

## Coronary nitric oxide production controls cardiac substrate metabolism during pregnancy in the dog

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**Williams JG, Ojaimi C, Qanud K, Zhang S, Xu X, Recchia FA, Hintze TH.** Coronary nitric oxide production controls cardiac substrate metabolism during pregnancy in the dog. *Am J Physiol Heart Circ Physiol* 294: H2516–H2523, 2008. First published April 18, 2008; doi:10.1152/ajpheart.01196.2007.—The aim of this study was to examine the role of nitric oxide (NO) in the control of cardiac metabolism at 60 days of pregnancy (P60) in the dog. There was a basal increase in diastolic coronary blood flow during pregnancy and a statistically significant increase in cardiac output ( $55 \pm 4\%$ ) and in cardiac NOx production ( $44 \pm 4$  to  $59 \pm 3$  nmol/min,  $P < 0.05$ ). Immunohistochemistry of the left ventricle showed an increase in endothelial nitric oxide synthase staining in the endothelial cells at P60. NO-dependent coronary vasodilation (Bezold-Jarisch reflex) was increased by 20% and blocked by  $N^G$ -nitro-L-arginine methyl ester (L-NAME). Isotopically labeled substrates were infused to measure oleate, glucose uptake, and oxidation. Glucose oxidation was not significantly different in P60 hearts ( $5.4 \pm 0.5$  vs.  $6.2 \pm 0.4$   $\mu$ mol/min) but greatly increased in response to L-NAME injection (to  $19.9 \pm 0.9$   $\mu$ mol/min,  $P < 0.05$ ). Free fatty acid (FFA) oxidation was increased in P60 (from  $5.3 \pm 0.6$  to  $10.4 \pm 0.5$   $\mu$ mol/min,  $P < 0.05$ ) and decreased in response to L-NAME (to  $4.5 \pm 0.5$   $\mu$ mol/min,  $P < 0.05$ ). There was an increased oxidation of FFA for ATP production but no change in the respiratory quotient during pregnancy. Genes associated with glucose and glycogen metabolism were downregulated, whereas genes involved in FFA oxidation were elevated. The acute inhibition of NO shifts the heart away from FFA and toward glucose metabolism despite the downregulation of the carbohydrate oxidative pathway. The increase in endothelium-derived NO during pregnancy results in a tonic inhibition of glucose oxidation and reliance on FFA uptake and oxidation to support ATP synthesis in conjunction with upregulation of FFA metabolic enzymes.

free fatty acid oxidation; glucose metabolism; gene chip; no metabolic vasodilation

THE CATABOLIC PHASE OF PREGNANCY is characterized by a shift in the maternal metabolism away from glucose and a decrease in the plasma concentration of glucose in the third trimester. This hypothetically results in a net increase in the amount of glucose available for the growing fetus. There is also an increase in the maternal tissue resistance to insulin, which decreases the rate at which glucose and lipids are removed from the circulation of the mother. There is an increase in vascular nitric oxide (NO) synthesis throughout pregnancy, and it has been theorized that there is a cause and effect relationship between plasma ovarian steroid levels and endothelial nitric oxide synthase (eNOS) expression and subsequent NO production. Estrogen treatment has been shown to increase uterine blood flow and increase uterine cGMP production (3).

The potential that NO modulates maternal cardiac metabolism during pregnancy has yet to be directly addressed.

The maternal metabolic rate is greatly increased during pregnancy (10). The oxygen needs of the heart vary with heart rate, blood pressure, ventricular mass, and contractility. Increases in cardiac and respiratory work during pregnancy lead to a 20–30% increase in basal oxygen consumption. This increase in oxygen consumption by the heart may be met partially by a small increase in oxygen extraction but mostly by an increase in coronary flow.

We have shown in a number of studies that endogenous NO, in addition to its classic vasodilator action, may regulate tissue oxygen consumption and substrate use by the heart (25) such that, when NO is present, the heart takes up primarily fatty acids, oxygen consumption is relatively low, and oxygen efficiency is high. The role of NO during pregnancy in the control of systemic vasodilation has been previously identified; however, the role of NO in the control of cardiac function and metabolism during pregnancy has not been fully determined (2). To this end, we examined the role of NO in the control of myocardial metabolism and substrate use during pregnancy in the conscious dog.

