Role of nitric oxide in the coupling of myocardial oxygen consumption and coronary vascular dynamics during pregnancy in the dog

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Williams JG, Rincon-Skinner T, Sun D, Wang Z, Zhang S, Zhang X, Hintze TH. Role of nitric oxide in the coupling of myocardial oxygen consumption and coronary vascular dynamics during pregnancy in the dog. Am J Physiol Heart Circ Physiol 293: H2479–H2486, 2007. First published July 20, 2007; doi:10.1152/ajpheart.00036.2006.—We examined the ability of cardiac endothelial nitric oxide synthase (eNOS) to couple myocardial oxygen consumption (MV˙O2) and oxygen delivery during pregnancy. Awake dogs were studied using echocardiography before and at 40 days, 50 days, and 60 days (60D) of pregnancy and at ∼14 days postpartum. Left ventricular eNOS, phosphorylated eNOS, and copper, zinc-superoxide dismutase (CuZnSOD or SOD-1) were determined by immunoblotting. MV˙O2 of left ventricular tissue samples was measured in vitro in response to increasing doses of bradykinin, enalapril maleate, and amlodipine. We examined the changes in passive diameter and flow-dependant arteriolar dilation of coronary arterioles. Echocardiography indicated increases in cardiac output (∼60%) during pregnancy. Myocardial eNOS (21 ± 4%), phosphorylated eNOS (19 ± 3%), and SOD-1 (61 ± 2.7%) protein levels were significantly increased at 60D. Bradykinin, enalapril maleate, and amlodipine (10−4 mol/l) decreased MV˙O2 in a nitric oxide-dependant manner (by 24 ± 1.3% in controls and 34 ± 2.2% at 60D; by 21 ± 1.1% in controls and 29 ± 1.1 at 60D; and by 22 ± 2.5% in controls and 26 ± 1.0% at 60D, respectively). Arterioles from pregnant dogs showed increased flow-dependant dilation in response to increased shear stress and larger passive diameter. Nitrite production was stimulated by bradykinin and carbachol in microvessels in vitro; pregnancy enhanced nitrite release. Myocardial eNOS, phosphorylated eNOS, and SOD-1 protein expression are increased during pregnancy, and this increase is associated with enhanced nitric oxide-dependant control of MV˙O2. Thus increases in eNOS and SOD-1 promote the coupling of oxygen delivery and efficiency in the heart during pregnancy.

PREGNANCY IN HUMANS IS ASSOCIATED WITH an early rise in plasma volume, which is increased further in the second trimester and then increases only slightly further until delivery. The average increase in plasma volume is 40% over normal values (12). Along with this volume overload state, there is an increase in cardiac output (20). In humans, cardiac output increases an average of 40% (from 5 to 7 l/min) in women at rest in the supine position. The increase in output is detectable starting at week 5 and then levels off at around week 20. A portion of the increase in cardiac output is attributable to an increase in stroke volume (3, 6). By midpregnancy, the stroke volume has increased upward of 30%. The increase in cardiac output is at least partially achieved by an enhanced myocardial performance (9) and a relative tachycardia. The basal heart rates of women rise significantly by the 4th wk of pregnancy and reach a peak at the 36th wk (4).

The increase in cardiac output is associated with a decrease in total peripheral resistance (23), which tends to be greatest in midpregnancy. This decrease in peripheral resistance, along with the increase in cardiac output, results in no net change in mean arterial pressure. The role of nitric oxide (NO) during pregnancy in the control of systemic vasodilation has been previously identified (2, 5); however, the role of NO in the control of cardiac function and metabolism during pregnancy has not been fully determined.

Endothelial cells produce a variety of substances that influence physiological functions, including thromboxane A2, NO, and arachidonic acid metabolites. There have been numerous studies indicating that NO production may change during pathophysiological conditions such as heart failure (13, 22, 29). Endothelium-dependent vasodilatory responses to ACh are enhanced in arteries from pregnant guinea pigs (28). There are several disease states, including preeclampsia and postpartum cardiomyopathy, that occur during pregnancy that may represent a disregulation of NO production. Agatston et al. (1) have shown that that women with a history of preeclampsia exhibit impaired endothelial function up to 1 yr postpartum. Thus NO may also play a role in modulating local vascular resistance during pregnancy.

