischemia/reperfusion in vivo, which demonstrate impaired vasorelaxation only after reperfusion (within minutes) (21). This rapid time course for generation of oxygen free radicals is also consistent with that shown in experiments using an electron paramagnetic resonance spin trap (22, 23).

To further evaluate the role of NO, direct measurements of NO were made in rat hearts subjected to preservation/heterotopic transplantation using a highly sensitive and specific NO-sensing electrode (13). The coronary vasculature of freshly explanted or preserved/transplanted hearts was flushed free of blood, perfused with both bradykinin and L-arg, and NO was measured by differential pulse voltammetry. NO concentration decreased from 2.1±0.4 μM (n = 5) for freshly explanted to 0.7±0.2 (n = 4) and 0.20±0.08 μM (n = 4) for hearts preserved for 19 and 38 h, respectively (Fig. 2, *A* and *B*). The perfusate from transplanted hearts consistently demonstrated lower NO levels than freshly explanted hearts, with levels augmented by the addition of SOD (Fig. 2 *C*). Addition of the competitive NOS inhibitor L-NMMA blocked further NO production, but the addition of the cGMP analogue (8-Br-cGMP) to the preservation solution did not alter NO levels after transplantation (data not shown). In situ endocardial measurements of NO using an implanted microsensor demonstrated a steady increase of NO concentration to a plateau of 0.67±0.06 (n = 3) and 0.17±0.05 μM (n = 3) after bradykinin challenge of freshly explanted and preserved (38 h) hearts, respectively (Fig. 3, *A* and *B*). These data indicate that NO levels are markedly suppressed after preservation/transplantation.

