Cardiac impairment and nitric oxide synthase activity in the chronic portal vein-stenosed rat

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Battarbee, Harold D., James H. Zavec, Matthew B. Grisham, Ronald E. Maloney, L. Judson Chandler, John W. Mercer, J. R., and Francois M. Cady. Cardiac impairment and nitric oxide synthase activity in the chronic portal vein-stenosed rat. Am. J. Physiol. 276 (Gastrointest. Liver Physiol. 39): G363–G372, 1999.—Decreased cardiac contractility and β-adrenergic responses have been observed in the chronic portal vein-stenosed (PVS) rat. Because nitric oxide (NO) may be increased in PVS and has been recognized as a negative inotropic agent, we investigated the induction of NO synthase (NOS2) and/or changes in constitutive NOS (NOS3) as factors in the cardiac dysfunction of the PVS rat. Ten to twelve days after portal vein stenosis or sham operation, cardiac function was evaluated in paced left ventricular papillary muscles (LVP) and right ventricular strips (RV). To determine if NO modulation of contractile function was altered in PVS, we examined the increase in developed tension produced by the effect of N-nitro-L-arginine (L-NNA) on the myocardial force-frequency relationship. Cardiac tissue NOS2 and NOS3 activities were assayed. Western blot analyses of NOS2 and NOS3 expression were performed, and circulating nitrate-nitrite (NOX) levels (an indicator of in vivo NOS activity) were assayed. Basal LVPm and RV contractile indexes were significantly reduced in PVS (30–50%), without a change in the relaxation rate. No between-group differences in the cardiac NOS2 or NOS3 enzymatic activities of PVS and sham-operated (SO) rats were observed. Western blots revealed no cardiac NOS2 expression in either SO or PVS rats. In contrast, NOS3 was expressed in both SO and PVS rats, but there was no quantitative difference in expression between the two groups. Changes in the cardiac force-frequency relationship (staircase effect) after L-NNA were consistent with NOS3 modulation of contractile function in both SO and PVS rats, but there was no between-group difference in the modulation. Circulating NOX concentrations did not differ between SO and PVS rats. In conclusion, protein expression with NOS3 modulation of contractile function in both SO and PVS rats, but there was no between-group difference in the

ALTERED HEMODYNAMICS, characterized by a hyperdynamic circulation with a decreased total peripheral resistance and an increased cardiac output, is one of the major sequelae of liver disease (16, 42). Recent studies in the carbon tetrachloride-induced cirrhotic rat (10, 43, 44), the chronic portal vein-stenosed (PVS) rat (32, 38, 43, 48), and in cirrhotic humans (16, 53) suggest that excess nitric oxide synthase (NOS) activity contributes to the circulatory changes associated with liver disease and portal hypertension. Circulating concentrations of endotoxin and cytokines that induce NO synthase have been reported to be elevated in cirrhotic patients, experimental cirrhotic models, and in the chronic PVS model. Intravenous administration of NO inhibitors such as Nω-monomethyl-L-arginine (L-NMMA) or Nω-nitro-L-arginine (L-NNA) increases peripheral resistance, mean arterial blood pressure, and splanchnic vascular resistance, decreases cardiac output, and restores responsiveness to pressor agonists (32, 45, 48). In addition to reducing the total peripheral vascular resistance, NOS activity may also be involved in altering myocardial contractility in liver disease. Both clinical and experimental research has established that liver disease can lead to impaired basal cardiac function, compromised contractile responses to cardiac preload and afterload, and attenuated chronotropic and inotropic responses to β-adrenergic receptor activation (6, 21, 33, 34, 58). As in the peripheral vasculature, two types of NOS activity have been described in cardiac muscle cells: a constitutive Ca2+-dependent isoform (NOS3) and a Ca2+-insensitive inducible isoform (NOS2). Expression of NOS2, which has a high capacity for NO production in cardiac muscle, has been associated with a persistent decrease in contractile force and attenuated β-sympathomimetic responsiveness (1, 3, 47). In these studies, myocardial cGMP and plasma nitrate-nitrite (NOX) levels were increased in parallel with the induction of NOS2, and pretreatment with dexamethasone (an inhibitor of NOS2 induction) prevented these changes (46).