The maternal cardiac metabolic rate is greatly increased during pregnancy (14). The oxygen needs of the heart vary with heart rate, blood pressure, ventricular mass, and contractility. There is a large increase in cardiac work during pregnancy: a 33% increase in basal myocardial oxygen consumption (MV˙O2). This increase in MV˙O2 may be met partially by a small increase in extraction and an increase in coronary flow. Thus there may also be a role for endothelial nitric oxide synthase (eNOS) expression in the modulation of MV˙O2 and cardiac efficiency during pregnancy. For instance, NO has been shown to regulate the activity of a number of mitochondrial electron transport chain components, including complexes I, II, and IV, along with aconitase.

The aim of our study was to determine the role of eNOS-derived NO in the modulation of mitochondrial respiration in the hearts of pregnant dogs. We also examined the changes in vasodilation induced by increases in shear stress and whether this increase is due to enhanced release of NO. We hypothesized that 1) myocardial eNOS protein expression is increased during pregnancy, 2) stimulation of eNOS will result in a
greater NO-dependent control of MV˙O2 in hearts from pregnant dogs, 3) there will be an enhanced NO-dependent dilatation in coronary arterioles in response to increases in shear stress, and 4) NO production will be increased in coronary microvessels.

METHODS

Experiments in pregnant conscious dogs. Female mongrel dogs (n = 22) were acquired from the vendor in either pregnant or control states. Pregnancy was assessed by the institutional veterinarian and based on the breeding schedule supplied by the vendor. The normal gestational period of the dog is ~65 days. Echocardiographic readings were taken at 40 days, 50 days, 60 days, and ~2 wk postpartum. We used 14 days postpartum because that is the first time point when we observed no significant difference in hemodynamic variables between control and postpartum dogs. The dogs were killed by injection of pentobarbital sodium (50 mg/kg), and the heart was harvested. The protocols were approved by the Institutional Animal Care and Use Committee of the New York Medical College. They conform to the guiding principles for the use and care of laboratory animals of the National Institutes of Health and the American Physiological Society.

Echocardiography. All studies were performed with a dedicated echocardiography machine (Accuson Sequoia 256) with conscious dogs lying on a laboratory table. M-mode recordings were made from short-axis views, with two-dimensional guidance. All dogs were in sinus rhythm, and the mean measurements from several consecutive beats covering at least one respiratory cycle were used for data analyses. Left ventricular (LV) chamber dimensions and wall thickness were measured in a plane below the mitral valve and perpendicular to the LV in an M-mode recording. LV chamber volumes were assessed in a two-dimensional parasternal long-axis view (26). LV volumes were calculated with the semi-cylindrical, semi-ellipsoid model. Stroke volume was calculated as the difference between LV end-systolic volume and LV end-diastolic volume. Cardiac output was calculated as the product of stroke volume and heart rate. Myocardial wall stress (WS) is the product of intraventricular pressure (LVP), radius of curvature (R), and wall thickness (h); WS = LVP × R/h.

Western blot. The protein expression of eNOS, phosphorylated eNOS, inducible nitric oxide synthase (iNOS), superoxide dismutase (CuZnSOD or SOD-1), SOD-2, and nitrotyrosine were analyzed by Western blot using specific antibodies followed by detection. Tissue samples from each heart were obtained and flash frozen in liquid nitrogen. Protein (75 μg) was separated on a 7.5% SDS-PAGE and electroblotted to a nitrocellulose membrane. The transferred proteins were incubated with a dilution of an antibody (polyclonal anti-eNOS from Affinity Bioreagents, SOD-1 and phosphorylated eNOS from Cell Signal, SOD-2 and iNOS from BD Biosciences, nitrotyrosine from Upstate) at 4°C overnight. Detection of the bound primary antibody was accomplished by exposure to a peroxidase-coupled anti-rabbit antibody (Amersham, dilution 1:1,000) followed by a chemiluminescent reaction using luminal reagent (SuperSignal West Pico; Pierce, Rockford, IL). Afterward, the membrane was exposed to a film, and the bands were analyzed by densitometry. We have used these techniques previously (17). The individual density values of each band were normalized to the LV free wall mass of each subject. This is used to compensate for the hypertrophy that occurs during pregnancy. We extracted the protein from preweighed LV samples from control and pregnant dogs and measured the ratio of protein obtained vs. wet weight (control vs. 60 days of pregnancy: 366 ± 2 vs. 368 ± 5 μg·ml⁻¹·g⁻¹). The ratio of protein per gram of wet weight tissue does not change during pregnancy. Because we load the same amount of protein in each well, we must normalize the results to the total mass of the LV free wall by multiplying the resulting density reading by the LV free wall mass divided by the control LV free wall mass.