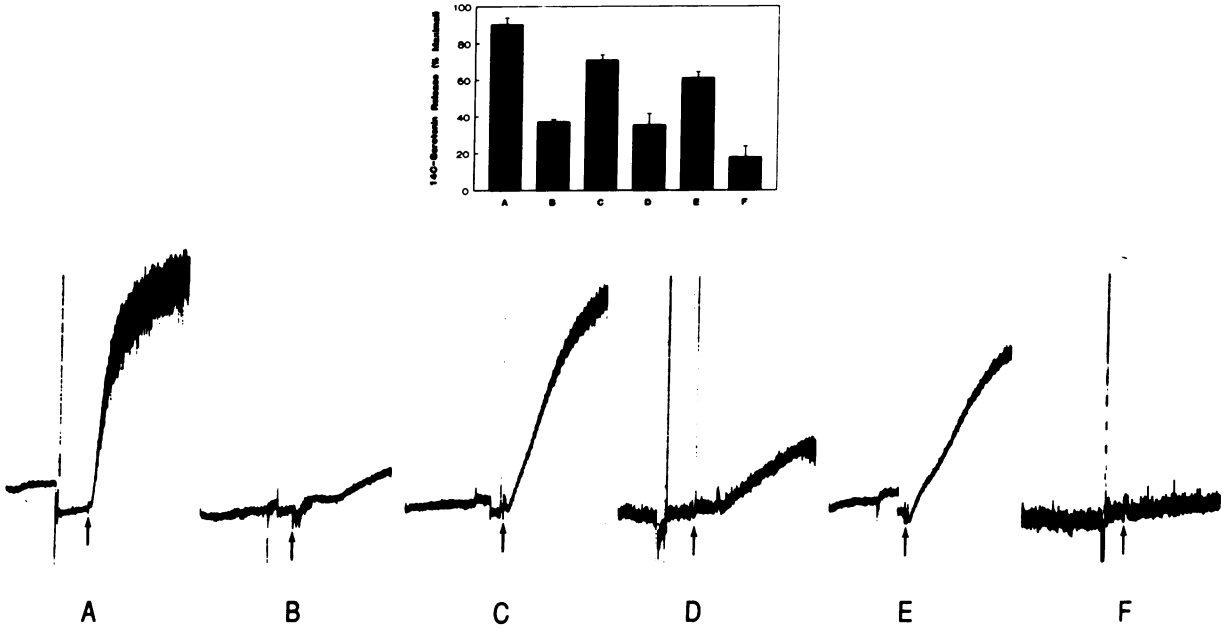

Figure 1. NO was measured by its ability to inhibit platelet aggregation and serotonin release (11). (*A*) Platelets aggregate in response to thrombin challenge (*arrow*). (*B*) This response is inhibited by the presence of normoxic ECs. (*C*) Hemoglobin prevents the antiaggregatory effect of ECs by quenching available NO. (*D*) ECs exposed to 16 h of hypoxia showed minimal reduction in antiaggregatory effect (indicating preserved NO generation), whereas (*E*) HUVECs exposed to 16 h of hypoxia followed by 15 min of reoxygenation fail to inhibit platelet aggregation. (*F*) SOD restores the antiaggregatory effect of reoxygenated ECs, indicating the potential role of free radicals in quenching available NO generated by the reoxygenated ECs. (*Bar graph*) Conditions are as indicated above, with platelet aggregation quantified by [^{14}C]serotonin release. Bar *E* represents ECs reoxygenated from 10 to 95 min. Peak NO quenching was noted at 10–15 min of reoxygenation. ($P < 0.01$ by ANOVA for: platelets alone vs platelets plus either normoxic or hypoxic ECs; hypoxic vs reoxygenated ECs; and reoxygenated ECs with vs without SOD).