The physiological role of the Ca2+-dependent isoform in cardiomyocytes is less clear. In isolated hamster papillary muscles, L-NMMA blocks the immediate negative inotropic effects of proinflammatory cytokines (12). More recently, it has been shown (26) that NOS3 modulates the force-frequency relationship (staircase effect) in rat papillary muscles. NOS3 activity is apparently modulated by Ca2+ concentrations within the normal cytosolic range during excitation-contraction coupling (26). It has been hypothesized that NOS3 modulates contractile function by acting as a countervailing negative inotropic signal in response to factors, such as β-adrenergic agonists, that increase L-type Ca2+ channel currents and the intracellular Ca2+ concentration (2). Taken together, this evidence suggests a

endotoxin; cirrhosis; liver disease; heart; constitutive nitric oxide synthase; calcium; contractility
causal relationship between NOS activity and myocardial dysfunction in liver disease. Because of our previous observations of impaired cardiac contractility and β-adrenoceptor responses in the chronic PVS rat (4, 58), we have investigated the hypothesis that augmented myocardial NOS2 and/or NOS3 activity depresses cardiac function in the PVS rat.

METHODS

Portal vein stenosis. Portal venous hypertension was produced by calibrated constriction of the hepatic portal vein. After fasting overnight, male Sprague-Dawley-derived rats (275 ± 50 g) were anesthetized with ketamine (100 mg/kg), a laparotomy was performed, the common portal vein was dissected free of surrounding tissue, and a ligature of 4-0 silk was placed around the vein. A blunt 22-gauge hypodermic needle was placed alongside the vein, and the ligature was tied snugly to the needle and vein. The needle was then removed, leaving a calibrated stenosis of the common portal vein. Sham-operated (SO) animals were treated similarly in that their common portal vein was exposed and freed from connective tissue, but their portal vein was not stenosed. The abdomen was closed in layers with 4-0 silk. Meperidine hydrochloride (1.25 mg sc) was immediately given to obtund any pain that might occur on recovery. Procaine penicillin G (275,000 U im) and dihydrostreptomycin (25 U im) were administered prophylactically against infection. When required, additional doses of meperidine were administered. Animals were caged individually and given water and food ad libitum until cardiac studies were conducted 10–12 days after surgery. Rats were randomly assigned to each respective group before surgery. Previous measurements have confirmed that this method leads to reproducible portal hypertension (14–17 mmHg) with extensive portosystemic shunting (>90%) (4, 58). By the end of each experiment, a graticule was used to measure the width and length of each tissue. Tissues were dried, and their weights recorded. These measurements were used to normalize the data for differences in tissue weight and dimensions. The best dimensional correlate for force development is cross-sectional area. For example, long and thin tissue specimens generate less force than short thicker specimens, even though their masses might be identical. Each tissue's mean cross-sectional area was calculated on the basis of muscle length at maximal basal developed tension (DT) and postexperimenal dry weight assuming a tissue density of 1.06 (19).

In the present study, contractile function of rat left ventricular papillary muscles and right ventricular strips was well maintained in accordance with the protocol described by Finkel and colleagues (12, 13) have demonstrated that when assessing force development (negative staircase effect). Finkel and colleagues (12, 13) have demonstrated that when assessing force development (negative staircase effect). Finkel and colleagues (12, 13) have demonstrated that when assessing force development (negative staircase effect). Finkel and colleagues (12, 13) have demonstrated that when assessing force development (negative staircase effect). Finkel and colleagues (12, 13) have demonstrated that when assessing force development (negative staircase effect). Finkel and colleagues (12, 13) have demonstrated that when assessing force development (negative staircase effect). Finkel and colleagues (12, 13) have demonstrated that when assessing force development (negative staircase effect). Finkel and colleagues (12, 13) have demonstrated that when assessing force development (negative staircase effect).
maintained for periods of 3–4 h; time-matched control tissue force development declined only ~5% when paced continuously at 0.5 Hz. When subjected to the force-frequency protocol used in the present study (0.5–5 Hz), time-matched control force development for two right ventricular tissues and two left ventricular papillary muscles declined 3.1, 2.5, 12.5, and 13.6%, respectively.

