Metabolic and Functional Protection by Selective Inhibition of Nitric Oxide Synthase 2 During Ischemia-Reperfusion in Isolated Perfused Hearts

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Background—Drugs that selectively block nitric oxide synthase (NOS) 2 enzyme activity by inhibiting dimerization of NOS2 monomers have recently been developed.

Methods and Results—To investigate whether selective inhibition of NOS2 is cardioprotective, rats were pretreated for 2 days with BBS2, an inhibitor of NOS2 dimerization, at 15 mg/kg SC. Isolated buffer-perfused hearts from treated (n=9) and control (n=7) hearts were subjected to 20 minutes of ischemia followed by 60 minutes of reperfusion. NOS2 protein was upregulated in all hearts at the end of ischemia and of reperfusion; NOS2 enzyme activity was 60% lower in hearts from the treated animals. In the treated hearts, the increase in end-diastolic pressure was significantly attenuated at the end of ischemia, and the return of developed pressure at reperfusion was greater (P<0.05). Creatine kinase release at reperfusion was lower in treated hearts than in controls (P<0.02). At the end of ischemia and of reperfusion, myocardial ATP levels were significantly higher in the treated hearts than in controls (P<0.05). In the treated hearts under ischemic conditions, lactate content was higher and the lactate/pyruvate ratio was lower than in controls (P<0.05); GAPDH activity was higher; and G-3-P and aldose reductase activity were lower. At reperfusion, in the treated hearts, there was less histological damage and less apoptosis of cardiac muscle cells.

Conclusions—Pretreatment with BBS2, a selective inhibitor of NOS2, improves contractile performance, preserves myocardial ATP, and reduces damage and death of cardiac myocytes during ischemia and reperfusion of isolated buffer-perfused rat hearts. (Circulation. 2004;109:1668-1673.)

Key Words: nitric oxide synthase • ischemia • reperfusion

Current knowledge about the roles of nitric oxide (NO) and the NO synthase (NOS) isoforms in the settings of ischemia/reperfusion injury and ischemic preconditioning of the heart is incomplete. In some studies of ischemia/reperfusion in blood- or buffer-perfused hearts, low levels of NO appeared to exert beneficial effects (studies using NO-donor drugs or administration of L-arginine or nonselective NOS inhibitors).1–4 Additional studies using semiselective NOS2 inhibitors or NOS2−/− mice yielded results that implied that large amounts of NO produced by NOS2 were deleterious to the heart.5–9 Studies in vivo in a setting of recurrent brief episodes of ischemia/reperfusion (ischemic preconditioning) or after NOS2 gene transfer to a percentage of myocardial cells was achieved yielded results that implied that the amounts of NO generated in those experiments were cardioprotective.10

Recently, McMillan et al11 reported the development of a new class of drugs that inhibit the dimerization of NOS2 monomers and thus produce highly selective blockade of the enzymatic activity of NOS2. Using a rat model of heterotopic abdominal heart transplantation, we reported that administration of one of these compounds (BBS1 or BBS2) was associated with significantly prolonged allograft survival and reductions of myocardial inflammation and of cardiomyocyte death by apoptosis.12 In the present study, we used BBS2 to test the hypothesis that selective inhibition of NOS2 improves contractile function, preserves myocardial ATP, and reduces cardiac myocyte damage during ischemia and reperfusion of the isolated and buffer-perfused rat heart.

Methods

Male Wistar-Furth rats (250 g) were purchased from Harlan Sprague-Dawley Inc (Indianapolis, Ind) and housed in the Columbia

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As an employee of Berlex, Dr Parkinson has a vested interest in the commercial development of the iNOS inhibitors that have been evaluated in Professor Cannon’s laboratory. The work in the present article, however, is preclinical and conceptual in nature.

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University Institute of Comparative Medicine. All studies were performed with the approval of the Institutional Animal Care and Use Committee at Columbia University, New York, and conform to the Guide for the Care and Use of Laboratory Animals (NIH publication No. 85-23, 1996).

