Altered cardiac metabolic phenotype after prolonged inhibition of NO synthesis in chronically instrumented dogs

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d’Agostino, Chiara, Volodymyr Labinskyy, Vincenzo Lionetti, Margaret P. Chandler, Biao Lei, Ken Matsuo, Michelle Bellomo, Xiaobin Xu, Thomas H. Hintze, William C. Stanley, and Fabio A. Recchia. Altered cardiac metabolic phenotype after prolonged inhibition of NO synthesis in chronically instrumented dogs. Am J Physiol Heart Circ Physiol 290: H1721–H1726, 2006. First published January 20, 2006; doi:10.1152/ajpheart.00745.2005.—Acute inhibition of nitric oxide (NO) synthase causes a reversible alteration in myocardial substrate metabolism. We tested the hypothesis that prolonged NO synthase inhibition alters cardiac metabolic phenotype. Seven chronically instrumented dogs were treated with Nω-nitro-L-arginine methyl ester (L-NAME, 35 mg·kg⁻¹·day⁻¹) for 10 days to inhibit NO synthesis, and seven were used as controls. Cardiac free fatty acid, glucose, and lactate oxidation were measured by infusion of [¹⁴C]glucose and [¹³C]lactate, respectively. After 10 days of L-NAME administration, despite no differences in left ventricular afterload, cardiac O₂ consumption was significantly increased by 30%, consistent with a marked enhancement in baseline oxidation of glucose (6.9 ± 2.0 vs. 1.7 ± 0.5 μmol·min⁻¹·100 g⁻¹, P < 0.05 vs. control) and lactate (21.6 ± 5.6 vs. 11.8 ± 2.6 μmol·min⁻¹·100 g⁻¹, P < 0.05 vs. control). When left ventricular afterload was increased by ANG II infusion to stimulate myocardial metabolism, glucose oxidation was augmented further in the L-NAME than in the control group, whereas free fatty acid oxidation decreased. Exogenous NO (diethylamine nonoate, 0.01 μmol·kg⁻¹·min⁻¹ iv) could not reverse this metabolic alteration. Consistent with the accelerated rate of carbohydrate oxidation, total myocardial pyruvate dehydrogenase activity and protein expression were higher (38 and 34%, respectively) in the L-NAME than in the control group. Also, protein expression of the constitutively active glucose transporter GLUT-1 was significantly elevated (46%) vs. control. We conclude that prolonged NO deficiency causes a profound alteration in cardiac metabolic phenotype, characterized by selective potentiation of carbohydrate oxidation, that cannot be reversed by a short-term infusion of exogenous NO. This phenomenon may constitute an adaptive mechanism to counterbalance cardiac mechanical inefficiency.

heart; metabolism; nitric oxide

BESIDES REGULATING CORONARY vessel tone, nitric oxide (NO) exerts various direct effects on cardiac muscle; one such effect is the modulation of energy substrate metabolism (13). Under aerobic conditions, the heart obtains most of the energy necessary for its contractile function from free fatty acid (FFA) oxidation and the remaining energy from carbohydrate oxidation (23). This balance is disturbed, and might play a pathophysiological role, in conditions such as cardiac ischemia, hypotrophy, and failure (20, 23, 27). Acute inhibition of NO synthesis not only reduces the ratio of cardiac work to O₂ consumption (19), but it also alters myocardial substrate selection in favor of higher glucose utilization (5, 11, 17). Conversely, acute administration of exogenous NO limits the increase in myocardial glucose uptake and nonoxidative metabolism during ischemia, when anaerobic glycolysis is accelerated (10). Although the short-term metabolic effects of NO synthase (NOS) blockade are well described, only one study has documented a marked increase in glucose uptake in crystalloid-perfused hearts isolated from endothelial NOS-knockout mice (26). Whether a prolonged reduction in NO synthesis affects myocardial utilization of FFA and carbohydrates in vivo is unknown. We previously showed a decreased cardiac mechanical efficiency in chronically instrumented dogs subjected to pharmacological NOS inhibition for 10 days (14). These findings prompted us to test the hypothesis that sustained NOS inhibition in dogs causes permanent changes in cardiac metabolic phenotype, characterized by abnormal cardiac substrate metabolism, at rest and during increases in left ventricular (LV) afterload. We also tested whether these alterations can be reversed by exogenously supplied NO. Large-animal models are particularly advantageous for this type of study, in that they allow withdrawal of blood samples from the coronary sinus (CS) for measurement of cardiac substrate metabolism in vivo. At the end of the experiments, freeze-clamped myocardial biopsies from the beating heart were used for quantification of activity and protein expression of key metabolic enzymes.