Measurement of NOS2 and NOS3 activity. NOS3 and NOS2 activities were measured in the combined left and right ventricular tissue from SO and PVS rats. Only the left ventricles of lipopolysaccharide (LPS)- and saline-injected rats (see Effect of endotoxin below) were assayed. On the day of the assay, the hearts were thawed, atra and major blood vessels were removed, and the tissue was homogenized using a Polytron homogenizer in ice-cold buffer (pH 7.2 at 20°C) containing (in mM) 320 sucrose, 10 HEPES, 0.1 EDTA, 1 dithiothreitol, 10 µg/ml leupeptin, 10 µg/ml soybean trypsin inhibitor, and 2 µg/ml aprotinin. The homogenate was centrifuged at 100,000 g for 30 min. The pellet was discarded, and the cytosolic fraction was placed on ice for immediate assay of NOS activities. Reactions were terminated by addition of 0.1 M HCl followed by addition of 0.1% BSA. After this incubation, the membranes were washed as described above, and the antigen-antibody-peroxidase complex was detected by enhanced chemiluminescence (Amersham) according to the manufacturer’s instructions and visualized by exposure to Amersham Hyperfilm. Film autoradiograms were analyzed and quantified by computer-assisted densitometry using a Bio-Rad molecular imaging system.

Gel electrophoresis and immunoblotting. For determination of NOS2 and NOS3 protein levels, left and right ventricles from SO, PVS, saline-injected controls, and LPS-injected rats were trimmed of their atria and great vessels, snap frozen, and transferred to liquid nitrogen for storage. Subsequently, the frozen tissues were mechanically pulverized on dry ice, the frozen tissue fragments placed in 2% SDS, probe sonicated, and boiled for 5 min. After centrifugation to remove any insoluble material, an aliquot was removed for determination of protein concentration by the bicinchoninic acid assay (Pierce, Rockford, IL) and a larger aliquot diluted with an equal volume of 2× electrophoresis sample buffer [final concn = 50 mM Tris·HCl (pH 6.7), 4% glycerol (wt/vol), 4% SDS, 1% 2-mercaptoethanol, and bromphenol blue (0.02 mg/ml)]. The 2× sample buffer contained 6% SDS to provide the final SDS concentration of 4%. Proteins were separated by size on a 7.5% SDS-polyacrylamide gel, using the buffer system of Laemmli (30) and transferred to polyvinylidene difluoride membranes in Towbin-SDS transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, and 0.01% SDS). After transfer, the blots were washed with PBS containing 0.05% Tween 20 (PBST) and blocked in PBST containing 5% nonfat dried milk (NFDM; Carnation) and 1% BSA for 24 h at 4°C with gentle agitation. The membrane was washed once with PBST and incubated with primary antibody diluted (NOS2, 1:10,000; NOS3, 1:2,500) in PBST containing 0.5% NFDM and 0.1% BSA for 1 h. Monoclonal NOS3 antibody and polyclonal NOS2 antibody were purchased from Transduction Laboratories (Lexington, KY). The membrane was then washed once for 5 min, once for 15 min, and then twice for 5 min in PBST followed by a 1-h incubation with agitation at room temperature with horseradish peroxidase-conjugated horse anti-mouse (NOS3) or anti-rabbit (NOS2) immunoglobulin G diluted 1:2,000 in PBST containing 0.5% NFDM and 0.1% BSA. After this incubation, the membranes were washed as described above, and the antigen-antibody-peroxidase complex was detected by enhanced chemiluminescence (Amersham) according to the manufacturer’s instructions and visualized by exposure to Amersham Hyperfilm. Film autoradiograms were analyzed and quantified by computer-assisted densitometry using a Bio-Rad molecular imaging system.

Effect of endotoxin. Endotoxin was injected into a separate set of rats, and these served as a positive control for the cardiac NOS2 activity assays, Western blots, contractility experiments, and circulating NOx assays. Male Sprague-Dawley rats (300 g) were fasted for 36 h to reduce plasma nitrates and nitrites derived from the diet. Eight rats were injected with pyrogen-free saline (ip) and another eight rats were injected (ip) with 4 mg/kg phenol-extracted LPS from S. typhosa (Sigma Chemical) and placed in individual cages. Six hours later, four saline controls and four LPS-treated rats were anesthetized with ketamine (100 mg/kg), a laparotomy was performed, and blood (1.5 ml) was collected from the inferior vena cava, portal vein, and aorta. Plasma was separated and stored at –20°C until assayed for nitrites. Immediately after the withdrawal of blood, the heart was quickly excised and rinsed in ice-cold saline. The left and right ventricles were dissected free, tissues harvested for contractility studies, and the remaining tissue was frozen in liquid nitrogen and stored at –70°C until assayed for NOS activities. After an additional 6 h, this procedure was repeated using the remaining controls and LPS-injected rats.