The inhibitor of NOS dimerization BBS2 was prepared in sterile water by Berlex Biosciences.11 BBS2 was administered to experimental animals at a dose of 15 mg/kg SC for 2 days before the ischemia/reperfusion protocol was instituted. Control animals received vehicle alone. The ischemia/reperfusion protocol was performed in an isovolumic isolated heart preparation as described in detail previously.11,12 All hearts were paced at 300 bpm with the use of right atrial pacing electrodes. Left ventricular developed pressure (LVDP) and LV end-diastolic pressure (LVEDP) were measured with a latex balloon in the left ventricle and were monitored continuously along with coronary perfusion pressure and flow.

After an equilibration period of 30 minutes, both control and treated animals were subjected to 20 minutes of global ischemia (zero-flow) followed by 60 minutes of reperfusion. At the end of the equilibration and the ischemic periods, some of the hearts were freeze-clamped for biochemical analysis. On completion of the reperfusion period, the remaining hearts were removed from the apparatus, and a coronal section of the LV was fixed in formalin for pathological examination. The remainder of the LV was frozen and was maintained at −80°C for biochemical measurements. Creatine kinase, tissue levels of ATP and glyceraldehyde-3-phosphate (G-3-P), and the activities of aldose reductase and GAPDH were measured as described previously.13

**Determination of NO Synthase Activity**

Perfused heart tissue was homogenized in ice-cold homogenization buffer containing 25 mmol/L Tris-HCl (pH 7.4), 1 mmol/L EDTA, and 1 mmol/L EGTA. Homogenates were centrifuged at 14 000g for 15 minutes at 4°C. The protein concentration of supernatant was determined by BCA protein assay (Pierce) with BSA as standard. NOS activity was measured with the NOS detects assay kit (Stratagene, Inc, catalog No. 2024500) according to its instructions from the conversion of [H]arginine to [H]citrulline.

**NOS2 mRNA by Real-Time Polymerase Chain Reaction**

Total RNA was isolated with TRIzol reagent (Gibco BRL); 1 μg of total RNA was used for reverse transcription reaction. Single-stranded cDNA was synthesized with AMV reverse transcriptase and oligo (dT) primer (Promega). Polymerase chain reaction primers were designed from the Genbank mRNA sequence of mouse NOS2.12 The samples were placed into the LightCycler instrument (Roche), and fluorescence curves were analyzed with LightCycler software to quantify NOS2 mRNA. Prediluted NOS2 cDNA isolated from stimulated macrophages was used for generating an experimental standard curve. Rat GAPDH specific primers were used as an internal control during quantification.

**NOS2 Protein by Western Blot**

Frozen ventricular tissue was homogenized at 4°C in RIPA-lysis buffer and centrifuged at 16 000g for 15 minutes at 4°C. The protein concentration of supernatants was determined by BCA assay (Pierce) with BSA as standard. Western blot for NOS2 protein was performed with GAPDH as a control for protein loading (~40 μg per lane).12

**Pathology**

Myocardium was obtained from each animal and fixed overnight in 10% phosphate-buffered formalin. Paraffin sections 4 μm thick were cut and stained with hematoxylin and eosin (H&E) and Masson’s trichrome technique. Apoptotic nuclei were detected with the labeling kit from Roche Diagnostics, which incorporates FITC-labeled nucleotides at the site of DNA fragmentation. Apoptotic nuclei were labeled blue by alkaline phosphatase.16 On the same sections, cardiac myocytes are stained brown with anti-desmin antibody (1:40; Sigma) and the conventional immunoperoxidase technique.15 The number of apoptotic desmin-positive myocytes and endothelial cells per square millimeter of myocardium was enumerated microscopically by use of a standardized grid (Olympus BX40). Serial sections were stained with anti-NOS2 monoclonal antibody (1:200, Transduction Laboratories) detected with the immunoperoxidase technique (Vector Laboratories) and 3,3′-diaminobenzidine.12

**Statistical Analysis**

The significance of differences in mean levels between groups for functional and biochemical measurements was assessed by unpaired t test. NOS2 protein levels and enzymatic activity were also analyzed by unpaired t test.

