Myocardial Glucose Uptake Is Regulated by Nitric Oxide via Endothelial Nitric Oxide Synthase in Langendorff Mouse Heart

Hideo Tada, Carl I. Thompson, Fabio A. Recchia, Kit E. Loke, Manuel Ochoa, Carolyne J. Smith, Edward G. Shesely, Gabor Kaley, Thomas H. Hintze

Abstract—Although the role of nitric oxide (NO) in the modulation of vascular tone has been studied and well understood, its potential role in the control of myocardial metabolism is only recently evident. Several lines of evidence indicate that NO regulates myocardial glucose metabolism; however, the details and mechanisms responsible are still unknown. The aim of this study was to further define the role of NO in the control of myocardial glucose metabolism and the nitric oxide synthase (NOS) isoform responsible using transgenic animals lacking endothelial NOS (ecNOS). In the present study, we examined the regulation of myocardial glucose uptake using isometrically contracting Langendorff-perfused hearts from normal mice (C57BL/6J), mice with defects in the expression of ecNOS [ecNOS (−/−)], and its heterozygote [ecNOS (+/−)], and wild-type mice [ecNOS (+/+)] (n=6, respectively). In hearts from normal mice, little myocardial glucose uptake was observed. This myocardial glucose uptake increased significantly in the presence of Nω-nitro-L-arginine methyl ester (L-NAME). Similarly, in the hearts from ecNOS (−/−), glucose uptake was much greater than in normal mice, whereas myocardial glucose uptake of ecNOS (+/−) and ecNOS (+/+) mice was not different from normal mice. In addition, myocardial glucose uptake of ecNOS (+/−) and ecNOS (+/+) mice increased significantly in the presence of L-NAME. At a workload of 800 g · beats/min, L-NAME increased glucose uptake from 0.1±0.1 to 3±0.4 μg/min · mg in ecNOS (+/−) mice and from 0.2±0.1 to 2.7±0.7 μg/min · mg in ecNOS (+/+) mice. Furthermore, in the hearts from ecNOS (−/−) mice, 8-bromoguanosine 3′:5′-cyclic monophosphate (8-Br-cGMP), a cGMP analog or S-nitroso-N-acetylpenicillamine (SNAP), a NO donor essentially shut off glucose uptake, and in hearts from ecNOS (+/−) mice, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), an inhibitor of cGMP, increased the glucose uptake significantly. These results indicate clearly that cardiac NO production regulates myocardial glucose uptake via a cGMP-dependent mechanism and strongly suggest that ecNOS plays a pivotal role in this regulation. These findings may be important in the understanding of the pathogenesis of the diseases such as ischemic heart disease, heart failure, diabetes mellitus, hypertension, and hypercholesterolemia, in which NO synthesis is altered and substrate utilization by the heart changes. (Circ Res. 2000;86:270-274.)

Key Words: cardiac work ■ mice, knockout ■ arginine ■ length-tension

Since the discovery of nitric oxide (NO), its actions on blood vessels have been extensively studied.1–3 In contrast to these vascular effects, the potential role of NO in the control of myocardial metabolism has received little attention. In recent years, several investigators indicated that NO may have an important role in the regulation of cardiac metabolism. Our previous studies have demonstrated that the inhibition of NO synthesis with nitric oxide synthase (NOS) inhibitors nitro- and arginine (NLA) or methyl ester of NLA (L-NAME) increased tissue oxygen consumption.4 The site of action is most likely cytochrome oxidase,5,6 and a recent elegant study by Clementi et al7 using cultured endothelial cells supports this conclusion. Furthermore, we have recently found that endothelial nitric oxide synthase (ecNOS) plays a pivotal role in the control of myocardial oxygen consumption using transgenic mice lacking ecNOS.8

In addition, inhibitors of NOS protect the heart against ischemic injury and improve the posts ischemic functional recovery in vitro.7 This protection may be related to stimulation of glucose uptake and glycolysis, resulting in a better maintenance of high-energy phosphates. These data suggest that NO plays an important role in the regulation of myocardial glucose metabolism. Although the use of arginine analogs has indicated a tentative role for NO in the regulation of...
myocardial glucose metabolism,9,10 and we have tentatively described a role for NO in the control of free fatty acid and glucose uptake in the heart during exercise and heart failure,11,12 questions remain as to which isoform of NOS is involved in this regulation. The aim of the present study was to further define the role of NO in the control of myocardial glucose metabolism using hearts from mice lacking ecNOS.

