Effects of Selective Inhibitors of Nitric Oxide Synthase-2 Dimerization on Acute Cardiac Allograft Rejection

Matthias J. Szabolcs, MD; Ji Sun, PhD; Ningsheng Ma, MD; Arline Albala, MS; Robert R. Sciacca, EngScD; Gary B. Philips, PhD; John Parkinson, PhD; Niloo Edwards, MD; Paul J. Cannon, MD

Background—Nitric oxide synthase-2 (NOS2) is expressed during acute cardiac allograft rejection in association with myocardial inflammation, contractile dysfunction, and death of cardiomyocytes by necrosis and apoptosis. Recently, allosteric inhibitors of NOS2 monomer dimerization that block NOS2 activity have been developed.

Methods and Results—To investigate effects of selective NOS2 blockade, 15 mg/kg of BBS-1 or BBS-2 was administered twice daily subcutaneously to rats starting the day of heterotopic heart transplantation. Cardiac allograft survival was increased significantly, from 6.8 days in controls to 13.3 and to 14.2 days in NOS2-inhibited allografts. At day 5 after heart transplantation, synthesis of NOx was reduced by 53%. There were significantly fewer T lymphocytes and macrophages in the inflammatory infiltrate, as well as less edema and cardiomyocyte damage, and the International Society of Heart and Lung Transplantation score fell from 5 to 4 and 3.5. NOS2 and nitrotyrosine immunostaining and the mean numbers of apoptotic cells and of apoptotic cardiomyocytes were significantly diminished in the treated allografts.

Conclusions—The data indicate that selective inhibition of NOS2 dimerization prolongs survival and reduces myocardial inflammation and cardiomyocyte damage in acute cardiac allograft rejection. (Circulation. 2002;106:2392-2396.)

Key Words: nitric oxide synthase ■ rejection ■ transplantation ■ apoptosis ■ inhibitors

Previous studies that used a rat heterotopic abdominal heart transplantation model have demonstrated that the expression of NOS2 (mRNA, protein, and enzyme activity) is upregulated during acute cardiac allograft rejection.1,2 The increased expression of NOS2 during acute rejection is associated with impaired ventricular performance, electrical instability, and death of heart muscle cells by both necrosis and apoptosis.2–4 Moreover, the severity of myocardial inflammation and cardiomyocyte damage, necrosis, and apoptosis are reduced during acute cardiac allograft rejection in mice in which the NOS2 gene has been deleted.3,5 Recently, McMillan et al7 and Blasko et al,8 using combinatorial chemistry, developed allosteric inhibitors of NOS2 monomer dimerization, which selectively block the enzyme activity of NOS2. The objective of the present study was to investigate in the rat heart transplantation model the effects of administration of these selective NOS2-blocking drugs on the survival and histopathology of acute cardiac allograft rejection.

Methods

Cardiac Transplantation and Drug Treatment

Male Lewis (TR-11) and Wistar-Furth (WF, RT-lu) rats, weighing 200 to 250 g each, were purchased from Harlan Sprague-Dawley Inc (Indianapolis, Ind). The animals were housed in the Columbia Institute of Comparative Medicine, an Association for Assessment and Accreditation of Laboratory Animal Care–approved facility, and they received humane care in compliance with National Institutes of Health standards. They were maintained on a 12-hour light/dark cycle and were fed a commercially available rat chow diet and tap water ad lib. Allogeneic (Lewis-Wistar-Furth) heterotopic abdominal cardiac transplantation was performed by one surgeon using the technique of Ono and Lindsay9 as previously described.1,2 The pyrimidylimidazole-based NOS2 dimerization inhibitors BBS-1 and BBS-2 were prepared by the Department of Medicinal Chemistry, Berlex Biosciences, Richmond, Calif. BBS-1 and BBS-2 correspond to Compounds 2 and 4, respectively, in Blasko et al.8 BBS-1 and BBS-2 were dissolved at 30 mg/mL in sterile water by addition of 1 molar equivalent of hydrochloric acid with gentle heating and stored at room temperature before use. The administration of BBS-1 and of BBS-2 was begun at a dose of 15 mg/kg in water subcutaneously twice daily on the day of transplantation and continued until rejection or until day 5 after transplantation.