Oxygen consumption. Myocardial muscle isolated from the LV free wall and the epicardium, endocardium, connective tissue, fat, and large arteries were removed and cut into 50- to 100-mg segments. Muscle segments were bathed at 37°C for 2 h in Krebs solution in which 21% O₂-5% CO₂-74% N₂ was bubbled continuously. Oxygen uptake by muscle slices was measured polarographically with a Yellow Springs Instrument apparatus consisting of the model 5300 biological oxygen monitor and a Clark-type oxygen electrode (model 5331). Tissue respiration was calculated as the rate of decrease in oxygen concentration after addition of muscle slices.

Bradykinin, enalapril maleate, or amloidipine, S-nitrosoguanidine (SNAP), and α-N-nitro-L-arginine methyl ester (l-NNAME) were added to the tissue bath in a cumulative concentration-dependent manner (each 10⁻⁷ to 10⁻⁴ mol/l), and the effects of oxygen consumption were recorded. Our group has used these techniques previously (18, 19).

Shear stress and passive diameter. Experiments were performed on coronary arterioles (~80 μm in diameter) isolated from LV free wall of 50-day pregnant dogs (n = 6) and nonpregnant dogs (n = 5). With the use of microscissors and an operating microscope (Olympus, Lake Success, NY), segments of subepicardial arterioles, ~1 mm in length, were separated from the adhering cardiac muscle by careful cutting and transferred to a vessel chamber containing Krebs bicarbonate-buffered physiological saline solution (PSS) at room temperature. We selected vessels that were approximately the same length and diameter to normalize the passive diameters. The vessel chamber contained two glass microcannulas, which were connected with silicone tubing to two pressure-servo syringe systems (Living Systems, Burlington, VT). The system was arranged with mirror symmetry so that the axis of symmetry was located perpendicular to the middle of the arteriolar segment. This resulted in equal resistance of the two sides of the system. The vessel chamber was connected to a reservoir through a suffusion pump. The total volume of PSS in the system was 100 ml suffused at a rate of 40 ml/min during the experiment.

The PSS used to perfuse the arteriole contained (in mM) 118 NaCl, 5 KCl, 2.5 CaCl₂, 1 MgSO₄, 1 KH₂PO₄, 10 glucose, 24 NaHCO₃, and 0.02 EDTA and was equilibrated with 21% O₂-5% CO₂-balance N₂ at pH 7.4. Intravascular pressure was maintained constant at 80 mmHg as previously reported (16). The change in passive diameter was measured by allowing the vessels to stabilize at a constant 80-mmHg pressure with no flow and then introducing an initial shear stress of 30 dyn/cm² and observing the change in diameter over time.

Arteriolar dilation induced by changes in the shear stress (flow) was compared before and after inhibition of NO and prostaglandin synthesis with N³-nitro-l-arginine methyl ester (l-NNAME, 2 × 10⁻⁴ mol/l) and indomethacin (10⁻⁵ mol/l), respectively, in hearts from dogs at 40, 50, and 60 days of pregnancy. Initial shear stress (10, 20, and 30 dyn/cm²) was established by a constant intraluminal flow (5–13 μl/min) calculated according to the basal diameter. Changes in diameter were continuously recorded for 4 min with a microscopical video caliper system.