MATERIALS AND METHODS

Surgical instrumentation. Fourteen adult, male, mongrel dogs (25–27 kg) were sedated with acepromazine maleate (1 mg/kg im), anesthetized with pentobarbital sodium (25 mg/kg iv), ventilated with room air, and instrumented as previously described (14, 17). Briefly, a thoracotomy was performed in the left fifth intercostal space. One catheter was placed in the descending thoracic aorta, and another was placed in the CS with the tip leading away from the right atrium. A solid-state pressure gauge (model P6.5, Konigsberg Instruments) was inserted into the LV through the apex. A Doppler flow transducer (Craig Hartley) was placed around the left circumflex coronary artery, and a pair of 3-MHz piezoelectric crystals was fixed on opposing endocardial surfaces at the base of the LV. A human, screw-type, unipolar myocardial pacing lead was fixed in the LV wall. Wires and catheters were run subcutaneously to the intracapsular region, the...
chest was closed in layers, and the pneumothorax was reduced. Antibiotics were administered after surgery, and the dogs were allowed to recover fully. After 7–10 days of recovery from surgery, the dogs were trained to lie quietly on the laboratory table. The protocol was approved by the Institutional Animal Care and Use Committee of the New York Medical College and conformed to the National Institutes of Health guidelines for the care and use of laboratory animals.

**Experimental protocol.** Chronic inhibition of NOS was obtained by administration of nitro-l-arginine methyl ester (L-NAME, 35 mg·kg⁻¹·day⁻¹ po; Sigma Aldrich) for 10 days. L-NAME was packed in capsules and administered once a day in the morning. We previously documented the efficacy of this dose in chronically treated dogs (14). Because it was necessary to harvest large cardiac biopsies at the end of the experiment, we used a separate group of seven dogs for control. Experiments were conducted in conscious dogs placed on the laboratory table after overnight fasting. Hemodynamics were recorded, and the isotopic tracers [9,10-3H]oleate, [U-14C]glucose, and [1,1-13C]lactate were continuously infused for the duration of the experiment through a peripheral vein following a protocol previously established by us (17). After 40 min of tracer infusion, paired blood samples were withdrawn from the aorta and CS. Then, to increase LV afterload, ANG II (20–40 ng·kg⁻¹·min⁻¹ iv) was infused for 30 min in both groups to reach a stable mean arterial pressure (MAP) of ~150 mmHg. Paired blood samples were withdrawn again from the aorta and CS. Finally, in the L-NAME-treated dogs, exogenous NO was supplied by infusion of the NO donor diethylamine nonoate (0.01 μmol·kg⁻¹·min⁻¹ iv), which caused an immediate drop of MAP to pre-ANG II (baseline) levels. Therefore, to maintain an MAP of 150 mmHg, we increased the dose of ANG II by five- to sixfold. Hemodynamics stabilized within 5–10 min, when the last set of blood samples was taken from the L-NAME-treated dogs. At the end of the experiment, the dogs were anesthetized with pentobarbital sodium (30 mg/kg iv), intubated, and ventilated. The fifth intercostal space was rapidly opened, and a large (25 g) tissue was removed from the left circumflex coronary artery, and LV diameter were measured in the L-NAME group (Fig. 1). The heart was then removed and weighed.

**Hemodynamic measurements.** LV and aortic pressure, blood flow in the left circumflex coronary artery, and LV diameter were measured and acquired, whereas the maximum rate of pressure development (dP/dtmax) and percent shortening of the LV diameter were calculated as previously described (14, 17).