Histology

Coronal sections of hearts were fixed in 10% phosphate-buffered formalin, embedded in paraffin, and 4-μm-thick sections were cut and mounted on silane-coated slides. For routine histological examination, sections were stained with hematoxylin and eosin to determine the extent and severity of rejection according to the International Society of Heart and Lung Transplantation (ISHLT).10

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From the Departments of Surgery (N.M., N.E.), Medicine (J.S., A.A., R.R.S., P.J.C.), and Pathology (M.J.S.), Columbia University College of Physicians and Surgeons, New York, NY, and Berlex Biosciences, Inc (G.B.P., J.P.), Richmond, Calif.
Correspondence to Paul J. Cannon, MD, Department of Medicine, Division of Cardiology, Columbia University, 630 W 168th St, New York, NY 10032. E-mail pjc4@columbia.edu
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Antibodies
Monoclonal antibodies (Abs) produced in mice were used to detect the distribution of macrophages (ED1) (1:50, Accurate Chemical, Teterboro, NJ), T cells (CD3) (1:100, Dako, Carpinteria, Calif), NOS2 (1:200, Transduction Laboratories Lexington, Ky) and nitrotyrosine (1:100; Upstate Biotechnology, Lake Placid, NY) in rat cardiac allografts harvested 5 days after transplantation. Cardiac myocytes were highlighted with a polyclonal anti-desmin Ab produced in rabbit (1:50; Sigma, St Louis, Mo) in combination with the TUNEL staining to demonstrate apoptotic cells as described previously.1

Immunohistochemistry
Immunoperoxidase labeling was performed on 4-μm-thick, 10% phosphate-buffered formalin-fixed, paraffin-embedded coronal sections of the cardiac allografts. Antigen retrieval for NOS2, nitrotyrosine, and CD3 was accomplished by boiling sections for 15 minutes in 0.1 mol/L citrate buffer (pH 6.0) in a microwave oven. Endogenous peroxidase activity was quenched with 1.5% H2O2 in phosphate-buffered saline (PBS). Nonspecific binding of the secondary Ab was blocked with 20% normal serum in PBS before overnight incubation at 4°C with the monoclonal Abs or 2 hours at room temperature for polyclonal Abs. The primary Ab was linked with a secondary biotinylated Ab (Vector Labs, Burlingame, Calif). The target antigen was detected with the use of an avidin-biotin-peroxidase kit (Vector Labs, Burlingame, Calif) and 3,3’ diaminobenzidine generating a brown reaction product. Mouse and rabbit IgG were used as negative controls. Sections were counterstained with hematoxylin.

TUNEL Labeling
Apoptotic cells were detected with the labeling kit from Roche Diagnostics, which incorporates fluorescein isothiocyanate conjugated (FITC)–labeled nucleotides at the sites of DNA fragmentation. An anti-FITC Ab linked to alkaline phosphatase was used to highlight apoptotic nuclei by demonstrating alkaline phosphatase activity with nitroblue tetrazolium reduction/6-chloro-3-indoxyl phosphate, yielding a blue reaction product. Thereafter, cardiac myocytes were highlighted with a polyclonal anti-desmin Ab according to the same immunoperoxidase method (brown reaction product) as mentioned above.

NOS2 Protein
The excised hearts were rinsed and flushed via the aorta with ice-cold saline to completely remove blood, then immediately frozen to −70°C. The frozen ventricular tissue was homogenized at 4°C in a microtome. Endogenous peroxidase activity was quenched with 1.5% H2O2 in phosphate-buffered saline. Nonspecific binding of the secondary Ab was blocked with 20% normal serum in PBS before overnight incubation at 4°C with the monoclonal Abs or 2 hours at room temperature for polyclonal Abs. The primary Ab was linked with a secondary biotinylated Ab (Vector Labs, Burlingame, Calif). The target antigen was detected with the use of an avidin-biotin-peroxidase kit (Vector Labs, Burlingame, Calif) and 3,3’ diaminobenzidine generating a brown reaction product. Mouse and rabbit IgG were used as negative controls. Sections were counterstained with hematoxylin.

Western Blot
Western blot for NOS2 protein. NOS2 protein in day-5 treated allografts (lanes 2 and 3) was lower than in day-5 control allografts (lane 1).