Microvessels. Isolation of coronary microvessels from the LV free wall of the canine heart was performed according to a previously described method (15) originally developed by Gerrissen and Printz (8). Microvessels were isolated at 37°C for 20 min, and nitrite release was measured. Formation of NO was measured as nitrite. Increasing concentrations of bradykinin and carbacol (10⁻¹⁰ to 10⁻⁵ mol/l), l-NNAME (10⁻⁴ mol/l), HOE-140 (10⁻⁵ mol/l), and atropine (10⁻⁵ mol/l) were studied.

Data calculation and statistical analysis. Data are presented as means ± SE. Echographic parameters were compared by one-way ANOVA followed by Dunnett’s post hoc test. Changes in MV˙O₂ and shear stress and passive diameter measurements were analyzed by two-way repeated-measures ANOVA followed by Tukey’s post hoc test. Nitrite production was analyzed by Student’s t-test. P < 0.05 was considered statistically significant.
Table 1. Changes in hemodynamics during pregnancy and postpartum

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>40D</th>
<th>50D</th>
<th>60D</th>
<th>PP</th>
</tr>
</thead>
<tbody>
<tr>
<td>LVEDD, cm</td>
<td>4.69±0.07</td>
<td>4.72±0.05</td>
<td>4.93±0.08*</td>
<td>5.08±0.10*</td>
<td>4.66±0.10</td>
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<tr>
<td>LVESD, cm</td>
<td>3.13±0.06</td>
<td>2.96±0.11</td>
<td>3.05±0.06</td>
<td>3.17±0.13</td>
<td>3.11±0.02</td>
</tr>
<tr>
<td>LVESV, ml</td>
<td>19.65±0.40</td>
<td>19.89±0.56</td>
<td>19.81±0.89</td>
<td>20.94±0.85</td>
<td></td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>70.80±5.72</td>
<td>75.33±1.61</td>
<td>88.75±1.17*</td>
<td>98.00±3.50*</td>
<td>69.75±1.02</td>
</tr>
<tr>
<td>Ejection fraction, %</td>
<td>62.68±0.98</td>
<td>60.49±2.16</td>
<td>64.76±0.46</td>
<td>65.92±1.47</td>
<td>59.89±0.06</td>
</tr>
<tr>
<td>LVFW mass, g</td>
<td>72.40±2.43</td>
<td>79.06±1.21*</td>
<td>84.34±4.55*</td>
<td>90.48±4.47*</td>
<td>78.73±3.04</td>
</tr>
<tr>
<td>MASS echo, g</td>
<td>73.42±1.02</td>
<td>83.12±6.95*</td>
<td>87.39±0.96*</td>
<td>94.36±2.15*</td>
<td>80.14±2.78</td>
</tr>
<tr>
<td>Stroke volume, ml</td>
<td>33.62±1.62</td>
<td>32.44±2.90</td>
<td>36.06±1.25*</td>
<td>37.83±1.08*</td>
<td>32.08±1.73</td>
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<tr>
<td>Fractional shortening, %</td>
<td>39.71±3.00</td>
<td>39.38±1.63</td>
<td>38.28±0.87</td>
<td>37.71±2.07</td>
<td>36.98±0.14</td>
</tr>
<tr>
<td>SWT LVFW, mm</td>
<td>0.89±0.02</td>
<td>0.91±0.01</td>
<td>0.97±0.03*</td>
<td>0.99±0.01*</td>
<td>0.88±0.02</td>
</tr>
<tr>
<td>DWT septum, mm</td>
<td>1.21±0.01</td>
<td>1.22±0.02</td>
<td>1.26±0.02*</td>
<td>1.32±0.01*</td>
<td>1.22±0.01</td>
</tr>
<tr>
<td>SWT septum, mm</td>
<td>0.89±0.02</td>
<td>0.90±0.01</td>
<td>0.96±0.04*</td>
<td>1.01±0.01*</td>
<td>0.88±0.02</td>
</tr>
<tr>
<td>Myocardial wall stress</td>
<td>2.63±0.09</td>
<td>2.60±0.05</td>
<td>2.56±0.12</td>
<td>2.62±0.08</td>
<td>2.62±0.11</td>
</tr>
</tbody>
</table>

Values are means ± SE. 40D, 40-day pregnant dogs; 50D, 50-day pregnant dogs; 60D, 60-day pregnant dogs; PP, postpartum; LVEDD, left ventricular end-diastolic diameter; LVESD, left ventricular end-systolic diameter; LVESV, left ventricular end-systolic volume; LVFW mass, left ventricular free wall mass; MASS echo, LVFW mass as calculated by echocardiography; DWT, diastolic wall thickness; SWT, systolic wall thickness. Myocardial Wall Stress calculated as (pressure × radius)/wall thickness (pressure is assumed to be constant). *P < 0.05 vs. control.