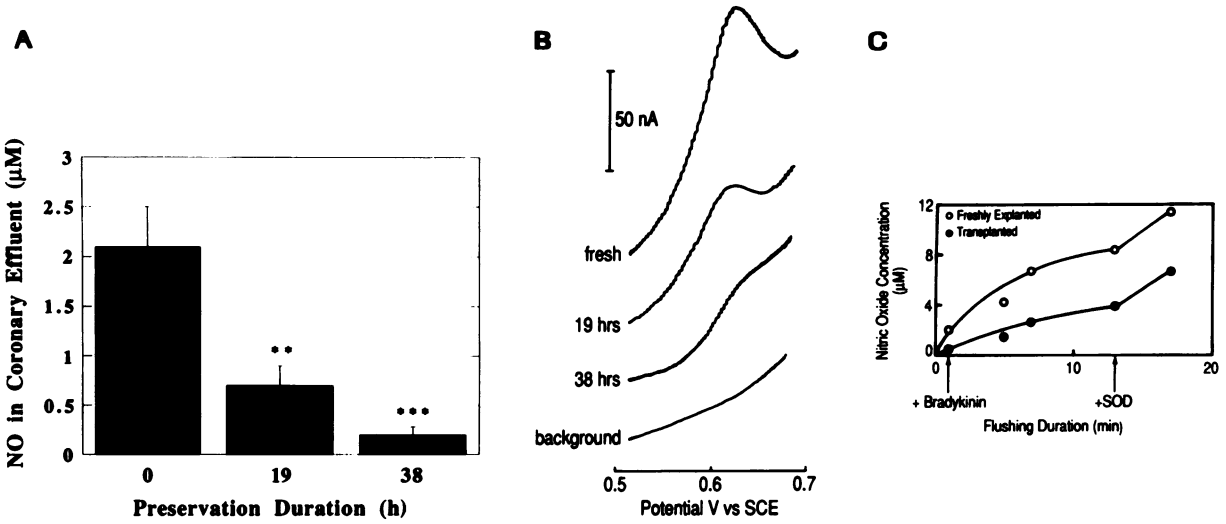

Figure 2. Direct measurement of coronary vascular and endocardial NO synthesis by differential pulse voltammetry or amperometry (13). (*A*) NO was measured by differential pulse voltammetry (sensor working electrode, saturated colomel electrode [SCE] reference, and platinum wire auxiliary electrode, pulse amplitude 40 mV) from aliquots of perfusate demonstrating the difference in production of NO by the coronary vasculature of freshly explanted ($n = 5$) and preserved hearts (19 and 38 h, $n = 4$ for each). $^{**}P < 0.01$, $^{***}P < 0.001$ vs 0 h of preservation. (*B*) Representative differential pulse voltammograms from these experiments. (*C*) NO concentration measured in the flushing solution at different time intervals (fresh heart, *open circles* and preserved heart [38 h], *solid circles*). Although NO is seen in the flush of a transplanted/reperfused heart, the levels are markedly diminished; augmentation is seen after the addition of SOD, suggesting the role of oxygen free radicals in the destruction of available NO.

Because SOD in the flush solution increased NO levels of transplanted hearts (Fig. 2 *C*), this suggested that NO was being quenched, but its synthesis remained unimpaired after reperfusion. To establish this further, NOS activity was assayed in extracts of freshly explanted, preserved, and preserved/transplanted hearts, as well as in preserved/transplanted hearts whose preservation solutions were supplemented with NTG or 8-Br-cGMP. Because the conversion of L-arg to L-citrulline is stoichiometric and reflects the production of NO (1), this conversion was measured using a modification of the TLC method described by Stuehr et al. (14). These results (Fig. 4) showed that the activity of NOS after transplantation is similar regardless of whether or not the LR has been supplemented with NTG or 8-Br-cGMP. There was, however, an increase in NOS activity when any group of transplanted hearts was compared with preserved hearts. The significance of this finding is unclear but it may reflect an increased level of NOS in the cells present in the transplanted organ or recruited during the reperfusion