Table 1. Cardiac Allograft Survival for Control and NOS2-Inhibited (BBS-1, BBS-2) Allografts

<table>
<thead>
<tr>
<th>Itemized Data, days</th>
<th>Mean±SD, days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n=6)</td>
<td>7, 7, 7, 6, 7</td>
</tr>
<tr>
<td>BBS-1 (n=6)</td>
<td>10, 21, 16, 10, 12, 11</td>
</tr>
<tr>
<td>BBS-2 (n=6)</td>
<td>9.1, 10, 9, 15, 21, 21</td>
</tr>
</tbody>
</table>

*Censored observation (experiment was terminated after 21 days).
†P<0.001 vs control.

Quantitative Evaluation
An experienced pathologist (M.S.), who had been blinded to the treatment, evaluated routinely stained sections (hematoxylin and eosin) with a semiquantitative scale (0 to 6) for myocyte loss and degree of inflammation (compatible with ISHLT grades in human allografts).3,10 According to this scale, 0 indicates no inflammation and myocyte loss (ISHLT grade 0); 1, perivascular inflammation (ISHLT grade 1A); 2, interstitial inflammation (ISHLT grade 1B); 3, inflammation with focal myocyte loss (ISHLT grade 2); 4, inflammation with multifocal myocyte loss (ISHLT grade 3A); 5, inflammation with confluent foci of myocyte loss (ISHLT grade 3B); and 6, inflammation with large areas of necrosis (>25% myocyte loss) and/or necrotizing vasculitis (ISHLT grade 4). The numbers of macrophages, T cells, apoptotic nuclei, and apoptotic cardiac myocytes (detected by double labeling for apoptotic nuclei and positive immunostaining for desmin) were also determined for an entire cardiac cross section with a standardized ocular grid (Olympus microscope). The results are expressed as number ED1+ cells, CD3+ cells, or apoptotic nuclei/mm² myocardium.

Statistical Analysis
NOS2 protein levels and enzyme activity were analyzed by ANOVA. The degree of apoptosis and amounts of T cells and macrophages were analyzed with the nonparametric Kruskal-Wallis procedure. Survival rates were compared by the log-rank test with the Bonferroni correction for multiple comparisons.

Results
The survival of the cardiac allografts in the animals treated with the selective NOS2 inhibitor BBS-1 was significantly prolonged, from 6.8±0.4 days in the control animals to 13.3±4.4 days (P<0.001) (Table 1). Survival of BBS-2 treated allografts was increased to 14.2±5.7 days (P<0.001). Treatment with the NOS2 inhibitor twice daily at 15 mg/kg subcutaneously was associated at day 5 after transplantation with a 53% reduction in myocardial NOS2 enzyme activity: 0.843 versus 1.807 (n=6, P<0.001); NOS2 protein was also significantly reduced at this time point (Figure 1). Figure 2a shows the hematoxylin and eosin section from a control cardiac allograft at day 5; there is a dense inflammatory infiltrate, marked interstitial edema, and abundant damage of cardiac muscle cells. Figure 2b shows a comparable section of a cardiac allograft at day 5 from a treated animal. There is
marked reduction in the number of inflammatory cells, as well as less interstitial edema and less damage of cardiomyocytes.

The numbers of T cells per square millimeter of myocardium at day 5 were reduced in the treated allografts from 364.1 to 202.8 (P<0.01) for BBS-1 and to 235.2 (P<0.01) for BBS-2. Macrophages were reduced from and 475.1 to 194.3 (P<0.001) for BBS-1 and 200.8 (P<0.001) for BBS-2 (Table 2).