**Blood analysis.** O2 content and total cardiac substrate concentrations were measured in arterial and CS blood samples. The concentration of O2, total and labeled substrates, and catabolites in arterial and coronary blood samples and mean coronary blood flow were used to calculate the rates of FFA, lactate, and glucose oxidation (17). The fractional O2 extraction was calculated at the arterial-CS difference in O2 concentration divided by the arterial O2 concentration. [13C]lactate enrichment was measured by mass spectroscopy, and simultaneous myocardial lactate uptake and output were calculated as previously described (17). With this method, total lactate uptake approximates total lactate oxidation (6), and the difference between tracer-measured lactate uptake and net lactate uptake corresponds to lactate output, an index of nonoxidative glycolysis. Myocardial O2 consumption (MVO2) and rates of substrate consumption were normalized by cardiac weight and expressed as micromoles per minute per 100 g of tissue.

**Insulin measurements.** Insulin was assayed by ELISA using a commercial kit for human insulin (American Laboratory Products, Windham, NH).

**Enzyme activities and metabolic products in cardiac tissue.** The activities of GAPDH and pyruvate dehydrogenase (PDH) were measured by the reverse of the procedure described by Molina y Vedia et al. (12) and the radioenzymatic method established by Sterk et al. (25), respectively.

**Western immunoblot analysis.** Protein was extracted from frozen tissue as previously described (9, 10). Fifty micrograms of total protein were separated by electrophoresis and transferred onto a polyvinylidene difluoride membrane. The membranes were incubated with specific antibodies against GLUT-1 (1:2,000 dilution), GLUT-4 (1:2,000 dilution), and GAPDH (1:3,000, all of which were obtained from Chemicon International. Antibodies against the E2 subunit of the PDH complex (1:300 dilution) and pyruvate dehydrogenase kinase-4 (PDK-4, 1:300 dilution) were purchased from Santa Cruz Biotechnology. After conjugation with the secondary antibody, the membranes were developed in Pierce SuperSignal chemiluminescence substrate solution. Successively, the membranes were reprobed for the constitutive cytosolic protein calsequestrin (1:2,000 dilution; Chemicon) as a control for uniform loading. Bands were visualized by autoradiography and quantified using commercially available software. Results are expressed as arbitrary units of density.

**RESULTS**

**Hemodynamics.** MAP in the two groups of dogs was similar at baseline and reached similar levels after 30 min of ANG II infusion (Fig. 1A). The augmented infusion rate of ANG II counterbalanced the effects of the NO donor in the L-NAME group and maintained a constant level of MAP. Mean blood flow in the left circumflex coronary artery increased in the control group during ANG II infusion and decreased slightly, although significantly, during NO donor infusion in the L-NAME group (Fig. 1B). The percent shortening of LV short-axis diameter (Fig. 1C) was reduced by approximately one-half in the L-NAME group compared with the control group, whereas LV end-diastolic diameter was not significantly different between the two groups (Fig. 1D).

Baseline values for LV dP/dtmax were 2,786 ± 168 and 2,525 ± 181 mmHg/s in the L-NAME and control groups, respectively [P = not significant (NS)], and did not change significantly within each group or among groups during ANG II or ANG II + NO donor infusion.

**Cardiac metabolism.** After 10 days of L-NAME administration, heart weight was 218.4 ± 9.0 g [P = NS vs. control (212.7 ± 12.0 g)]. Arterial O2 content was not different between the two groups (data not shown), but CS PO2 was significantly lower in the L-NAME than in the control group at baseline (20.9 ± 19 vs. 24.9 ± 0.6 mmHg) and during ANG II infusion (16.8 ± 1.4 vs. 21.4 ± 0.7 mmHg), reflecting a higher O2 extraction in the L-NAME group. At baseline, fractional O2 extraction was 0.79 ± 0.01 and 0.69 ± 0.01 in the L-NAME and control groups, respectively, and increased during ANG II infusion to 0.85 ± 0.01 and 0.78 ± 0.02, respectively (both, P < 0.05). However, the percent increase in O2 extraction during ANG II infusion was significantly lower in the L-NAME than in the control group (7.6 ± 0.9 vs. 13.7 ± 2.5%). These data were consistent with the finding that baseline MVO2 was ~30% higher in the L-NAME than in the
control group (Fig. 2A), whereas ANG II infusion caused a significant increase only in the control group.