Materials and Methods

Animals
Normal mice (C57BL/6J) obtained from the Jackson Laboratory (Bar Harbor, Maine) were used as controls. Heterozygote ecNOS (+/−) mice, originally developed by Shesely et al,13 were interbred to generate ecNOS heterozygotes (+/−), homozygous (+/+), and wild-type (+/+ mice).8 The ecNOS mice were genotyped by Southern blot analysis of DNA from tail snips as described previously (Figure 1), to discern ecNOS (+/−), (−/−), and (+/+) mice. All protocols were approved by the Institutional Animal Care and Use Committee of New York Medical College and conform to the current National Institutes of Health and American Physiological Society guidelines for the use and care of laboratory animals.

Isolated Mouse Heart Preparation

Isolated heart preparation was performed using a method modified for the mouse heart.14 Mice (either sex) were anesthetized with sodium pentobarbital (65 mg/kg IP), and heparin (100 U) was injected intravenously. The thorax was opened. The heart was quickly excised, placed in ice-cold saline, and immediately mounted, via the ascending aorta, onto a perfusion apparatus. The heart was perfused with a nonrecirculating perfusate at a constant flow (0.1 mL/min) and pressure of 60 mm Hg (Langendorff preparation). The perfusate was a modified Krebs-Henseleit solution containing (in mmol/L) NaCl 117.4, KCl 4.7, MgSO4 1.1, KH2PO4 1.2, glucose 5.5, CaCl2 2.5, ascorbate 0.1, and l-arginine 1.0. The perfusate was equilibrated with 5% CO2/95% O2 at 37°C, and pH was adjusted with NaHCO3 (20 to 25 mmol/L) to 7.40. The perfusate leaving the reservoir was continuously pumped through a 3-μm filter to prevent particulate matter from entering the coronary circulation. A metal hook was inserted into the apex of the heart to control and record tension and heart rate. Tension was measured using a FTO3C transducer (Grass Instrument Co, Quincy, Mass) and recorded on a Dynograph recorder R511A (Sensor Medicis, Anaheim, Calif). In some studies, 10−9 mol/L of Nω-nitro-L-arginine methyl ester (L-NAME), 10−4 mol/L of 8-bromoguanosine 3′,5′-cyclic monophosphate (8-Br-cGMP), 50 μmol/L of S-nitroso-N-acetylpenicillamine (SNAP), or 10−6 mol/L of HF-1-[2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) (Sigma) were added to the perfusate.

Cardiac Glucose Uptake

Hearts were perfused at up to three different workloads to stimulate glucose utilization (peak-systolic tension from 1 to 9 g). Heart work was estimated as the product of developed tension (peak-systolic tension−end-diastolic tension) and heart rate. Perfusate leaving the heart was collected over 1 minute for the measurement of glucose during steady state at each level of work. Glucose in perfusate was measured using Glucose [HK] 10 from Sigma. Glucose uptake was calculated by the following equation:

\[
\text{Glucose Uptake} = \frac{[\text{Glucose}]_{\text{in}} - [\text{Glucose}]_{\text{out}}}{\text{Flow Rate}} \cdot \frac{1}{\text{Heart weight}}
\]

\[
[\text{Glucose}]_{\text{in}}: \text{glucose concentration, inflow (μg/mL)},
\]

\[
[\text{Glucose}]_{\text{out}}: \text{glucose concentration, outflow (μg/mL)}.
\]

Pacing Procedure

In some studies, the hearts were paced at 200 bpm to keep the heart rates the same. One electrode was attached to the metal hook, and the other one was inserted into the perfusion apparatus just above the ascending aorta, and then the heart was stimulated (33 Hz, up to 10 V, 1-ms duration) using a Grass S44 stimulator (Grass Instrument Co).

Statistical Analysis

Data were calculated as mean±SEM. Linear regression was performed using least-squares analysis (Microsoft Excel). Graphs were produced using Microsoft Excel. Statistical analyses of heart work and myocardial glucose uptake were performed using unpaired t test, and that of linear regression lines were performed using ANOVA. P<0.05 was considered statistically significant.

Results

The age, body weight, and sex of all mice are shown in Table 1.