### METHODS

#### Surgical Preparation

Pregnant dogs ( $45 \pm 2$  days pregnant,  $n = 20$ ) and control dogs ( $N = 6$ ) were sedated with a mixture of ketamine and valium (1 ml of each, 100 mg/ml ketamine and 5 mg/ml valium), and anesthetized with compressed oxygen ( $\sim 2$  L/min at 1 BAR) mixed with 2% isoflurane. A thoracotomy was performed, and catheters (Tygon) were placed in the aorta, left atrium, and coronary sinus (CS). A left ventricular (LV) pressure gauge (P 6.5; Konigsberg Instruments, Pasadena, CA), a Doppler flow transducer (Craig Hartley; Ultrasonic Instrumentation for Cardiovascular Research), a pair of piezoelectric crystals, and a pair of pacing electrodes were implanted as described previously (30). The dogs were allowed to fully recover, and all measurements were taken at  $60 \pm 2$  days pregnant. Because the canine pregnancy last for  $\sim 65$  days, this corresponds to roughly month 8 of a human pregnancy. The control animals were approximately the same age as the pregnant dogs. All dogs were impregnated at the vendor and are a hound mix of random source. All protocols were approved by the Institutional Animal Care and Use Committee of New York Medical College.

#### Hemodynamic Recordings

Arterial pressure, LV pressure, left circumflex coronary blood flow (CBF), and internal diameters were measured; heart rate (HR), mean

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arterial pressure, and mean CBF were derived in conscious dogs, as we described previously (32). All dogs were trained to lie quietly on the laboratory table for a minimum of 4 h. Measurements were recorded continuously throughout the course of the experiment. Baseline measurements were taken after the dog was resting quietly on the table for 40 min. To assess the effects of pregnancy on NO-dependent coronary vasodilation (the Bezold-Jarisch reflex), veratrine at a dose of 5  $\mu\text{g/kg}$  was administered as a bolus injection (1 ml) in the left atrium through the implanted catheter with HR maintained via an implanted pacer at a constant 150 beats/min. The Bezold-Jarisch reflex is a cardiovascular depressor reflex involving a marked increase in vagal (parasympathetic) efferent discharge to the heart, elicited by stimulation of chemical receptors, primarily in the left ventricle. This causes a bradycardia and dilatation of the peripheral blood vessels with resulting lowering of the blood pressure. NO synthase was blocked using a nonselective inhibitor of NO synthase, *N*<sup>G</sup>-nitro-L-arginine methyl ester (L-NAME, 35 mg/kg iv), and measurements were recorded continuously before and after injection. The dose of L-NAME was chosen based on the well-known efficacy of 35 mg/kg in dogs (21).