RESULTS

Echocardiographic variables. The LV end-diastolic diameter was significantly elevated at 50 days and 60 days of gestation vs. control (Table 1). LV end-diastolic volume was significantly higher in 50- and 60-day pregnant dogs (P < 0.05; Fig. 1). LV free wall mass was also significantly larger in hearts from 50- and 60-day pregnant dogs (P < 0.05 vs. control), indicating ventricular hypertrophy. Ejection fraction was not significantly different. Heart rate steadily increased during the course of the pregnancy and was significantly higher at 50 and 60 days of pregnancy (P < 0.05 vs. control). Stroke volume was also significantly elevated at 50 and 60 days of pregnancy (P < 0.05 vs. control). Cardiac output was increased at 50 and 60 days of pregnancy, respectively, due to the tachycardia and increased stroke volume (Fig. 1). All changes had resolved post partum.

Protein expression. There is an increase in the eNOS protein in LVs from 50- and 60-day pregnant dogs compared with control animals (Fig. 2A). Phosphorylated eNOS protein levels were increased in hearts at 40, 50, and 60 days of pregnancy (Fig. 2B). SOD-1 levels significantly increased in both the 50- and 60-day pregnant dogs (Fig. 2C). All bar graphs are normalized to LV free wall mass. There was no significant difference in the amount of nitrotyrosine, iNOS, or SOD-2 staining in control vs. pregnant dogs.

Effect of bradykinin on MV2O2. Cumulative doses of bradykinin caused dose-dependant decreases in MV2O2 in LV tissue from nonpregnant dogs (Fig. 3A). The response to bradykinin was increased during all three gestational periods of pregnancy but was only statistically significant (P < 0.05 vs. control) at high doses (10^-4 mol/l). The enhanced effect of bradykinin was lost post partum, and MV2O2 returned to control levels. Bradykinin-induced reduction in MV2O2 was significantly attenuated by L-NAME in all groups.

Effect of enalapril maleate. The angiotensin-converting enzyme inhibitor enalapril maleate caused a reduction in MV2O2 in cardiac muscle from female dogs. This reduction occurred in a dose-dependant manner (Fig. 3B). High doses (10^-4 mol/l) of enalapril maleate produced a significantly larger (P < 0.05 vs. control) decrease in MV2O2 in pregnant dogs at all three time points. The decrease in MV2O2 to enalapril maleate returned to control in dog hearts at ~2 wk after giving birth. L-NAME had similar effects as in the bradykinin groups.

Effect of amlodipine. Cumulative doses of amlodipine caused dose-dependant decrease in oxygen consumption in tissue from dogs (Fig. 4A). The response to amlodipine was increased
of the arterioles was significantly larger in arterioles from pregnant hearts than from controls (Fig. 5). In arterioles from control hearts, shear stress (dyn/cm²) was reduced from 30 to 18 ± 1.6 in control arterioles (Fig. 6). The administration of L-NAME resulted in a decrease from 30 to 21 ± 2.1 dyn/cm², and L-NAME plus indomethacin resulted in a decrease from 30 to 24 ± 1.2 dyn/cm² (P < 0.05 vs. control). Arterioles obtained from hearts at 40 days of pregnancy demonstrated a reduction in shear stress from 30 to 20 ± 1.7 dyn/cm² under control conditions (Fig. 6). The administration of L-NAME attenuated the decrease from 30 to 24 ± 0.8 dyn/cm², whereas L-NAME + indomethacin resulted in a reduction from 30 to 24 ± 1.8 dyn/cm² (P < 0.05 vs. control). Arterioles obtained from hearts at 50 days of pregnancy under control conditions showed a reduction in shear stress from 30 to 12 ± 2.5 dyn/cm². L-NAME administration resulted in a decrease from 30 to 17 ± 1.9 dyn/cm², whereas L-NAME plus indomethacin resulted in a decrease from 30 to 26 ± 3.5 dyn/cm² (P < 0.05 vs. control, P < 0.05 vs. L-NAME alone, respectively). Arterioles obtained from hearts at 60 days of pregnancy under control conditions showed a reduction in shear stress from 30 to 13 ± 1.6 dyn/cm². L-NAME administration resulted in a decrease from 30 to 19 ± 2.3 dyn/cm², whereas L-NAME plus indomethacin showed a reduction from 30 to 18 ± 6.4 dyn/cm² (P < 0.05 vs. control).