Isolated Mouse Heart

Initially, each mouse heart was stretched to 1 gram-tension and allowed to equilibrate. To ensure that each heart re-

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### TABLE 1. Characteristics of Mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Age, d</th>
<th>Weight, g</th>
<th>Heart Weight, mg</th>
<th>Sex (Male/Female)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>70±8</td>
<td>18.8±1.0</td>
<td>99±5</td>
<td>0/6</td>
</tr>
<tr>
<td>Normal with L-NAME</td>
<td>80±11</td>
<td>17.5±1.0</td>
<td>107±12</td>
<td>0/6</td>
</tr>
<tr>
<td>ecNOS (+/−)</td>
<td>302±22</td>
<td>31.0±3.8</td>
<td>142±13</td>
<td>4/2</td>
</tr>
<tr>
<td>ecNOS (+/−) with L-NAME</td>
<td>313±22</td>
<td>30.7±1.9</td>
<td>158±10</td>
<td>1/5</td>
</tr>
<tr>
<td>ecNOS (+/−) with ODQ</td>
<td>417±28</td>
<td>25.1±2.2</td>
<td>136±13</td>
<td>0/6</td>
</tr>
<tr>
<td>ecNOS (+/+)</td>
<td>128±25</td>
<td>30.4±2.8</td>
<td>138±10</td>
<td>4/2</td>
</tr>
<tr>
<td>ecNOS (+/+) with L-NAME</td>
<td>138±23</td>
<td>30.3±4.3</td>
<td>144±17</td>
<td>3/3</td>
</tr>
<tr>
<td>ecNOS (+/−)</td>
<td>253±30</td>
<td>28.7±1.3</td>
<td>191±19</td>
<td>5/7</td>
</tr>
<tr>
<td>ecNOS (+/−) with 8-Br-cGMP</td>
<td>298±51</td>
<td>24.3±0.6</td>
<td>131±8</td>
<td>4/2</td>
</tr>
<tr>
<td>ecNOS (+/−) with SNAP</td>
<td>421±12</td>
<td>24.4±0.7</td>
<td>122±7</td>
<td>0/6</td>
</tr>
</tbody>
</table>

Data are mean±SEM.
sponded in a similar fashion, end-diastolic tension was increased in one to two additional steps (Figure 2A). To ensure that hearts from different genotypes responded to stretch, the relationship between end-diastolic tension and peak-systolic tension was plotted (Figure 2B). There was a linear relationship between end-diastolic tension and peak-systolic tension in all genotypes of mice and in the presence of L-NAME (Table 2). There was no significant difference among the groups.

Role of NO in the Regulation of Myocardial Glucose Metabolism

The effects of changes in cardiac work versus glucose uptake are shown in Figure 3A. In the hearts from normal mice, little glucose uptake was observed at any level of heart work. However, myocardial glucose uptake of normal mice increased in the presence of L-NAME (10^-4 mol/L), an inhibitor of NOS. The average changes in heart work and glucose uptake are shown in Figure 3B. L-NAME markedly increased glucose uptake in hearts from normal mice.

Significance of ecNOS in the Regulation of Myocardial Glucose Metabolism

Myocardial glucose uptake of ecNOS (-/-) mice was much greater than that of normal mice (Figures 3A and 3B), whereas myocardial glucose uptake of ecNOS (+/-) and ecNOS (+/+) mice were not different from normal mice (Figures 3A and 3B). In addition, myocardial glucose uptake of ecNOS (+/-) and ecNOS (+/+) mice increased significantly in the presence of L-NAME (10^-4 mol/L) (Figures 3A and 3B).

Role of cGMP in the Regulation of NO-Mediated Myocardial Glucose Metabolism

8-Br-cGMP (10^-4 mol/L), a cGMP analog, or SNAP (50 μmol/L), a NO donor, essentially shut off glucose uptake in the hearts from ecNOS (-/-) mice (Figure 4). Furthermore, ODQ (10^-4 mol/L), a selective and potent inhibitor of cGMP, increased glucose uptake remarkably in the hearts.

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**Figure 2.** Original recording of tension (A) of the mouse heart in our preparation. Diastolic and systolic tension increased as a result of stretch; hence, heart work also increased. The linear relationships between end-diastolic tension and peak-systolic tension (B) are shown. There was no difference between the 7 groups.