### Cardiac Metabolites

Blood samples from aorta and CS were collected in plastic syringes treated with heparin or EDTA both before and after administration of L-NAME. Blood gases, lactate, and glucose were measured with a blood gas analyzer (IL-682 CO-Oximeter). Free fatty acid (FFA) analysis was performed on plasma from EDTA-treated samples with a colorimetric assay (NEFA C kit; Wako Diagnostics, Richmond, VA) as described previously (20, 17). Oxygen content and total cardiac substrate concentrations were measured in arterial and CS blood samples. The concentration of oxygen, total and radioactively labeled substrates in arterial and coronary blood samples, and mean CBF were used to calculate the rates of FFA and glucose oxidation as previously described (21). Briefly, the following isotopically labeled substrates were infused in the dogs: [9,10-<sup>3</sup>H]oleate and [U-<sup>14</sup>C]glucose. [<sup>3</sup>H]oleate bound to albumin was infused at a rate of 0.7  $\mu\text{Ci/min}$ . The bolus-continuous infusion method was used for [<sup>14</sup>C]glucose (20  $\mu\text{Ci} + 0.3 \mu\text{Ci/min}$ ). Plasma [<sup>3</sup>H]oleate concentration was measured by extracting fatty acids from 1 ml of plasma in 4 ml of heptane-isopropanol (3:7) and counting the radioactivity of the organic phase with a liquid scintillation counter (model LS 6500; Beckman). <sup>3</sup>H<sub>2</sub>O concentration was measured by distilling 2 ml of plasma in custom-made, modified Hickman stills (Kontes Glass; Custom Shop). We developed this method and tested it to exclude possible contamination of the distillate with [<sup>14</sup>C]- or [<sup>3</sup>H]oleate. Blood samples for [<sup>14</sup>C]glucose measurements were first deproteinized in ice-cold 1 M perchloric acid (1:2 vol/vol). The acidity of the extract was then neutralized with K<sub>2</sub>CO<sub>3</sub>, and the neutral solution was run through ion-exchange resin columns (Dowex 1 and 50) to separate secondary labeled [<sup>14</sup>C]lactate, -pyruvate, and -alanine. Total glucose concentration and <sup>14</sup>C activity were then measured in the eluate to calculate [<sup>14</sup>C]glucose specific activity. Blood samples (3 ml) were placed in a sealed flask with a center well containing 800  $\mu\text{l}$  of 1 M NaOH. The blood was acidified with 1 ml of concentrated lactic acid (98%), and the flask was placed on a rotating table at room temperature for 3 h. All of the CO<sub>2</sub> so displaced from the blood combined with solution contained in a well inside the flask to form bicarbonate. The newly formed solution of bicarbonate was then collected to count the activity of <sup>14</sup>C. The fractional oxygen extraction was calculated at the arterial-CS difference in oxygen concentration divided by the arterial oxygen concentration.

### Cardiac NO Metabolites

NO metabolite (NO<sub>x</sub>, i.e., nitrate, nitrite, and NO) concentration in micromoles per liter was measured in plasma obtained from aortic and CS blood samples after centrifugation at 1,000 g for 15 min. Plasma

was stored at  $-20^{\circ}\text{C}$  until the day of analysis. The method for NO<sub>x</sub> analysis has been described in detail (30). NO<sub>x</sub> production was calculated as a product of CBF and arteriovenous (A-V) NO<sub>x</sub> differences.

### Immunohistochemical Examination

Tissue samples were cut into  $\sim 3$ - to 4-mm thickness and fixed in 10% neutral formalin solution (Sigma) for 24 h. After being rinsed overnight, the samples were dehydrated with ethanol, followed by xylene. The tissue samples were impregnated with paraffin in an oven heated to  $60^{\circ}\text{C}$  and were embedded. Sections of 5  $\mu\text{m}$  thickness were cut and transferred to the oven and heated to  $60^{\circ}\text{C}$ . This was followed by deparaffinization of xylene I, a series of ethanol baths, tap water, and distilled water for rehydration. Sections were brought to boiling in 10 mM sodium citrate buffer, pH 6.0. Next, endogenous peroxidase was quenched with 0.3% H<sub>2</sub>O<sub>2</sub> for 30 min and washed in PBS. The sections were incubated in 2% normal horse serum in PBS buffer for 30 min. The sections were incubated overnight with 1:50 dilution of anti-eNOS primary antibody (Affinity Bioreagents) at  $4^{\circ}\text{C}$  temperature and then incubated for 30 min with 1:500 diluted biotinylated affinity-purified antirabbit IgG secondary antibody (Vector Laboratories). The slides were incubated with avidin-biotin peroxidase complex (Vector Laboratories) and then incubated with diaminobenzidine stock solution for  $\sim 2$ –5 min. The sections were stained with hematoxylin and ethanol for 5–10 min, xylene for 10 min, and then mounted with cytooseal mounting medium.