Effects of bradykinin and acetylcholine on NO production. Bradykinin and carbachol increased nitrite release in control microvessels (Fig. 7). Microvessels from pregnant hearts showed an increased nitrite release in response to bradykinin or
carbachol. The basal levels were higher during pregnancy ($P < 0.05$), as were the peak values ($P < 0.05$). L-NAME and HOE-140 blocked the effects of bradykinin, whereas administration of atropine blocked the effects of carbachol on nitrite release.

**DISCUSSION**

Our main findings are as follows: 1) pregnancy is associated with an increased cardiac workload; 2) myocardial eNOS, phosphorylated eNOS, and SOD-1 protein expression are increased in 60-day pregnant dogs compared with results shown in controls despite hypertrophy; 3) the elevation of eNOS protein expression is associated with an enhanced kinin-medi
ated and NO-dependant modulation of MV$_2$ that returns to control postpartum; 4) there is a greater shear stress-induced, flow-dependant dilation during pregnancy and that increase is partially NO dependant; and 5) nitrite production from coronary microvessels is increased during pregnancy. All of these adjustments may lead to a better coupling between oxygen delivery and consumption in the heart during pregnancy.

**Pregnancy and cardiac function.** Pregnancy is a chronic volume-overload state that necessitates maternal cardiovascular adaptations to support fetal development. The increases in heart rate are most likely due to a withdrawal of vagal tone and not due to an increase in sympathetic activity since heart rate increased from 70 ± 6 to 98 ± 4 beats/min. Although ejection fraction did not change, the stroke volume increased as a result of the increased LV end-diastolic volume, a Frank-Starling effect. The combination of an increased stroke volume and heart rate led to an increase in cardiac output. We also observed increases in LV end-diastolic diameter and LV free wall mass at 50 and 60 days of pregnancy, which is indicative of physiological hypertrophy. Similarly, there was no change in the ratio of diastolic wall thickness to systolic wall thickness, which is again consistent with physiological hypertrophy. We did note that the LV diastolic diameter increased, whereas the LV wall thickness increased, which would lead to an underestimation of the hypertrophy of the LV. These results support previous studies in humans (21) and support the concept that pregnancy is associated with an increased maternal cardiac workload.

**Myocardial eNOS expression.** Numerous studies have shown an increase in eNOS expression in various vascular beds in pregnancy (10, 30). We demonstrate that there is an elevated eNOS protein in the myocardium of dogs at 50 and 60 days of pregnancy compared with control. Our study did not detect any changes in iNOS in the myocardium. This is not surprising because iNOS expression in the myocardium appears to be only important under inflammatory conditions (11). We also detected an increase in phosphorylated eNOS at 40, 50, and 60 days of pregnancy, although the ratio of activated eNOS to nonactivated eNOS most likely remains constant.