The higher baseline MV˙O2 in the L-NAME group was not paralleled by a significant difference in FFA oxidation rate between the two groups (Fig. 2B) but, rather, by a fourfold increase in the rate of glucose oxidation (Fig. 2C). Also, total cardiac lactate uptake, an index of lactate oxidation rate, was significantly higher (~2-fold) at baseline in the L-NAME than in the control group. ANG II infusion caused a significant decrease in FFA oxidation in the L-NAME, but not in the control, group. Conversely, glucose oxidation increased significantly in both groups, and total lactate uptake doubled in the control group. Changes in arterial level and uptake of the three cardiac substrates are reported in Table 1. The fall in FFA oxidation in the L-NAME group was paralleled by a similar change in arterial FFA levels and FFA uptake. Circulating glucose and cardiac glucose uptake did not change significantly as a function of time in either group, nor were there differences between groups during ANG II and ANG II + NO donor infusions. Finally, arterial lactate concentration increased slightly, but significantly, in the control group during ANG II infusion and doubled in the L-NAME group during ANG II + NO donor infusion. Lactate release, an index of the rate of nonoxidative glycolysis, did not display significant changes, except for a significant decrease in the L-NAME group, during ANG II infusion.

We tested whether higher glucose oxidation in the L-NAME group was due, at least in part, to changes in plasma insulin levels. Plasma insulin concentration was 4.3 ± 1.2 μU/ml at baseline, before initiation of L-NAME administration, and
Table 1. Changes in arterial FFA, glucose, and lactate concentration and in FFA and glucose uptake and lactate release

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>ANG II</th>
<th>ANG II + NO Donor</th>
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<tr>
<td>FFA, mM</td>
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<td></td>
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<tr>
<td>Control</td>
<td>0.63 ± 0.04</td>
<td>0.58 ± 0.04*</td>
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<tr>
<td>t-NNAME</td>
<td>0.51 ± 0.06</td>
<td>0.36 ± 0.04†</td>
<td>0.310 ± 0.049*</td>
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<tr>
<td>Glucose, mM</td>
<td></td>
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<tr>
<td>Control</td>
<td>4.43 ± 0.24</td>
<td>3.95 ± 0.28</td>
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<tr>
<td>t-NNAME</td>
<td>4.48 ± 0.20</td>
<td>4.44 ± 0.17</td>
<td>4.91 ± 0.28</td>
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<tr>
<td>Lactate, mM</td>
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<tr>
<td>Control</td>
<td>0.75 ± 0.10</td>
<td>1.05 ± 0.16*</td>
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<tr>
<td>t-NNAME</td>
<td>1.11 ± 0.27</td>
<td>1.30 ± 0.24</td>
<td>2.20 ± 0.27*</td>
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<td>FFA uptake, μmol/min</td>
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<tr>
<td>1-100 g^-1</td>
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<tr>
<td>Control</td>
<td>7.27 ± 0.5</td>
<td>8.28 ± 1.164</td>
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<tr>
<td>t-NNAME</td>
<td>7.30 ± 1.4</td>
<td>5.80 ± 1.2*</td>
<td>4.0 ± 1.0*</td>
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<td>Glucose uptake, μmol/min</td>
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<td>1-100 g^-1</td>
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<tr>
<td>Control</td>
<td>9.68 ± 5.29</td>
<td>12.22 ± 2.57</td>
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<tr>
<td>t-NNAME</td>
<td>13.23 ± 5.47</td>
<td>23.02 ± 9.43</td>
<td>19.81 ± 4.17</td>
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<tr>
<td>Lactate release, μmol/min</td>
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<td>1-100 g^-1</td>
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</tr>
<tr>
<td>Control</td>
<td>7.04 ± 1.97</td>
<td>9.63 ± 2.30</td>
<td></td>
</tr>
<tr>
<td>t-NNAME</td>
<td>4.67 ± 1.22</td>
<td>3.57 ± 1.63*</td>
<td>6.98 ± 1.83</td>
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Values are means ± SE (n = 7 per group). FFA, free fatty acid; NO, nitric oxide donor (diethylamine nonoate); t-NNAME, N-nitro-l-arginine methyl ester.*P < 0.05 vs. baseline; †P < 0.05 vs. control.

5.4 ± 1.5 μU/ml after 10 days of NOS blockade (P = NS). It did not change significantly during ANG II infusion (3.7 ± 1.01 μU/ml) or during infusion of ANG II + NO donor (4.9 ± 2.2 μU/ml).