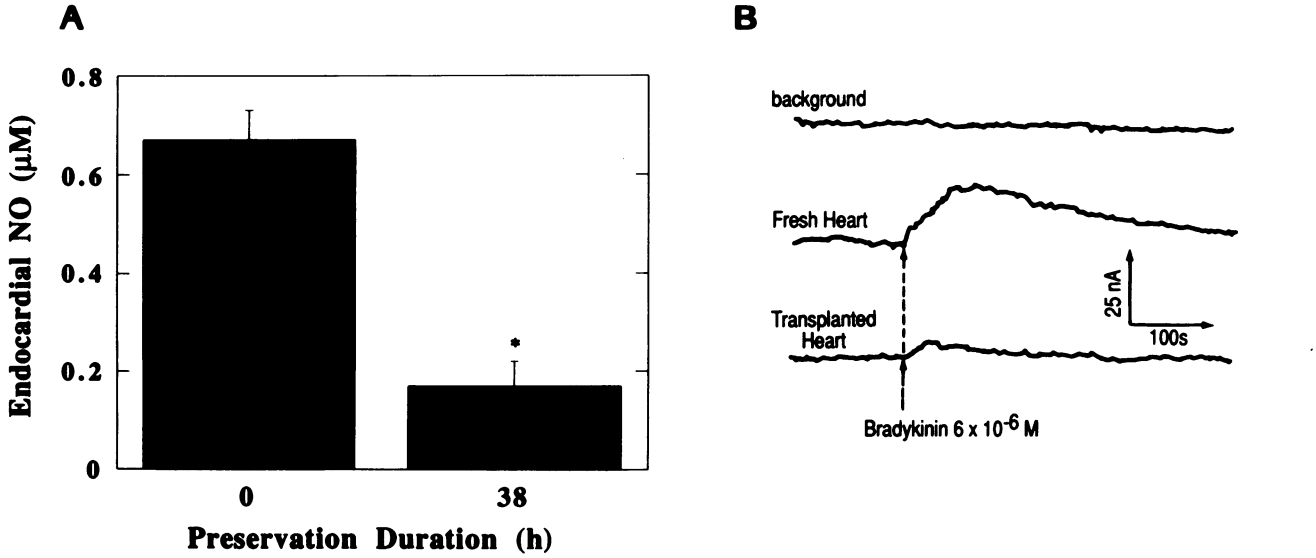

Figure 3. Measurement of endocardial NO. (*A*) The porphyrinic sensor was implanted in the septal endocardium of freshly explanted ($n = 3$) or preserved/transplanted hearts ($n = 3$), and after baseline stabilization 6×10^{-6} M bradykinin was applied, and NO release was monitored continuously by amperometry at a constant potential of 0.63 V. Peak NO levels are shown. $^{*}P < 0.05$ vs 0 h of preservation. (*B*) Representative amperometric tracing demonstrating NO levels after bradykinin challenge. Although the endocardium of a preserved/transplanted heart generates an analytic signal after bradykinin challenge, it is significantly less than that of a freshly explanted heart.

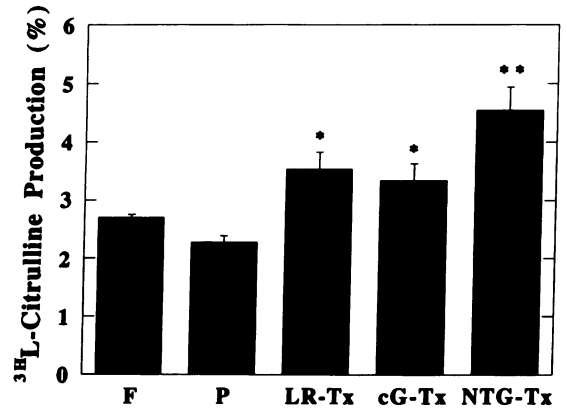

Figure 4. Cardiac NOS activity. NOS activity was measured in cardiac extracts by measuring the conversion of [^{3}H]L-arg to [^{3}H]L-citrulline, as described in the text. Hearts were either freshly explanted (*F; n* = 3), preserved for 12 h in LR (*P; n* = 3), or preserved for 12 h in LR alone and transplanted (*LR-Tx; n* = 3) or LR supplemented with 0.5 mM 8-Br-cGMP (*cG-Tx; n* = 3) or 0.1 mg/ml NTG (*NTG-Tx; n* = 3). $*P < 0.05$, $**P < 0.01$ vs preserved but nontransplanted hearts.

period. This increase in graft capacity to generate NO after transplantation was not translated into increased NO availability (as measured with the NO electrode). These data, together with the hypoxic/reoxygenated endothelial cell data described previously, support the hypothesis that NO synthesis continues after reimplantation, but NO availability during reperfusion is reduced because of its rapid destruction.

Based on these observations, we hypothesized that supplementation of the NO pathway by addition of NO/cGMP agonists during preservation would extend preservation and enhance vascular function. Cardiac grafts were assessed by both ECG and a scoring system based on blinded evaluation of color, turgor, and contractility. The duration of reperfusion after transplantation was chosen as 10 min because pilot studies demonstrated that hearts that did not survive became dark, hard, and failed to contract (by visual inspection with absent depolarizations by ECG) within 1–2 min after reperfusion. Failed grafts never regained function, and surviving grafts continued to function for several hours of observation. Because of these observations, as well as those of others (23) suggesting that oxygen free radicals are generated within minutes by reperfused hearts, we evaluated grafts at 10 min of reperfusion. Only 17% of hearts survived a 12-h storage period when preserved in LR alone (Fig. 5 *A*). In sharp contrast, addition of the endothelium-independent vasodilator (NTG 0.1 mg/ml) resulted in 92% survival, with scores closely paralleling survival (Fig. 5 *A*) in all experiments. This beneficial effect of NTG on cardiac preservation was dose dependent (Fig. 5 *B*) and was also evident when NTG was added to the clinical standard UW solution (24–26); only 35% of hearts preserved with UW alone survived a preservation period of 24–28 h, whereas the addi-