**Results**

**Myocardial Function**

LVDP was similar in treated and control hearts under baseline conditions (Table 1). Global ischemia resulted in cessation of LVDP in both groups. Reperfusion resulted in a greater recovery of LVDP in BBS2-treated hearts compared with controls (Table 1). LVEDP was similar in both groups of hearts at the start of ischemia (Table 1). During ischemia, the rise in EDP, ie, contracture, was attenuated in the hearts treated with BBS2 compared with controls (Table 1). Reperfusion resulted in much higher EDP in controls than hearts treated with BBS2 (Table 1).

Myocardial oxygen consumption was similar in both groups under baseline and reperfusion conditions and was unaffected by BBS2 treatment (Table 1).

**Creatine Kinase Release During Reperfusion**

Creatine kinase release from the control hearts on reperfusion was 1061±395 IU, a value significantly higher than the 312±56 IU released on reperfusion from the BBS2 treated hearts (Figure 1, P=0.02).

**Enzymatic Changes and ATP Levels**

GAPDH activity at the end of ischemia was higher in BBS2-treated hearts than in controls (Table 2). G-3-P, a substrate for GAPDH, was lower in the BBS2-treated hearts than controls under ischemic conditions (Table 2).

Aldose reductase activity, known to be stimulated by NO, was also significantly reduced at the end of ischemia in BBS2-treated hearts (Table 2). The data on changes in GAPDH activity, G-3-P levels, and aldose reductase activity

### Table 1. Hemodynamic Values From Isolated Rat Hearts Subjected to Ischemia and Reperfusion

<table>
<thead>
<tr>
<th>Measure</th>
<th>Equilibration</th>
<th>End Ischemia</th>
<th>Reperfusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LVDP</td>
<td>99±12</td>
<td>...</td>
<td>37±15</td>
</tr>
<tr>
<td>EDP</td>
<td>5±2</td>
<td>43±11</td>
<td>46±16</td>
</tr>
<tr>
<td>MV0₂</td>
<td>6.8±1.0</td>
<td>...</td>
<td>6.6±0.8</td>
</tr>
<tr>
<td>BBS2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LVDP</td>
<td>102±16</td>
<td></td>
<td>84±13*</td>
</tr>
<tr>
<td>EDP</td>
<td>3±2</td>
<td>8±3*</td>
<td>5±1*</td>
</tr>
<tr>
<td>MV0₂</td>
<td>6.9±1.2</td>
<td>...</td>
<td>6.3±1.6</td>
</tr>
</tbody>
</table>

Values are mean±SD. LVDP and EDP are expressed in mm Hg; MV₀₂ in μmol·g wet wt⁻¹·min⁻¹).

*P<0.05 vs controls.
are consistent with less inhibition of glucose metabolism during ischemia because of inhibition of NOS2 by BBS2.

There was no significant difference in the baseline measurements of myocardial ATP during the equilibrium period in the control and treated hearts (Table 2). ATP levels in the myocardium at the end of the ischemic period were depressed in both groups of rat hearts. However, the ATP level in the treated hearts was significantly higher than the level in the untreated controls ($P < 0.05$, Table 2). At the end of the reperfusion period, myocardial ATP was also significantly higher in the treated hearts (data not shown).

**Lactate/Pyruvate Ratio**

The lactate/pyruvate ratio, which has been used as an index of the cytosolic redox state, was not significantly different between the control and BBS2-treated hearts during baseline perfusion or at the end of reperfusion. At the end of the ischemic period, however, lactate levels were higher and the lactate/pyruvate ratio was significantly lower in the treated hearts than in the untreated control hearts ($P < 0.05$, Table 2).