**Figure 3.** Scatter plots (A) of the relationships between double product and myocardial glucose uptake are shown. Calculated mean values showing the relationship between double product and myocardial glucose uptake (B) are also shown. In the absence of ecNOS [ecNOS (-/-)] or in the presence of L-NAME, glucose uptake was increased. Data are mean±SEM.

**TABLE 2.** Relationship Between End-Diastolic Tension and Peak-Systolic Tension

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Equation</th>
<th>R</th>
<th>P Value of R</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>y = 1.48x + 2.22</td>
<td>0.69</td>
<td>0.0002</td>
</tr>
<tr>
<td>Normal with L-NAME</td>
<td>y = 1.29x + 1.58</td>
<td>0.75</td>
<td>0.01</td>
</tr>
<tr>
<td>ecNOS (+/-)</td>
<td>y = 1.69x + 1.52</td>
<td>0.81</td>
<td>0.003</td>
</tr>
<tr>
<td>ecNOS (+/-) with L-NAME</td>
<td>y = 1.49x + 1.71</td>
<td>0.62</td>
<td>0.001</td>
</tr>
<tr>
<td>ecNOS (+/-)</td>
<td>y = 1.65x + 2.32</td>
<td>0.60</td>
<td>0.002</td>
</tr>
<tr>
<td>ecNOS (+/-) with L-NAME</td>
<td>y = 1.99x + 0.67</td>
<td>0.84</td>
<td>0.04</td>
</tr>
<tr>
<td>ecNOS (-/-)</td>
<td>y = 1.05x + 1.80</td>
<td>0.73</td>
<td>0.002</td>
</tr>
</tbody>
</table>
known,1–3 the potential role of NO in the control of myocardial glucose uptake is shown. In the hearts from ecNOS (−/−) mice, myocardial glucose uptake was essentially shut off in the presence of cGMP analog or NO donor. Furthermore, inhibition of cGMP increased myocardial glucose uptake in the hearts from ecNOS (+/−) mice. The heart rate in these protocols was kept at 200 bpm. Data are mean ± SEM.

from ecNOS (+/−) mice (Figure 4). In these protocols, every heart was paced at 200 bpm throughout the experiment. To ensure that these drugs had no effects on cardiac function, the relationships between end-diastolic tension and peak-systolic tension were plotted again. There was no significant difference among the groups (data not shown).

Discussion

Although the effects of NO on blood vessels are well known,1–3 the potential role of NO in the control of myocardial metabolism has only recently been appreciated. In the present study, we have demonstrated clearly that NO regulates myocardial glucose metabolism via cGMP in the mouse heart and for the first time that ecNOS is a key enzyme in this regulation. Our studies have clearly shown that removal of NO production using a NOS inhibitor or gene deletion in ecNOS (−/−) mice or inhibition of cGMP in heterozygote mice markedly increases glucose uptake. This is independent of changes in cardiac work, given that the large glucose uptake after L-NAME or in ecNOS (−/−) mice occurred at low cardiac work, i.e., the double product, in our studies.

There are some differences in heart work among groups in our study due to the variation of heart rate. However, we can exclude the contribution of heart rate to our results, because all hearts were paced at the same heart rate in studies of the role of cGMP. In addition, even in other protocols, the highest glucose uptake occurred at the lowest workloads. Furthermore, myocardial glucose uptake was much greater in ecNOS (−/−) mice and normal, ecNOS (+/−), ecNOS (+/+) mice with L-NAME than in normal ecNOS (+/+) or ecNOS (+/−) mice. To be certain that a gross difference in cardiac function in mouse hearts of different genotypes was not responsible for the altered glucose uptake, we constructed a length-tension relationship. There was no difference in the relationship between passive and active tension across groups. Therefore, the altered glucose uptake cannot be explained by altered function. Furthermore, although heart rate was low in the ecNOS (−/−) heart and normal heart after L-NAME, this cannot account for the difference in glucose uptake, because high glucose uptake occurred at low workloads. Finally, there was no significant difference in the increase in heart work in ecNOS (+/+ +) and ecNOS (+/−) mice before and after L-NAME, and yet a significant increase in myocardial glucose uptake occurred after inhibition of NOS.