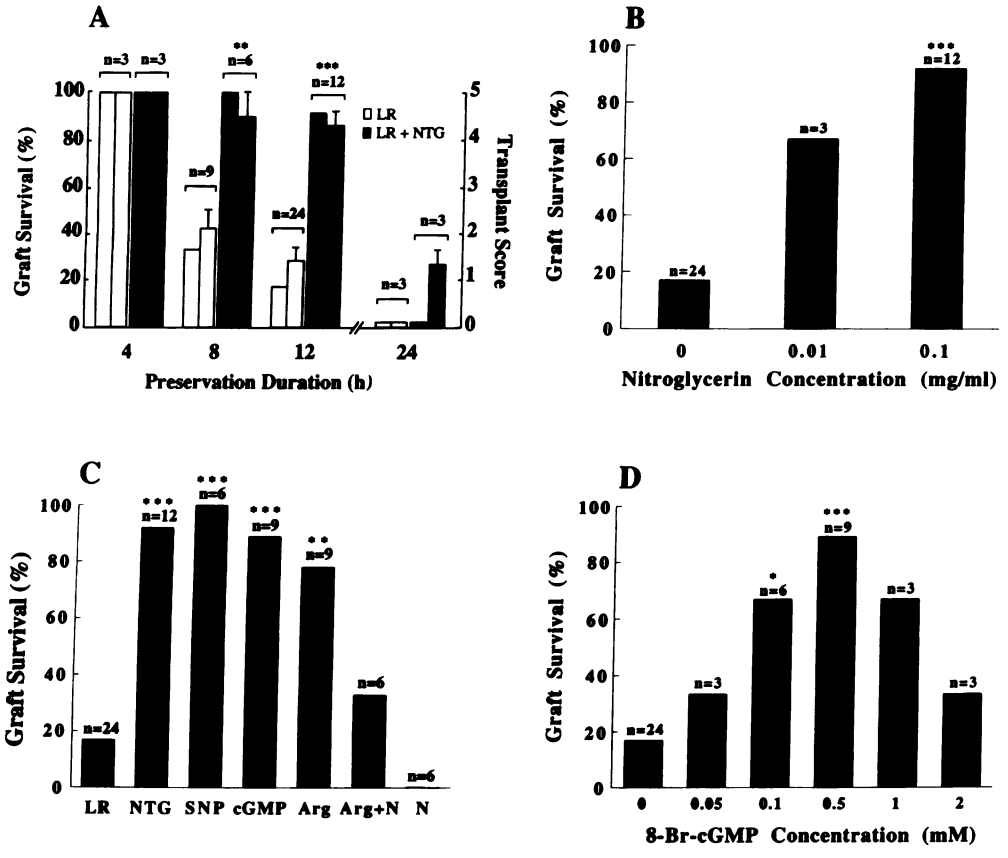

Figure 5. The role of the NO pathway in cardiac preservation and transplantation. A heterotopic rat cardiac transplant model was used (8), with harvest, preservation, transplantation, and assessment performed as described in Methods. (*A*) Time course of NTG effect. NTG (0.1 mg/ml, *closed bars*) added to LR enhanced survival (*leftmost bars at each condition/time point*) and scores (*rightmost bars at each condition/time point*) compared with LR alone (*open bars*). (*B*) NTG dose–response at 12 h of preservation followed by transplantation. Maximal effect is seen at 0.1 mg/ml. (*C*) Role of the NO pathway in enhancing graft preservation. At 12 h of preservation with LR, NTG (0.1 mg/ml), nitroprusside (*SNP*, 0.01 mg/ml), 8-Br-cGMP (*cGMP*, 0.5 mM), and L-arg (*Arg*, 2 mM) all enhanced preservation, whereas a fivefold molar excess of N^G-monomethyl-L-arg (*N*) antagonized the beneficial action of 2 mM L-arg. There was no survival of cardiac grafts preserved for 12 h with N^G-monomethyl-L-arg alone. (*D*) Dose-response of 8-Br-cGMP at 12 h of preservation in LR demonstrated maximal benefit by 0.5 mM. Control experiments at 12 h of preservation were performed throughout the experimental period (n = 24 transplants total) and are shown in panels *A-D* for illustrative purposes. * $P < 0.05$, ** $P < 0.01$, and *** P 0.001, experimental group vs LR alone at the identical preservation duration.