**Nitric Oxide Synthase 2**

NOS2 mRNA normalized to GAPDH (internal control) was expressed in both control and BBS2-treated hearts at the end of the reperfusion period ($16.8 \pm 3.4$ versus $10.8 \pm 1.2$, $P < 0.01$). Only minimal amounts of NOS2 protein were detected in hearts before the ischemic period (Figure 2). NOS2 protein was expressed in both control and BBS2-treated hearts at the end of the ischemic and reperfusion periods (Figure 2), indicating that the NOS2 expression was upregulated. Despite slightly higher NOS2 protein after reperfusion (Figure 2), the NOS2 activity of myocardial homogenates was reduced by 60% below control values in the hearts treated with the inhibitor of NOS2 (Figure 3).

**Pathology**

In the H&E sections of myocardium from ischemic and/or reperfused control hearts, there was irregular waving of the muscle fibers and contraction bands indicative of early damage (Figure 4a). These alterations were less apparent in sections from reperfused hearts of rats treated with the

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**TABLE 2. Metabolic and Enzymatic Changes in BBS-Treated Hearts Before and at the End of Ischemia**

<table>
<thead>
<tr>
<th>Sample/Time Point</th>
<th>n</th>
<th>Controls</th>
<th>BBS2 Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>G-3-P</td>
<td>6</td>
<td>89±12</td>
<td>96±16</td>
</tr>
<tr>
<td>Before ischemia</td>
<td></td>
<td>178±39</td>
<td>118±27*</td>
</tr>
<tr>
<td>GAPDH</td>
<td>3</td>
<td>4.2±0.5</td>
<td>4.8±0.7</td>
</tr>
<tr>
<td>Before ischemia</td>
<td></td>
<td>2.9±0.6</td>
<td>4.9±0.9*</td>
</tr>
<tr>
<td>Aldose reductase</td>
<td>5</td>
<td>3.4±0.4</td>
<td>2.9±0.6</td>
</tr>
<tr>
<td>Before ischemia</td>
<td></td>
<td>6.3±0.7</td>
<td>3.9±0.5*</td>
</tr>
<tr>
<td>ATP</td>
<td>6</td>
<td>17.6±3.2</td>
<td>15.9±4.6</td>
</tr>
<tr>
<td>Before ischemia</td>
<td></td>
<td>1.64±0.4</td>
<td>7.3±1.8*</td>
</tr>
<tr>
<td>Lactate/pyruvate</td>
<td>6</td>
<td>17.2±4.8</td>
<td>19.6±6.2</td>
</tr>
<tr>
<td>Before ischemia</td>
<td></td>
<td>456±56</td>
<td>306±29*</td>
</tr>
<tr>
<td>Lactate</td>
<td>6</td>
<td>5.6±1.1</td>
<td>6.1±1.8</td>
</tr>
<tr>
<td>Before ischemia</td>
<td></td>
<td>128.5±16.2</td>
<td>181.6±21.1*</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>6</td>
<td>0.32±0.06</td>
<td>0.38±0.09</td>
</tr>
<tr>
<td>Before ischemia</td>
<td></td>
<td>0.28±0.04</td>
<td>0.59±0.10*</td>
</tr>
</tbody>
</table>

G-3-P, ATP, lactate, and pyruvate are expressed as μmol/g dry wt; GAPDH is expressed as μmol NADH·min⁻¹·mg protein⁻¹; and aldose reductase activity is expressed as μmol NADPH·min⁻¹·mg protein⁻¹.

*P<0.05 vs controls in their respective time point.
inhibitor of NOS2 (Figure 4b). Similar numbers of apoptotic endothelial cells were apparent in control (12.3±5.3/mm²) and treated (7.4±4.7/mm²) reperfused hearts. Apoptotic cardiomyocytes were observed only in sections from control animals (2.7±1.1/mm²), and none were present in BBS2-treated hearts (Figure 4, e and f). NOS2-positive immunostaining was observed in myocytes and endothelial cells of both sets of animals (Figure 4, e and f).