Statistics. Because there were only two treatment groups of animals, ANOVA was used as an omnibus screening test followed by the appropriate univariate t-test to evaluate the data. The Mann-Whitney U test was used when a distribution-free statistical procedure was required, i.e., normalized force-frequency data (51). All values are expressed as means ± SE. P < 0.05 was considered statistically significant.

RESULTS

Basal contractile and relaxation functions. Table 1 shows basal force development and relaxation data.
acquired at a pacing frequency of 2 Hz in right ventricular strips and left ventricular papillary muscles from SO and PVS rats. Data were obtained from several animal groups over the past 2 years. Basal DT and dT/dt in right ventricular strips and left ventricular papillary muscles were reduced 30–50% in PVS compared with SO rats. Stenosis did not significantly affect −dT/dt. The tissue dimensions used for the normalization of the contractile data are given in Table 2.

Cardiac inotropic and lusitropic responses to pacing. A negative staircase effect or negative force-frequency relationship has been described repeatedly for the rat and hamster heart (12, 13, 31, 40, 47, 50). In the current studies, when the pacing frequencies of SO and PVS cardiac tissues were incrementally increased, there was an immediate and transient increase in force development at frequencies ≥2 Hz compared with the force development immediately preceding the increment (positive staircase effect). This increase was followed by decreased force development (negative staircase effect). To determine the effect of NOS3 on DT, the immediate peak in force development was used as the response for a given pacing frequency. Choosing the immediate response after an increment in frequency and comparing it with the preceding stable tension development value permitted the assessment of NOS3 modulation of contractile function at each pacing frequency and minimized any compensatory changes in sarcoplasmic reticulum Ca²⁺ transport that might interfere with observing Ca²⁺-dependent NOS3 activity (13).

No between-group differences were found in force development of SO and PVS rats in either left ventricular papillary muscles or right ventricular strips at pacing frequencies between 0.5 and 5.0 Hz before L-NNA. L-NNA did not affect force development at lower pacing frequencies (<2 Hz). As Fig. 1 demonstrates, at pacing frequencies above 2 Hz, force development in left ventricular papillary muscles (Fig. 1A) and right ventricular strips (Fig. 1B) significantly increased after L-NNA treatment in both groups. However, SO and PVS rats exhibited no between-group differences at any pacing frequency after L-NNA.

The effect of pacing frequency on relaxation of left ventricular papillary muscles and right ventricular strips in SO and PVS rats was also determined. The relaxation rate was not significantly affected by portal vein stenosis, and L-NNA did not alter the frequency-relaxation curves (Fig. 2). Similar effects were observed in right ventricular strips (data not shown).

Table 2. Isolated RV and LVPM dimensions

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<th>Length, mm</th>
<th>Width, mm</th>
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<tr>
<td>RV</td>
<td>12.0 ± 0.9</td>
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<td>LVPM</td>
<td>5.1 ± 0.5</td>
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Values are means ± SE.

Cardiac NOS activity. The ventricular NOS2 and NOS3 activities of SO and PVS rats were measured. These enzymatic assay results are shown in Table 3. There were no significant differences in the activities of the constitutive and inducible NOS isoforms between the two treatment groups. To verify the assays, NOS3 and NOS2 activities were measured 6 h after LPS or vehicle injections in normal rats. LPS induced a 760% increase in left ventricular NOS2 activity (P < 0.05),

![Figure 1](http://ajpgi.physiology.org.org/) Effects of pacing rate on force development in isolated left ventricular papillary muscles (A) and right ventricular strips (B) from sham-operated (SO) and portal vein-stenosed (PVS) rats before (open symbols) and after L-nitro-L-arginine (L-NNA) (filled symbols). Values are means ± SE (n = 6). Where SE values are not shown, the values fell within the treatment group symbol.
but no change in NOS3 activity was found compared with saline-injected controls.