tion of NTG (0.1 mg/ml) increased survival to 100% (n = 20 and 6, respectively, $P < 0.025$). These data are consistent with the observation that there is impaired endothelium-dependent vasodilation after the use of UW for cardiac preservation (27). Another EC-independent vasodilator (nitroprusside) also enhanced preservation, as did L-arg, the endogenous substrate for NOS (Fig. 5 *C*). The competitive NOS antagonists $N\omega$-nitro-L-arg or L-NMMA blocked the beneficial effects of L-arg, and grafts preserved in the presence of L-NMMA alone had the poorest survival of any group (0%, n = 6; Fig. 5 *C*). Consistent with an integral role of the NO pathway in cardiac preservation, 8-Br-cGMP, a membrane-permeable cGMP analogue which has its site of action distal to NO (i.e., cGMP-dependent protein kinase) (28), enhanced cardiac preservation in a dose-dependent manner (Fig. 5 *D*).

To elucidate the mechanism(s) underlying NO/cGMP enhancement of cardiac preservation, blood flow after transplantation was quantified in a series of both successful and failed grafts by infusion of latex microspheres (9) with histologic confirmation of their deposition in the microvasculature. Grafts that failed after implantation had four times less flow than those that were successful (Fig. 6 *A*). Stimulation of the NO/cGMP pathway with either NTG, nitroprusside, L-arg, or 8-Br-cGMP was associated with enhanced blood flow, whereas antagonism of L-arg with L-NMMA was associated with decreased blood flow (Fig. 6 *B*). Because infiltration of neutrophils into ischemic and reperfused myocardium contributes significantly to tissue damage (29–31), the role of the NO pathway was also explored. NTG in the preservation solution was associated with a marked decrease in neutrophil infiltration after cardiac transplantation, as determined by histology (Fig. 6 *C*) and by quantification of the neutrophil enzyme myeloperoxidase (Fig. 6 *D*).

These data emphasize the importance of the NO/cGMP pathway in vascular homeostasis. Because hypoxia also suppresses EC (32) and smooth muscle cell (8) cAMP levels, thus impairing another endogenous vasodilator system which also modulates vascular permeability and leukocyte–EC interactions (33, 34), we have designed an organ storage solution containing agonists of both pathways (dibutyryl-cAMP, 2 mM and NTG, 0.1 mg/ml). This is a highly effective preservation solution in both heterotopic rat and orthotopic baboon cardiac transplant models (35), the latter involving cardiopulmonary bypass (identical to human heart transplantation). Since baboon hearts were preserved successfully for an unprecedented 24 h with simple hypothermic storage in our supplemented