There were also some differences in age and sex among groups. The ages of ecNOS (−/−) mice were higher than those of normal mice, but the ages of ecNOS (+/−) or (+/+) mice were also higher than those of normal mice. However, glucose uptake of these ecNOS (+/−) or (+/+) mice was not different from normal mice. There were no males in the normal or the normal with L-NAME group. However, myocardial glucose uptake of normal with L-NAME was much higher than those of normal mice. Therefore, we can conclude that age or sex had no effect on these results.

An inhibitory action of NO on glycolysis was first demonstrated in a model of chronic liver inflammation.15 Other investigators have suggested a stimulatory role for NO in the control of glucose metabolism.16,17 Recently, Depre et al18 reported that inhibitors of NOS protect the heart against ischemic injury and improve the postischemic recovery of cardiac function. This protection was related to stimulation of glucose uptake and glycolysis after NOS inhibition. Depre et al proposed that the stimulation of glycolysis after NOS inhibition was mediated by increased glucose transport, because the flux through phosphofructo-1-kinase was smaller than through the transporter, and hence hexose-6-phosphates accumulated. Depre et al19 in another study also demonstrated that cGMP might be involved in this mechanism because 8-Br-cGMP and a NO donor inhibited glucose uptake in Langendorff rat heart. These studies are confirmed by our results and extended further given that ODQ markedly increased glucose uptake in the hearts from ecNOS (+/−) mice.

Recently, we have reported a significant increase in myocardial glucose uptake and decrease in free fatty acid (FFA) uptake associated with significant decrease in cardiac NO production in conscious dogs with pacing-induced heart failure.11 This switch in myocardial substrate utilization also occurred after acute pharmacological blockade of NO production with NLA in normal conscious dogs. In addition, this NLA-induced switch in myocardial substrate utilization from FFA to glucose was rapidly reversed by a NO donor,10 suggesting that cardiac NO production acutely regulates substrate utilization of the myocardium. Furthermore, bovine polymerized hemoglobin-based oxygen-carrying (HBOC) solution increased myocardial glucose and lactate consumption with a significant decrease in FFA consumption in conscious normal dogs,20 again suggesting that HBOCs may improve metabolic efficiency of the heart by shifting metabolism from FFA to glucose and lactate as a result of its ability to scavenge NO. The effects of NLA or HBOCs were not due to altered afterload because infusion of angiotensin II to cause similar hemodynamic actions did not alter FFA or glucose uptake by the heart in the same conscious dogs. Taken together, the present study in mice and our previous studies in chronically
instrumented conscious dogs indicate that cardiac NO production may regulate myocardial glucose, lactate, and FFA metabolism.

There are some other and more subtle implications of our study. First, as in our study, most in vitro preparations use only glucose as a substrate because it is difficult to use FFAs. If NO is present, then glucose uptake will be limited and may be reflected in the stability of the preparation. In tissue culture, particularly in isolated cells, where only myocytes are present and NO production is low, the cardiac myocytes will take up glucose. This in no way reflects the normal physiological state and NO production is low, the cardiac myocytes will take up particularly in isolated cells, where only myocytes are present reflected in the stability of the preparation. In tissue culture, is present, then glucose uptake will be limited and may be glucose as a substrate because it is difficult to use FFAs. If NO synthesis is attenuated and cardiac substrate metabolism.

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Using arginine analogs or HBOCs in previous studies, we were unable to determine which isoform of NOS is involved in the control of substrate uptake by the heart. In the present study, we have provided direct evidence that cardiac NO production regulates myocardial glucose uptake via a cGMP-dependent mechanism, and that the constitutive isoform of ecNOS plays a pivotal role in this regulation. Our study was not able to determine a role for cardiac myocyte ecNOS, although neuronal NOS seems to be unimportant. The knowledge generated from the present study may contribute to a better understanding of the normal physiological control of cardiac substrate utilization and the pathogenesis of diseases such as ischemic heart disease, heart failure, diabetes mellitus, hypertension, and hypercholesterolemia, in which NO synthesis is attenuated and cardiac substrate or oxygen consumption is altered.

Acknowledgments

This study was supported by grants from the National Heart, Lung and Blood Institute: HL 50142 and PO-1 43023 (to T.H.H.) and R-29 54081 (to C.J.S.). H.T. was supported by a fellowship from the Biopure Co and K.E.L. by fellowship 9820046T from the American Heart Association NY State Affiliate.

References

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Circ Res. 2000;86:270-274
doi: 10.1161/01.RES.86.3.270

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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