(Table 2) Table 3 shows that the average ISHLT score decreased from 5 in control to 4 for BBS-1- and to 3.5 for BBS-2–treated allografts (P<0.01). Figure 2c and 2d show myocardial sections from cardiac allografts at day 5. The sections have been stained for apoptosis (TUNEL technique, labeled blue) and with desmin (highlighting myocytes, labeled brown). In the allograft from the control animal there are abundant apoptotic inflammatory cells and cardiomyocytes (Figure 2c). In the day-5 allograft from a treated animal, there is a marked reduction in apoptotic nuclei, including apoptotic cardiomyocytes (Figure 2d). The mean number of apoptotic cells was reduced in the treated animals from 102.6 to 51.9 (P<0.001) for BBS-1 and to 60.3 (P<0.001) for BBS-2, and the number of apoptotic cardiomyocytes was reduced from 17.7 to 7.3 (P<0.001) for BBS-1 and 8.0 (P<0.001) for BBS-2 (Table 2). Figure 2e shows a myocardial section immunostained for NOS2 from a day-5 control allograft; there is strongly positive immunostaining for NOS2 in the inflammatory infiltrate (ie, macrophages) and in cardiomyocytes located near the inflammatory infiltrates. In a similar day-5 section from a treated animal (Figure 2f), there is marked reduction of the NOS2 immunoreactivity compared with the control graft, mostly because of the reduction of the inflammation. Figure 2g and 2h demonstrate that immunostaining for nitrotyrosine, a marker for the presence of peroxynitrite, was present in macrophages and cardiomyocytes in sections from the day-5 control allografts and was diminished in day-5 allografts from treated animals.

**Discussion**

The drugs used to inhibit NOS2 enzyme activity in the present study, BBS-1 and BBS-2, have demonstrated a 1000-fold selectivity for the NOS2 isofrom in vitro and, at a dose of 15 mg/kg subcutaneously twice daily, achieved a 53% inhibition of NOx production by NOS2 in the cardiac allografts of treated animals at day 5 after transplantation.

Table 2

<table>
<thead>
<tr>
<th>Itemized Data</th>
<th>Control (n=9)</th>
<th>BBS-1 (n=6)</th>
<th>BBS-2 (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T cells</td>
<td>364.1±81.3</td>
<td>202.8±97.7*</td>
<td>235.2±48.0*</td>
</tr>
<tr>
<td>Macrophages</td>
<td>475.1±77.2</td>
<td>194.3±80.3†</td>
<td>200.8±56.7†</td>
</tr>
<tr>
<td>Apoptosis</td>
<td>102.6±17.0</td>
<td>51.9±7.9†</td>
<td>60.3±16.9†</td>
</tr>
<tr>
<td>Apoptosis (myocytes)</td>
<td>17.7±3.3</td>
<td>7.3±1.3†</td>
<td>8.0±3.6†</td>
</tr>
</tbody>
</table>

Values are expressed as mean±SD per square millimeter (n=6 for BBS-1 and BBS-2, and n=9 for control).

*P<0.01 vs control; †P<0.001 vs control.

Table 3

<table>
<thead>
<tr>
<th>Itemized Data</th>
<th>Mean±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n=9)</td>
<td>5.0±0.7</td>
</tr>
<tr>
<td>BBS-1 (n=6)</td>
<td>4.0±0.9</td>
</tr>
<tr>
<td>BBS-2 (n=6)</td>
<td>3.5±0.5*</td>
</tr>
</tbody>
</table>

*P<0.01 vs control.
The compounds allosterically bind to a heme-containing NOS2 monomer to form an inactive NOS2 monomer-heme complex in a pterin- and L-arginine-independent manner that inhibits the normal dimerization of NOS2 monomers and blocks NO synthesis from L-arginine by the enzyme. The BBS-1 and BBS-2 are not subject to substrate antagonism (eg, with L-arginine) and are more potent than aminoguanidine, which is only semiselective for NOS2, acts as an antioxidant, and also has biochemical effects unrelated to NO such as inhibition of aldolase and of diamine oxidase. The new compounds inhibit the dimerization of NOS2 monomers more effectively than they inhibit the dimerization of NOS1 and NOS3 monomers. Aortic rings from rats treated with 30 mg/kg BID of BBS-1 or BBS-2 had normal basal and acetylcholine NOS3-dependent vascular responses, which were blocked by the nonselective NOS inhibitor L-NAME. Homogenates of gastrocnemius muscle from the treated animals had normal levels of calcium-dependent NOS1 activity by the 14C-L-arginine to 14C-citrulline assay. No pathological or biochemical evidence of cardiovascular toxicity was found with doses (30 mg/kg BID) of BBS-1 and BBS-2 twice as high as those used in this study (Drs Philips and Parkinson, unpublished observations).