**Myocardial SOD expression.** An increased production of NO by eNOS upregulation could lead to increases in peroxynitrite production. Peroxynitrite is a reactive oxidant that is produced from the reaction of NO with superoxide anion and impairs cardiovascular function through multiple mechanisms, including activation of matrix metalloproteinases and nuclear enzyme poly(ADP-ribose) polymerase. However, the myocardium has a series of defense mechanisms, including the enzymes SOD, catalase, and glutathione peroxidase plus other endogenous antioxidants such as vitamin E, ascorbic acid, and cysteine, to protect the cell against cytotoxic oxygen metabolites. CuZnSOD or SOD-1 is a cytosolic, antioxidant enzyme that scavenges potentially damaging free radicals such as peroxynitrite. Studies performed by Fukai et al. (7) have found that treadmill exercise training increases eNOS and SOD-1 expression in wild-type mice but had no effect on mice lacking eNOS. This suggests that upregulation of SOD-1 in response to chronic exercise is mediated by NO. This may represent an important mechanism by which NO stimulates SOD-1 expression to decrease superoxide-mediated degradation of NO. We did not find an increase in the amount of nitrotyrosine staining during pregnancy, and we did not detect any changes in SOD-2 expression.

**Impact of hypertrophy.** During pregnancy, there is a large increase in the LV free wall mass of ~25%. We determined that the amount of protein for any given wet weight of tissue remains constant throughout pregnancy. Thus the hypertrophy occurring during pregnancy does not represent a merely physical increase in size but also an increase in the amount of protein present. Because most Western blots are performed by placing a set amount of protein in each well (i.e., 75 μg), it seems clear that to accurately describe the amount of protein measured one must normalize the data to the overall wet weight of the tissue measured. If normalization is not performed, the data actually underestimate the total amount of protein from that tissue. In our previous work on pregnant rats, we failed to account for the hypertrophy occurring during pregnancy and perhaps underestimated the amount of increase in eNOS protein expression (17). Although there was a significant difference between eNOS, phosphorylated eNOS, and SOD-1, protein expression was not significantly different until it was normalized to LV mass (4 ± 3% vs. 21 ± 4%, 5 ± 3% vs. 18 ± 3%, and 18 ± 8% vs. 61 ± 3%, respectively). The increase in NO production acts synergistically with the increase in SOD-1; thus there is both an increase in NO production and a decrease in NO scavenging. SOD-2, iNOS, and nitrotyrosine do not change even after normalization to LV mass.

MV$_2$. Our findings clearly indicate that agents that stimulate the release of endogenous NO such as bradykinin, enalapril maleate, and amlodipine significantly reduce MV$_2$ by a NOS-dependant mechanism in hearts from dogs. The increased response to bradykinin or substances that increase local kinin production and bioavailability during pregnancy.

Increasing doses of bradykinin reduced MV$_2$ in a dose-dependant manner in all groups of dogs. Angiotensin-converting enzyme inhibitors like enalapril maleate lead to an accumulation of endogenous kinins via kininase II inhibition. Amlodipine stimulates the release of NO from blood vessels via a kinin-dependant mechanism (32). Kinins bind to the bradykinin B$_2$ receptor and stimulate the release of NO (15, 31). Bradykinin, enalapril maleate, and amlodipine caused a dose-dependant reduction in MV$_2$ in all groups of dogs. This reduction was enhanced in hearts from the 40-, 50-, and 60-day pregnant dogs and confirms the increase in myocardial NO production and bioavailability during pregnancy.

SNAP reduced MV$_2$ in a dose-dependant, NOS-independant manner in pregnant and control dogs. There was no significant difference in the SNAP-induced reduction in tissue oxygen consumption between pregnant and control tissue. This indicates that there is no increased sensitivity of myocardial tissue to NO but rather an enhanced NO-dependant regulation of MV$_2$.

It has been previously shown that inhibition of eNOS results in increases in MV$_2$ at all levels of cardiac work (24). The increase in MV$_2$ is not related to changes in contractile performance. The elevation of MV$_2$ was accomplished without changes in either the ATP content or the ATP synthesis rate. Thus it appears that NO produced by the endothelium...
increases myocardial efficiency, allowing the myocardium to produce the same amount of work with less oxygen consumed. This would be particularly important during pregnancy when the heart must pump an estimated 300,000 more liters of blood compared with that shown in control situations. This increase in blood pumped is primarily a result of the increased stroke volume coupled with an increased heart rate, which if NO were absent would require an increase in the amount of oxygen consumed. NO has also been shown to have some control over substrate utilization of the myocardium. High levels of NO tend to promote free fatty acid metabolism, whereas low NO favors glucose metabolism. Because free fatty acid metabolism produces far more ATP per mole of oxygen consumed, high levels of NO would be a definite advantage to the heart during pregnancy.