Discussion

BBS2 binds allosterically to heme-containing monomers of NOS2 in a pterin- and L-arginine–independent manner; this forms an inactive complex that inhibits the homodimerization of the NOS2 monomers and the enzymatic synthesis of NO from L-arginine and oxygen. In cell-based dimerization assays, the IC₅₀ of BBS2 for NOS3 was >1500-fold greater and the IC₅₀ for NOS1 was 600-fold greater than the IC₅₀ for NOS2 (J.F. Parkinson and G.B. Phillips, unpublished observations). This high pharmacological specificity of BBS2 for NOS2 in vitro has been confirmed in vivo. Enkhabaatar et al found that BBS2 had no adverse effects on cardiac and pulmonary hemodynamics in sheep with and without burn and smoke inhalation injury. Ichinose et al demonstrated that treatment with BBS2 prevented systemic, myocardial, and pulmonary dysfunctions that result from NOS2 expression after administration of Escherichia coli endotoxin to mice. The protective effects of BBS2 were associated with inhibition of formation of NOS2 homodimers in heart and lung and blockade of endotoxin-induced increase in plasma NOx. The selectivity of BBS2 for NOS2 was also shown by the lack of effects of BBS2 on baseline pulmonary and systemic hemodynamics. In addition, administration of BBS2 to rats for 10 days at the dose used in the present study showed no difference in agonist-stimulated vascular reactivity in aortic rings between BBS2- and vehicle-treated animals, which reflects lack of effect of BBS2 on NOS3. BBS2 lacks the multiple biochemical activities of semiselective NOS2 inhibitors such as aminoguanidine. Selective inhibition of NOS2 by BBS-2 allows potentially beneficial synthesis of NO by other NOS isoforms to remain unimpeded. Measurements made after completion of the ischemia/reperfusion protocol indicated that NOS2 enzyme activity in the myocardium was reduced in the treated animals to a level 60% of that found in the untreated control hearts. The slight reductions of NOS2 mRNA in the myocardium of the treated animals are unexplained but might indicate an additional small effect of BBS2 on the translation of NOS2.

Cardiac function during the ischemia/reperfusion protocol was significantly better in the hearts from rats treated with BBS2 than in hearts from control rats. Inhibition of NOS2 attenuated the marked rise in EDV during the period of ischemia and reperfusion. This reduction in the compliance of the ventricle (“ischemic contracture”) is thought to result from increases of intracellular calcium in cardiac muscle cells as a consequence of ATP depletion. In hearts from control rats, the recovery of systolic function during reperfusion after ischemia (assessed by the magnitude of LVDP) was only to a value that was 38% of the LVDP during the equilibration period. In hearts from the rats treated with BBS2, the recovery of LVDP was significantly greater, to a value that was 83% of that during equilibration.

Effects on myocardial energy metabolism were also observed in hearts from rats treated with BBS2. In the equilibration period, ATP levels were not significantly different in control and treated animals. At the end of the period of global ischemia, myocardial ATP was reduced significantly in all hearts; however, the mean ATP level in the hearts from rats treated with BBS2 was significantly higher than that of the untreated controls. Synthesis of ATP by oxidative phosphorylation ceases during global ischemia because of reduced coronary flow and reduced oxygen delivery, and in this situation, ATP production results from anaerobic glycolysis. The observation that ATP was significantly higher at the end of the ischemic period in hearts from animals treated with the
selective inhibitor of NOS2 implies that anaerobic glycolysis in the untreated control hearts during the ischemic period was inhibited to some extent by NO synthesized by NOS2.

Two possible mechanisms could explain this NO-mediated inhibition of anaerobic glycolysis. Several groups have reported that GAPDH, a critical enzyme in the glycolytic pathway, can undergo S-nitrosylation and auto-ADP ribosylation by NO, leading to inhibition of its enzymatic activity. The findings that myocardial GAPDH activity was reduced and the level of G-3-P at the end of the ischemic period was significantly higher in control than in treated hearts are consistent with the idea that inhibition of GAPDH by NOS2-produced NO during ischemia was and was reduced in hearts from animals that received BBS2.