Enzyme activity and protein expression in cardiac tissue. GAPDH activity was not significantly different between the two groups (Fig. 3); however, both total PDH activity and the active form of this enzyme were ~40% higher in the t-NNAME than in the control group. GLUT-1, but not GLUT-4, protein expression was increased 46% in the t-NNAME group, and subunit E2 of PDH was also upregulated, consistent with higher activity of this enzyme (Fig. 4). Paradoxically, GAPDH protein expression was diminished after NOS inhibition (Fig. 4). Finally, PDK-4 protein expression was not significantly different from control (Fig. 4).

**DISCUSSION**

Our study shows that prolonged inhibition of NO synthesis in the heart causes a fourfold and a twofold increase in basal glucose and lactate oxidation, respectively, compared with the control group and is not associated with significant changes in FFA oxidation. A higher afterload imposed on the LV further stimulated glucose and lactate oxidation, likely due to a simultaneous decrease in FFA utilization. Exogenous NO could not reverse these metabolic alterations. Finally, consistent with the metabolic measurements in vivo, prolonged NOS inhibition resulted in an increase in protein expression of GLUT-1, the constitutively active glucose transporter, and in protein expression and total activity of PDH, the enzyme that catalyzes the rate-controlling step in the carbohydrate oxidative pathway.

Marked alterations in FFA and glucose metabolism were previously shown by us in conscious dogs subjected to acute inhibition of NO synthesis (17). In that study, we found a 30% reduction in FFA oxidation associated with a fourfold increase in glucose oxidation over the first 2 h following intravenous administration of t-NAME. These changes were significantly different from those in a control group of dogs in which ANG II was infused to mimic the vasopressor effects of t-NAME. However, it is known that a more prolonged inhibition of NO synthesis leads to compensatory adaptations, as indicated, for instance, by the normal levels of arterial blood pressure that we observed after 10 days of t-NAME administration (14). Although compensatory mechanisms at the vascular level have been well characterized (15, 28), there are no reports on myocardial metabolic adaptations to chronic reduction in NO bioavailability. This is particularly important if we consider that in some pathological conditions of the cardiovascular system, such as cardiac hypertrophy and failure, the reduced availability of NO is chronic, rather than transient (7, 8). Prior studies in NOS-knockout mice explored cardiac mechanical function in vivo (2); however, energy metabolism could not be determined in parallel, and the activity of enzymes of intermediary metabolism has not been assayed in myocardial tissue. Only one study from our group showed a marked enhancement in glucose uptake, reversed by infusion of exogenous NO and cGMP, in hearts isolated from endothelial NOS-knockout mice (26). In the present study, by employing three different isotopes to label the main cardiac substrates in vivo, we found a normal rate of FFA oxidation, whereas glucose oxidation was higher than in the control group, indicating that, in contrast to acute NOS inhibition, a prolonged reduction in NO availability causes a selective alteration in carbohydrate metabolism. The higher basal rate of glucose oxidation met the ~30% increase in MV02 at rest compared with control dogs. We previously showed that 10 days of systemic NOS inhibition causes an increased MV02-to-LV work ratio in the heart, i.e., lowers LV mechanical efficiency and leads to the same hemodynamic adaptations that we describe in the present study (14). We now provide evidence that, consistent with a higher O2 extraction,
hearts subjected to a prolonged period of NO deprivation display an elevated energy turnover fueled by higher rates of glucose and lactate oxidation. In this regard, it is noteworthy that the ATP-to-O₂ ratio is higher for glucose and lactate than for fatty acid oxidation (24).