Arteriole dilation. We have demonstrated previously that canine coronary arterioles respond to increases in shear stress by dilating (25). Coronary arterioles from the normal dog were sensitive to increases in shear stress, reaching near maximal dilation at shear stresses of 30 dyn/cm². There is an enhanced dilation in response to shear stress in coronary arterioles from 50- and 60-day pregnant dogs. The inhibition of NO synthase using L-NAME significantly decreased the shear stress-induced dilation of coronary arterioles. This inhibition was significantly greater in coronary arterioles from 50- and 60-day pregnant dogs. There was a component of the shear stress-induced dilation that was attributable to prostaglandins, but this appeared to be significant in only 50-day pregnant dogs. In the 50-day group, 35% of the reduction of shear stress was attributable to NO, whereas the remaining 65% was due to prostaglandins. Interestingly, at 60 days of pregnancy, 100% of the reduction in shear stress was attributable to NO, and administration of indomethacin actually inhibited the reduction in shear stress. Previous work by Conrad et al. (5) has shown that NO was found to mediate renal vasodilation. Our results suggests that, at some stages, perhaps late, during pregnancy, NO may exert a greater control over the shear stress-induced dilation, whereas at other stages prostaglandins may partially mediate the response. This increased arteriolar dilation results in an increased flow through the coronary arterioles, thereby supporting an increased oxygen delivery to the myocardial tissue.

Nitrite production. We found that bradykinin and carbachol significantly increased nitrite release from isolated canine coronary microvessels. These effects were dramatically reduced by L-NAME, an NO synthase inhibitor, indicating that the release of nitrite by all these agents is dependent on NO release. NO production caused by bradykinin was substantially reduced by the B2 kinin receptor antagonist HOE-140, suggesting that in part the release of nitrite is dependent on activation of the B2 kinin receptor by endogenous kinins. Atropine blocked the release of nitrite in response to carbachol. These results demonstrate that vascular kinin formation and muscarinic receptor activation both play a role as mediators in the control of NO production by coronary microvessels.

In response to pregnancy, a period of increased cardiac work, the cardiovascular system responds by both increasing the flow to the myocardial tissue and increasing the efficiency of MV02, both NO dependent. Walsh et al. (27) showed the vascular overexpression of eNOS enhanced the regulation of MV02 by NO. NO produced by the capillary endothelial cells is freely diffusible into the neighboring myocytes. The increased NO produced during pregnancy has the ability to both control vascular tone in the smooth muscle and oxygen consumption by the myocytes. The increased flow results in an increased oxygen delivery, which is coupled to the increased efficiency of the myocytes, yielding a decrease in oxygen consumption. Thus the pregnant heart is able to perform greater amounts of work for any level of oxygen consumed.

**Therapeutic implications.** The detection and clinical management of hypertension in pregnant women are complicated by concerns for fetal development and survival, as well as for the health of the mother. Preeclampsia describes a common syndrome that occurs in the second half of pregnancy, often manifesting with hypertension and proteinuria. It occurs in up to 10% of all pregnancies. Antihypertensive drug therapy is recommended for pregnant women with systolic blood pressures of 160 to 180 mmHg or higher and diastolic blood pressures of 105 to 110 mmHg or higher. Although evidence concerning the potential adverse effects of most antihypertensive drugs has been poorly quantified, use of many of these agents is contraindicated during pregnancy. Enalapril for example is strongly contraindicated in pregnancy because it can result in injury and even death to the developing fetus. No evidence of teratogenicity or other fetal/embryo toxicities have been found in laboratory animals given amlodipine.

**Limitations of study.** Our study indicates that there is a role for NO in the coupling of cardiac metabolism and arteriolar dilation during normal pregnancy. However, we have not yet elucidated the site responsible for the increase in NO release. The change could occur anywhere from eNOS expression, signal transduction, superoxide scavenging by SOD-1, to NO biosynthesis. Further studies should be performed to answer this question.

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**REFERENCES**