Expression of NOS2 and NOS3 proteins. Western blots of the left and right ventricular tissues of SO and PVS rats detected no NOS2 protein expression in either group. However, tissues from LPS-injected rats were positive for NOS2 expression (Fig. 4). In contrast to NOS2 expression, NOS3 expression was readily observed in all tissues. However, densitometry readings for SO left and right ventricles (48 ± 9 and 52 ± 5, respectively; n = 4 for each group) and PVS left and right ventricular tissues (51 ± 13 and 56 ± 6, respectively; n = 4 for each group) did not differ significantly. In hearts from LPS-treated rats, the increase in NOS2 was associated with a decline in NOS3 expression. For example, in Fig. 4 compare the bands representing left ventricular tissue after LPS treatment with the corresponding bands from SO and PVS rats.

Circulating NOX. Table 4 compares aortic, portal vein, and inferior vena caval NOX concentration in SO and PVS rats. No differences in plasma NOX levels within or between groups were observed. On the other hand, LPS injections increased the mean inferior vena caval NOX concentration 950 and 1,300% at 6 and 12 h, respectively.

DISCUSSION

NO generation by a constitutive, Ca2+-dependent NOS plays an important role in the control of normal blood pressure, regional blood flow regulation, and the modulation of cardiac contractility (26, 47). In addition to a constitutive enzyme, cytokines (TNF-α and IL-1β) and endotoxin induce a Ca2+-independent NOS in endothelial cells, vascular smooth muscle cells, and cardiomyocytes. Expression of this enzyme leads to sustained vasodilation and hypersresponsiveness to vasoconstrictor agonists, and, in cardiomyocytes, persistent

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<th>Enzyme</th>
<th>Left and Right Ventricular NOS</th>
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<tr>
<td></td>
<td>SO</td>
<td>PVS</td>
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<tr>
<td>NOS3</td>
<td>2.22±0.31</td>
<td>2.23±0.37</td>
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<tr>
<td>NOS2</td>
<td>0.11±0.06</td>
<td>0.17±0.14</td>
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<td>n</td>
<td>10</td>
<td>9</td>
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Values are means ± SE. Enzymatic activity is expressed as pmol·min⁻¹·mg protein⁻¹ of [14C]citrulline formed. Lipopolysaccharide (LPS) was given at a dose of 4 mg/kg. Constitutive nitric oxide synthase (NOS3) and inducible NOS (NOS2) activities were measured 6 h after saline or LPS injection in normal rats. *P < 0.05 compared with saline-injected animals.
increases in the inotropic state and attenuated responses to β-adrenergic agonists (3, 26, 46).

Elevated NOS activity is thought to be an important contributor to the altered hemodynamics observed in liver disease (53). Because NO acts only locally and is not a circulating hormone, NOS-mediated hemodynamic alterations are hypothesized to be due to widespread NOS2 induction by some as yet unidentified factor(s), possibly endotoxin or cytokines. Chronic liver disease and/or portosystemic shunting are known to allow substances from the intestine to escape hepatic inactivation and enter the systemic circulation, and endotoxin and cytokine levels are elevated in some hepatic patients (53) and, reportedly, in the PVS and cirrhotic rat models of liver disease (9, 11). Observations that NOS inhibition increases the blood pressure, total peripheral resistance, and splanchnic vascular resistance while decreasing cardiac output and restoring splanchnic and peripheral responsiveness to pressor agonists in hepatic models are consistent with the hypothesis that induced NOS activity is responsible for the altered hemodynamics (10, 32, 45, 48). This hypothesis, however, is not unequivocally supported by the evidence (11, 24, 27). Recent evidence suggests that NOS3 is at least partly responsible for the hemodynamic disturbances (8, 11, 14, 39, 43). When NOS2 induction is prevented with dexamethasone, a hyperdynamic circulation still develops after portal vein stenosis and NOS inhibition still reduces gastric blood flow (11). In addition, NOS3 activity and expression are increased in the aorta and superior mesenteric artery of cirrhotic and PVS rats (8, 43). Studies by Karatapanis et al. (24) suggest that upregulation of NOS3, instead of NOS2 induction, may be an important contributor in the vascular hyporesponsiveness observed in chronic portal vein stenosis. Western blots and RT-PCR studies in PVS and cirrhotic rats indicate that in both these hepatic models NOS3, not NOS2, appears to be the major determinant of vascular NO production (39, 43). Despite these studies indicating increased NOS2 and NOS3 activities in liver disease, and despite reports of associated cardiac impairment, there have been no studies conducted on the role of NOS isoforms in the altered cardiac performance that has been observed.