To test whether LV stress could unmask an additional alteration in FFA oxidation, we infused ANG II to mimic the hemodynamic state that typically follows acute NOS blockade. This intervention led to two interesting phenomena: 1) Coronary blood flow increased in control, but not in L-NAME-treated, dogs. This could be due to the prolonged loss of NO synthesis that reduced coronary reserve or to an altered match between metabolic demand and blood flow supply. 2) We found a significant decrease in FFA oxidation at elevated blood pressure, although part of it was likely attributable to the reduced arterial concentration of this substrate. As mentioned above, less O₂ consumption is required for a given amount of ATP synthesis in carbohydrate than in fatty acid oxidation, which could explain why MV̇O₂ did not increase significantly, as in the control group, in response to higher cardiac workloads. Endogenous NO is known to be a reversible inhibitor of mitochondrial respiration (3); therefore, it is possible that the enhanced utilization of glucose could be an adaptive mechanism to limit the inefficiency of hearts subjected to a prolonged period of NO deprivation. On the other hand, the systemic administration of exogenous NO could not reverse the metabolic alterations caused by 10 days of NOS inhibition, although the effect of the NO donor diethylamine nonoate on hemodynamics was so strong as to require a four- to fivefold increase in the rate of ANG II infusion to readjust arterial blood pressure. Cardiac metabolic alterations did not revert, on the other hand, the systemic administration of exogenous NO could not reverse the metabolic alterations caused by 10 days of NOS inhibition, although the effect of the NO donor diethylamine nonoate on hemodynamics was so strong as to require a four- to fivefold increase in the rate of ANG II infusion to readjust arterial blood pressure. Cardiac metabolic alterations did not revert, in our previous findings in dogs with acute NOS blockade (16). Evidently, a short-term increase in NO availability was not sufficient to suppress the rate of carbohydrate oxidation potentiated by long-term mechanisms.

Biochemical analysis of tissue biopsies suggests that chronic NOS inhibition upregulates constitutive glucose transport and pyruvate oxidation. Myocardial glucose uptake is determined by the transporters GLUT-1, which is constitutively active, and GLUT-4, which is recruited in response to insulin or stress (4, 18). The protein expression of GLUT-1 was increased 46% after sustained NOS inhibition. Moreover, plasma insulin levels were not affected by L-NAME administration or by ANG II and ANG II + NO donor infusion, remaining within the typical range previously found by us in dogs after overnight fasting. Taken together, these findings indicate that the increased cardiac glucose consumption was not caused by elevated insulin levels. However, we cannot exclude changes in insulin sensitivity as found by other authors in skeletal muscle of rats with chronic NOS blockade (1) or of endothelial and neuronal NOS-knockout mice (22). The oxidative glycolytic pathway was also potentiated by a 33% increase in protein expression and an 80% increase in the active, dephosphorylated form of PDH, the enzyme that catalyzes the rate-controlling, key irreversible step in carbohydrate oxidation (23). Whereas the active form of PDH reflects the enzyme activity in vivo, the total activity corresponds to the catalytic reserve available during augmented metabolic demand. Total PDH activity was significantly increased 40%, likely accounting for the dramatic increase in glucose and lactate oxidation during elevations in LV afterload induced by ANG II. PDH is under the inhibitory control of PDK-4; however, the protein expression of PDK-4 was not significantly different between the two groups. Finally, protein expression, but not activity, of GAPDH was significantly reduced in dogs with NOS inhibition, suggesting a complex regulatory cross talk between these two enzymes. We previously described a similar paradoxical alteration, inconsistent with increased glucose consumption, in the failing heart (9).

Several limitations of the present study should be pointed out. 1) We could not determine possible alterations in intracellular signaling pathways, e.g., NO-Akt-dependent regulation of substrate metabolism, which could mediate the metabolic changes induced by sustained NOS blockade. Such mechanistic studies are best made in more controlled experimental systems in vitro, not in conscious large animals. 2) The comparisons between the two groups in the activation of PDH.
are limited, because the l-NAME group was treated with an NO donor, whereas the control group was not. For ideal assessment of the effects on PDH, the study design should have included an additional group of dogs that were chronically treated with l-NAME but not the NO donor. Despite this limitation, the higher total PDH activity and expression indicated an augmented carbohydrate oxidative capacity of the NOS-blocked heart. 3) Different types of acute stress, e.g., exercise or dobutamine infusion, may have given results different from those obtained with ANG II.

In conclusion, a sustained reduction in cardiac NO synthesis leads to a long-term potentiation of the carbohydrate oxidative pathway, which could constitute, at least in part, a compensatory mechanism to limit O2 consumption in a less efficient heart. Whereas previous studies showed reversible alterations in substrate consumption in response to acute changes in NO bioavailability, the present findings indicate that a prolonged reduction in NO synthesis induces a sustained alteration of cardiac metabolic phenotype.

GRANTS
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