The finding that cardiac allograft survival was prolonged and that the intensity of myocardial inflammation and damage to heart muscle cells (ISHLT score, apoptosis and immunostaining for nitrotyrosine) were reduced in the animals treated with the selective NOS2 inhibitor is consistent with data obtained previously. Worrall and coworkers observed a modest prolongation of allograft survival, reduced histopathologic changes, and improved electrical stability and ventricular performance of rat cardiac allografts in animals treated with aminoguanidine. Both Koglin et al and Szabolcs et al studied acute cardiac allograft rejection in strains of mice in which the gene for NOS2 had been functionally deleted. The allografts implanted into NOS2−/− mice exhibited longer survival, a reduction of ISHLT scores by one full grade, less myocardial inflammation, less apoptosis, and less nitrotyrosine immunostaining than those implanted into NOS2+/− mice. The present results obtained with selective and unique NOS2 inhibitors show more significant reduction of rejection and more prolonged allograft survival compared with the slight changes in survival and histopathology observed in cardiac allografts in recipients that were treated with relatively nonselective NOS2 inhibitors. The more marked effects observed in the present study may relate to the greater potency, the new compounds to inhibit NOS2 and to their greater selectivity against NOS2, allowing any uninhibited NOS1 and NOS3 to exert beneficial (ie, antiinflammatory) effects.

In the untreated cardiac allografts undergoing acute rejection at day 5 after transplantation, NOS2 expression was upregulated in endothelial cells, inflammatory cells (ie, macrophages), and cardiomyocytes. Increased NOS2 expression during acute cardiac allograft rejection in rats, mice, and humans has been associated with apoptosis of macrophages and of heart muscle cells. Loss of heart muscle cells over time may (in addition to the negatively inotropic effects of NO) contribute to the impaired ventricular contractile performance that characterizes the rejecting heart. Studies in vitro by several groups have indicated that the large amount of NO synthesized by NOS2 in activated macrophages or by isolated purified cardiac myocytes in which NOS2 expression was induced by administration of cytokines has the capability to cause the death of cardiomyocytes by an autocrine or paracrine NO-dependent mechanism.

In other settings, however, NO has been shown to inhibit apoptosis of hepatocytes, cardiac myocytes subjected to stretch, and vascular endothelial cells. The different responses to NO are determined by a variety of factors, including the cells and NOS isoforms synthesizing NO, its diffusion, the NO target molecules in the area, and the redox state of the pertinent microenvironment.

Increased NOS2 expression has also been associated with nitration of proteins in cardiac cells manifested by positive immunostaining for nitrotyrosine. Nitrotyrosine in tissues is a marker for the presence of peroxynitrite, a strong toxic oxidant formed by combination of NO and superoxide that is capable of interfering with function of cardiac proteins and of causing DNA strand breaks and poly ADP ribose synthase activation, which in turn can trigger death of cells by apoptosis and by necrosis. In the present study of a selective NOS2 inhibitor, as in our prior studies of genetically manipulated mice, the amount of positive immunostaining for nitrotyrosine of myocardial inflammatory cells and cardiomyocytes was reduced significantly in the animals treated with the NOS2 inhibitor or in the animals that were NOS2−/−.

The present study was not designed to investigate the effects of the selective NOS2 inhibitor on the transplant-associated vasculopathy due to chronic rejection. Ravalli et al and Russell et al have reported that NOS2 is upregulated in the vascular smooth muscle cells of the coronary vessels, which manifest an occlusive vasculopathy during chronic allograft rejection. The latter group found that the transplant-associated vasculopathy was more extensive and severe in allografts in mice in which the NOS2 gene was deleted.

In summary, the administration of BBS-1 and of BBS-2, selective inhibitors of NOS2 dimerization, to rats that had undergone heterotopic abdominal heart transplantation was associated with significantly prolonged cardiac allograft survival and significant reductions in the myocardial inflammation and cardiomyocyte death by apoptosis and necrosis. The data suggest that these compounds (and/or other selective NOS2 inhibitors) may provide useful additions to the current armamentarium of treatments for acute cardiac allograft rejection. They may also prove beneficial in diseases such as myocarditis, myocardial infarction, and dilated cardiomyopathy in which the myocardium is inflamed and NOS2 is expressed.

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References
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