Although at least three isoforms of NOS have been described in mammalian tissues, only NOS2 and NOS3 are expressed in the adult rat heart (44). Activation of either of these isoforms decreases basal force development and the positive inotropic response to β-adrenergic agonists (1–3, 13, 26). Several groups have shown that NOS2 induction by cytokines decreases contractile performance in a manner that is reversible with NOS antagonists (3, 26, 52). Some of these effects occur far too rapidly for gene transcription and NOS2 expression and appear to result from enhanced activity of the constitutive NOS isoform in the myocardium (12). cDNA hybridization, RT-PCR, immunohistochemical detection, and Western blot studies have shown that under normal circumstances NOS3 is readily detectable in rat cardiomyocytes (2, 26). In contrast, NOS2 is not normally detectable but is readily detected after pretreatment with endotoxin (28) and the proinflammatory cytokines IL-1β and TNF-γ (26). Recently, it has also been demonstrated that NOS3 modulates the cardiac force-frequency relationship in the normal rat cardiomyocyte. Increases in enzymatic activity were associated with decreased force generation as the pacing frequency was increased (26). Intracellular Ca2+ measurements using fura 2 have associated increasing pacing rates with progressive increases in systolic and diastolic Ca2+ and greater force generation. This larger force generation at increased pacing rates occurs concomitantly with increased NO production and intracellular cGMP. In these studies, nitrate and cGMP release by paced cells could be attenuated by treatment with an intracellular Ca2+ chelator [1,2-bis(2-aminophenoxy)-ethane-N,N,N′,N′-tetraacetic acid] or NOS inhibition with arginine analogs. Methylene blue, nitro-arginine, and LY-83583 (a guanylyl cyclase inhibitor) all increased the amplitude of myocyte shortening at paced frequencies >3 Hz (26). Thus, at pacing frequencies encompassing the range of the normal rat heart rate, myocyte contractile function appears to be modulated by the cytosolic Ca2+ concentration, activation of the Ca2+-dependent NOS isoform, and cGMP generation (26).

In previous studies, our laboratory demonstrated that right ventricular and left ventricular contractile function is reduced by 40–60% in chronic PVS rats (4, 58). In addition, inotropic responses to β-adrenoceptor agonists are decreased an average of 50–60%, apparently through effects at postreceptor sites (4, 58). Because NOS activity is thought to be increased in portal vein stenosis and because these enzymes modulate cardiac contractile force development, we have extended our studies to include the roles of NOS isoforms in the cardiac depression associated with chronic portal vein stenosis. The possibility that increased NOS activity might be involved was especially

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<th>Treatment Group</th>
<th>Saline</th>
<th>LPS</th>
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<tr>
<td>Aorta</td>
<td>SO 16.3±1.2</td>
<td>17.4±2.4</td>
<td>176.4±24.8*</td>
</tr>
<tr>
<td></td>
<td>PVS 15.4±1.0</td>
<td>18.2±2.8</td>
<td>193.0±36.1*</td>
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<tr>
<td>Portal vein</td>
<td>14.5±1.1</td>
<td>18.3±2.5</td>
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<td>Inferior vena cava</td>
<td>14.5±1.1</td>
<td>18.3±2.5</td>
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Values are means ± SE in μmol/l of circulating nitrate-nitrite (NOX) concentration. LPS was given at a dose of 4 mg/kg. * P < 0.005 compared with saline controls.
of which can affect cytosolic $\text{Ca}^{2+}$

stenosis on the circulating NOX concentrations and has been shown, using intraperitoneally injected and SO rats at the time of maximal Na$^{+}$/Ca$^{2+}$ exchange (36), and sarcolemmal ATP-dependent Ca$^{2+}$ transport (55), each of which can affect cytosolic Ca$^{2+}$ and ventricular contractile function.