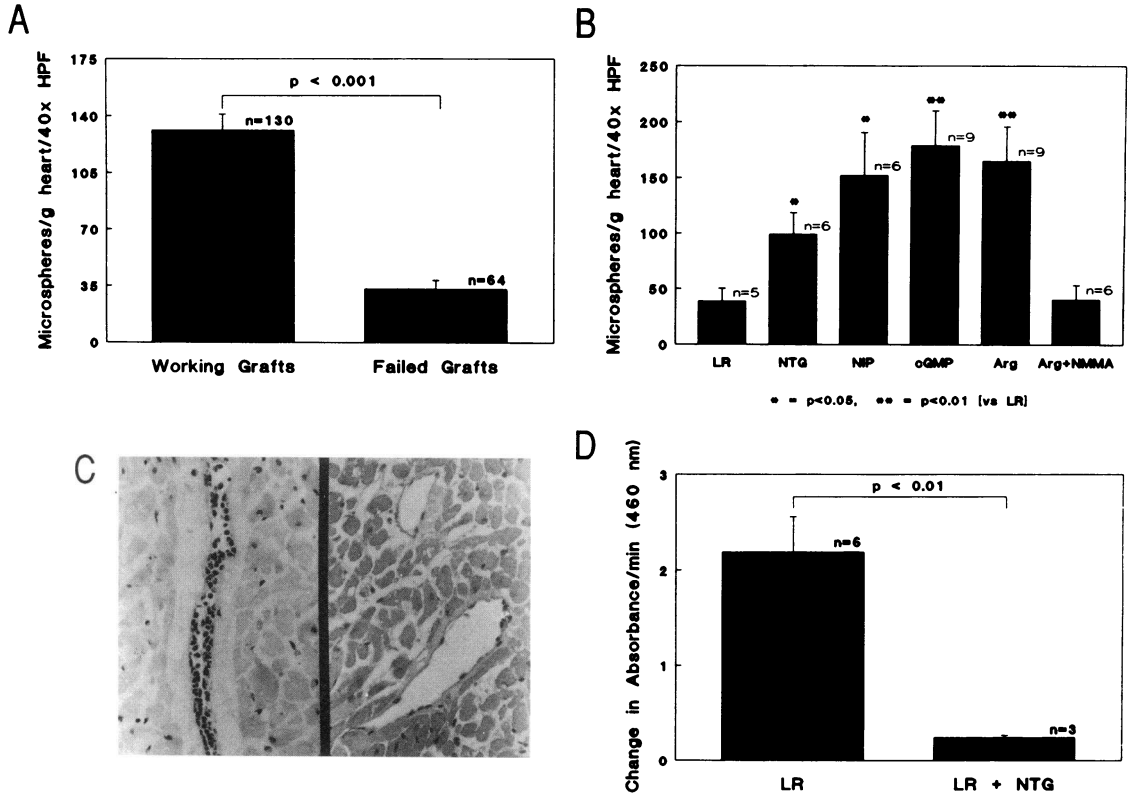

Figure 6. Effect of the NO pathway on graft blood flow and leukostasis. Blood flow to transplanted hearts was evaluated by injecting 10-μm latex microspheres into the donor aortic root and quantifying as described in Methods. (*A*) Hearts that failed after transplantation had fewer microspheres per gram of ventricle than those that survived the preservation/transplantation process. (*B*) Preservation/transplantation as described in Fig. 5 *D* above demonstrated that stimulation of the NO cGMP pathway was associated with higher blood flows in cardiac grafts. In contrast, antagonism of this pathway was associated with lower blood flows. (*C*) Transplanted hearts preserved for 12 h in LR alone demonstrated a prominent band of neutrophils adherent ot the vasculature (*left*), whereas the addition of NTG (0.1 mg/ml) to the preservation solution under identical preservation/transplantation conditions was not associated with neutrophil accumulation (*right*). (*D*) Quantification of cardiac neutrophil deposition by assessing myeloperoxidase activity as described (10) normalized to venticular weight. Hearts preserved for 12 h in LR alone showed an $\approx$ 10-fold increased accumulation of leukocytes after transplantation compared with hearts preserved similarly in LR supplemented with NTG (0.1 mg/ml).

solution, this suggests the potential significance of maintaining the integrity of intra/intercellular second messenger pathways in ischemic and reperfused tissue. Our data are consonant with those of other cardiac models, which infer by indirect measurements that the coronary vasculature produces NO (36) and that the NO pathway is depressed in the setting of ischemia and reperfusion (37, 38). This study represents the first application of these principles to the setting of organ preservation for transplantation, with direct measurements demonstrating that coronary vascular and endocardial NO levels are diminished after transplantation and that supplementation of the NO pathway significantly enhances graft survival after prolonged preservation. These results are likely to apply to all vascular organs and suggest novel ways to improve organ preservation for transplantation as a strategy to treat human disease.

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