It is also known that increases in myocardial NO during ischemia (because of enzymatic biosynthesis by NO synthase isoforms and/or by nonenzymatic formation from nitrite) activate aldose reductase, the first enzyme in the polyol pathway. Increased activity of aldose reductase utilizes NADH and is associated with an increase of the cytosolic NADH/NAD+ ratio. The myocardial tissue lactate/pyruvate ratio is an indirect measure of the cytosolic NADH/NAD+ ratio. This ratio rose markedly in all of the hearts by the end of the ischemic period. However, the rise in the ratio was attenuated significantly in the hearts from rats treated with BBS2. This suggests that during the period of ischemia, inhibition of NOS2-mediated synthesis of NO by BBS2 reduced NO-mediated inhibition of aldose reductase activity. Deinhibition of aldose reductase during the period of ischemia may have contributed to the preservation of the cytosolic NADH/NAD+ ratio, thereby enhancing anaerobic glycolysis and preserving the myocardial stores of ATP during ischemia. At the completion of the reperfusion period, the lactate/pyruvate ratio had returned to values similar to those in the equilibration period in all hearts. Myocardial ATP levels at this time were increased above ischemic values in both groups; however, myocardial ATP was significantly higher after reperfusion in the BBS2-treated hearts than in untreated controls. Because oxygen consumption during reperfusion was not different in the 2 groups, this difference in ATP stores may reflect the fact that net ATP synthesis by aerobic metabolic pathways was higher in the treated hearts during reperfusion as a consequence of less ischemic damage to the heart muscle cells.

During the period of reperfusion, the release of creatine kinase from the heart (a marker of ischemic injury to cardiac muscle cells) was significantly lower in hearts treated with BBS2 than in untreated control hearts. Pathological examination of the hearts at the end of the reperfusion period confirmed that there was less cardiac muscle fiber damage and fewer apoptotic cardiomyocytes in the hearts from the rats treated with the inhibitor of NOS2 than was observed in untreated control animals. Although many mechanisms may be involved in these differences in myocardial pathology, it is likely that inhibition of the synthesis of NO by NOS2 plays a major role. The present results are consistent with previous studies of acute ischemia and reperfusion in which administration of semiselective inhibitors of NOS2 or experimental use of NOS2−/− mice was associated with enhanced cardiac performance and/or reduction of myocardial infarct size. Other studies by several groups have indicated that increased myocardial expression of NOS2 and increased NO synthesis by this isoform occur during acute cardiac allograft rejection and are associated with contractile dysfunction, electrical instability, increased nitration of cardiac proteins, and death of cardiomyocytes by necrosis and by apoptosis. These changes are reduced if the heart transplantation is performed by use of NOS2−/− animals or by the administration of inhibitors of NOS2 enzymatic activity.

In summary, the data from the present study indicate that predadministration of BBS2, a highly selective inhibitor of NOS2 that blocks dimerization of NOS2 monomers, improves contractile function, preserves myocardial ATP, and reduces damage and apoptotic death of cardiac myocytes during ischemia and reperfusion of the isolated buffer-perfused rat hearts. Experiments were not performed in blood-perfused hearts to avoid alterations of NOS2 expression in the isolated heart transplantation. Nor did we determine the effects of administration of BBS-2 after induction of ischemia and reperfusion or in relationship to beneficial consequences of NOS2 expression in the late phase of ischemic preconditioning. The protective effects of selective inhibition of NOS2 by BBS2 observed in the present study indicate that NO synthesized by NOS2 contributes to the adverse biochemical and functional consequences of acute ischemia and reperfusion in buffer-perfused rat hearts. The data suggest that after additional studies, inhibitors of NOS2 dimerization may provide novel adjunctive therapy for the protection of ischemic myocardium.

Acknowledgments

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