In the present study, the effect of chronic portal vein stenosis on the circulating NOX concentrations and constitutive and inducible NOS activities was assessed. The plasma NOX concentration has been shown to be a sensitive indicator of in vivo NOS activity (14, 55, 56), and our LPS experiment confirmed this. LPS injections dramatically increased circulating NOX levels. However, NOX levels in SO and PVS rats were not different in inferior vena caval, portal venous, or aortic blood, suggesting that NOS activity is not altered 10–12 days after portal vein stenosis. There have been only two other reports of circulating NOX levels in PVS rats: a study by Hori et al. (20) and one by Murakami and colleagues (41). Murakami et al. (41) reported no difference between circulating and urinary NOX concentrations in PVS and SO rats at the time of maximal Na$^{+}$ retention; thus, their data are consistent with our own. In contrast, the findings by Hori et al. (20) showed increased urinary and circulating NOX 10–12 days after portal vein stenosis. Because circulating NOX levels were also measured 10–12 days after stenosis in our study, differences in the time frame of the study cannot explain the incongruity of the data. Interestingly, in the study by Hori et al. (20), a correlation was found between circulating and urinary NOX and the amount of portosystemic shunting. This positive correlation suggests that shunting of visceral venous blood contributed to the increased NOX levels they observed (20) (portosystemic shunting exceeds 90% in this model). In our study, rats were fasted for 36 h before blood samples were collected, whereas the rats used in the studies by Hori et al. (20) and Murakami et al. (41) were not fasted beforehand. The action of intestinal flora on NOX concentrations is significant (57), and prolonged fasting or antibiotic treatment significantly reduces these levels. Perhaps these differences can be explained by the shunting of NOX-rich visceral blood into the systemic circulation of fed PVS rats.

Differences in renal function may also contribute to the elevated circulating NOX levels in the PVS rat. It has been shown, using intraperitoneally injected $^{15}$Na$^{15}$NO$_2$, that 60–70% of $^{15}$N is excreted in the urine in the rat, the main metabolites being $^{15}$NO$_2$ and $^{15}$N urea (54). Thus, the major route for the excretion of vescicularly derived NOX is via the urine. After portal vein stenosis, the renal glomerular filtration rate and filtration fraction are reduced ~30%, at a time that coincides with maximal Na$^{+}$ retention and maximal vasodilation (41). Although renal function beyond the initial few postoperative days has not been systematically assessed in PVS rats, there is good evidence it is affected. Renal blood flow is comparable to controls at postsurgery day 20, and renal resistance is reduced 30% (49). Collectively, these studies suggest that the elevated NOX levels observed in portal vein stenosis are more complex than a simple increase in NOS activity and that circulating and urinary levels may be affected by the intestinal flora and fasting, portosystemic shunting, and/or renal function. Ours is the only report to date in the PVS rat in which an attempt was made to minimize the contribution of the intestinal flora before assessing circulating NOX.

It might seem perplexing that there have been several hemodynamic studies that suggest NOS activity is increased in the chronic PVS rat, whereas our circulating NOX data indicate NOS activity is not affected. There have, however, been a number of other hemodynamic studies that indicate NOS activity is not involved (11, 23, 43). In most of these conflicting reports, investigators have focused on hemodynamic and vascular changes after pharmacological NOS inhibition with arginine analogs. Endogenous NO production or its metabolites were almost never measured. Several possibilities come to mind that might explain these seeming incongruities. 1) PVS rats may be more sensitive to the effects of NO. Heinemann and Stauber (18) have demonstrated that the PVS rat’s visceral vascular bed, in which blood flow is increased 40%, is more sensitive to the vasorelaxive effects of NO. If vascular bed resistance vessels are indeed more sensitive to NO, then an NOS-dependent hyperdynamic circulation could develop without changes in the circulating NOX concentration or in NOS expression. This observation may also explain why different vascular beds may be vasodilated either with or without an increase in NOX expression in the stenosed model. A heterogenous pattern of vascular tissue NOS3 expression has been demonstrated in portal vein stenosis (23, 43). 2) When NO or its metabolites from a discrete vascular bed with increased NOS activity are diluted in the systemic circulation, NO production differences may not be obvious. 3) Increased NOS activity may not be responsible for the hyperdynamic circulation. The role of NOS in the hyperdynamic circulation is a contentious one. There have been several hemodynamic studies that have suggested a role for NOS in the PVS rat’s hyperdynamic circulation (23, 32, 45). Other investigators, however, have found no such relationship (11, 23, 43). In some studies, no between-group differences in hyperemic tissue NOS3 or NOS2 levels have been found compared with suitable controls (11). Still others have observed that visceral blood flow changes in response to NOS inhibition are less in the stenosed rat (22) or that NOS inhibition increases vascular bed responses to vasoconstrictor agonists in both controls and stenosed animals but does not change the response differences between the groups, suggesting that NOS is not involved in the hyperresponsiveness to pressor agonists (27). These observations have been interpreted by some to indicate that NOS plays an important role in splanchnic circulatory control, but its effect...
on the hyperdynamic circulation in portal vein stenosis is insignificant (22).

Measurement of cardiac NOS3 enzymatic activity in the present study demonstrated constitutive activity in both SO and PVS rats, but no between-group difference in activity was evident. NOS2 enzymatic activity was not detectable in either group. These observations suggest that changes in NOS activity are not responsible for the cardiodepression we have observed in the chronic PVS rat. However, because NOS3 is a Ca$^{2+}$-dependent isoform and enzymatic assay conditions were optimized for maximal activity, the observation of no change in NOS3 activity with portal vein stenosis does not preclude the possibility that Ca$^{2+}$-dependent differences in cardiomyocyte NOS3 activity might exist between treatment groups. To test this hypothesis, a functional NOS assay was conducted using paced cardiac tissues. In the normal cardiomyocyte, changes in the pacing rate have been associated with frequency-dependent changes in cytosolic Ca$^{2+}$ concentrations, changes in NOS3 activity, and an NOS3-dependent decrease in contractile function. Thus the pacing frequency can be used to manipulate the cytosolic Ca$^{2+}$ concentration and therefore NOS3 activation (31, 40). Using this functional assay, we compared pacing frequency-induced changes in the cardiac contractility of SO and PVS rats, using right ventricular strips and left ventricular papillary muscles before and after NOS inhibition with L-NNA. No between-group differences were observed at lower pacing frequencies (0.5–1.0 Hz), and NOS inhibition with L-NNA did not affect tension development. At higher pacing frequencies, however, tension development significantly increased after L-NNA (Fig. 1). These inotropic changes are consistent with observations by others (13, 26) that Ca$^{2+}$-dependent NOS activity affects cardiac contractile performance in the rat. The absence of a difference between SO and PVS rats before and after NOS inhibition indicates that the degree of NOS3 modulation of cardiac contractile function is not different in these two groups.

When the effects on cardiac relaxation were examined, no frequency-dependent differences were found between SO and PVS rats and incubation with L-NNA did not significantly alter the lusitropic state (Fig. 2). In some cardiac anomalies, changes in myocardial relaxation properties have been associated with altered sarcoplasmic reticulum Ca$^{2+}$ transport, Ca$^{2+}$-ATPase isoform shifts, and alterations in the sarcoplasmic reticular membrane architecture and density of the Ca$^{2+}$ pump molecules (25). Because no between-group differences in lusitropism were observed and since L-NNA did not affect myocardial relaxation in either group, these data suggest that chronic portal vein stenosis does not affect any of the aforementioned lusitropic factors and that NOS plays no modulatory role in the relaxation process.

To further test the hypothesis that differences in NOS activity are responsible for the cardiac impairment observed in portal vein stenosis, we examined the expression of NOS2 and NOS3 proteins in left and right ventricular tissues, using a Western blot technique. No evidence of inducible NOS protein expression was found in either SO or PVS rats. In contrast to NOS2 expression, NOS3 expression was readily observed in both left and right ventricular tissues. However, cardiac NOS3 expression in PVS rats did not differ significantly from that found in SO rats. LPS-injected rats, which were used as a positive control for NOS2, expressed the NOS2 protein. In addition, Western blots of NOS2 and NOS3 in LPS-injected rats displayed a reciprocal expression of proteins for the two isoforms. NOS2 protein expression was associated with a dramatic attenuation of NOS3 expression when immunoblots were compared with those of both SO and PVS rats. These results are consistent with the observations recently made in brain, lung, and heart by Liu et al. (37).

In conclusion, protein expression data, enzymatic assays, end-product assays, and functional data strongly suggest that between-group differences in NOS2 and NOS3 are not responsible for the impairment of cardiac function that we have observed in the chronic PVS rat. NOS2 protein expression in SO and PVS rats was below detection limits in both groups, and circulating NOX concentrations did not differ between treatment groups. Unlike NOS2, Western blots for NOS3 expression, NOS3 enzymatic assays, and contractility studies indicate that this isoform was expressed in both SO and PVS rats and that it does modulate cardiac contractile performance. However, no between-group differences in NOS3 expression, enzymatic activity, or contractile modulation could be discerned.

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