### RNA Isolation and Microarray Analysis

Total cardiac RNA was extracted from the LV of the control and pregnant dogs ( $n = 8$ ) as described previously (16). RNA quality was assessed by electrophoresis with the Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA). RNA from each sample was used to generate a high-fidelity cDNA with the labeling protocols for sample preparation recommended by Affymetrix. The microarray labeling, hybridization, and analysis procedures have been described previously in more detail (16). Each sample was individually subjected to gene expression analysis via the Affymetrix Canine Genome Array. The GeneChip Canine Genome Array was used and contains  $>23,813$  *Canis familiaris* probe sets to monitor gene expression for  $>16,000$  full-length transcripts. Sequence information for the GeneChip Canine Genome Array includes public content from GenBank (release 137.0, August 2003), dbEST (October 2003), and proprietary beagle sequence content licensed from LION Bioscience. All of the hybridization data have been submitted to the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus database (<http://www.ncbi.nlm.nih.gov/geo>) with GEO Accession Numbers for series GSE5260.

### Data Analysis

Triple product (TP), an index of work performed, was calculated as LV systolic pressure multiplied by  $dP/dt_{\text{max}}$  and HR ( $\text{mmHg} \times \text{mmHg/s}$ ) times beats per minute and expressed in units. Stroke volume (SV) was calculated using the ventricular diameter crystals and modeling the ventricle as a cylinder, assuming the long axis was two times the base diameter. This approximation allows for calculations of end-systolic and -diastolic volumes, SV, and cardiac output ( $\text{SV} \times \text{HR}$ ) (15). Respiratory quotient ( $\text{RQ} = \text{CO}_2 \text{ produced} / \text{O}_2 \text{ consumed}$ ) and cardiac production of CO<sub>2</sub> were calculated as previously described (20). Oxygen content of the blood was calculated by the following formula:  $\text{oxygen content} = 1.39 \times (\text{O}_2\text{Hb}/100) \times \text{THb} + (0.003 \times \text{PO}_2)$ . Myocardial oxygen consumption ( $\text{MVO}_2$ ) was calculated as the arterial-CS oxygen difference multiplied by two times the mean circumflex blood flow. Efficiency was calculated as the TP divided by  $\text{MVO}_2$ . Predicted total ATP synthesis levels in both control and pregnancy were calculated by the theoretical ATP yield from each substrate as follows (mol ATP/mol substrate): 36 glucose, 18 lactate, and 129 palmitate.

Table 1. Effects of L-NAME on hemodynamics during pregnancy

	Control	P60	P60 + L-NAME
LV systolic pressure, mmHg	124.6±3.4	118.9±2.5	134.5±2.4
Mean arterial pressure, mmHg	105.2±4.5	97.2±3.9	112.6±3.7†
LV systolic diameter, mm	25.1±1.09	26.1±0.97	26.3±1.2
LV diastolic diameter, mm	39.5±1.39	44.0±1.5*	40.1±1.5
LV systolic volume, ml	19.6±1.09	21.2±0.97	20.7±1.1
LV diastolic volume, ml	49.0±1.39	60.8±1.6*	48.1±1.6†
Diastolic CBF, ml/min	32.8±1.57	41.2±2.8*	39.6±2.7
Heart rate, beats/min	68.0±7.00	107.2±7.0*	71.0±5.0†
LV dP/dt, mmHg/s	3,108.1±365.5	3,390.4±87.5*	3,241.2±60.0
Triple product, units	27.6±1.4	39.3±2.8*	24.2±2.3†
Stroke volume, ml	29.4±1.2	39.6±0.9*	28.2±1.4†
Cardiac output, ml/min	2038±34	4234±51*	1981±27†
Total peripheral resistance, units	0.051±.02	0.023±.01	0.056±.02†
MV̇O <sub>2</sub> , ml/min	9.6±0.4	11.0±1.1	16.4±0.7†
Efficiency (TP/MV̇O <sub>2</sub> )	2.87±0.2	3.57±0.3*	1.47±0.2†

Values are means ± SE. P60, 60 days of pregnancy; L-NAME, N<sup>G</sup>-nitro-L-arginine methyl ester; LV, left ventricular; TP, triple product; MV̇O<sub>2</sub>, myocardial oxygen consumption. \**P* < 0.05 vs. control (\*) and vs. P60 (†).

All data are presented as means ± SE. In the *in vivo* studies, the responses are the peak after administration of veratrine. Statistical significance of differences was determined with Student's *t*-test for each peak response, and differences between groups were determined with repeated-measures ANOVA. Changes are considered significant at a value of *P* < 0.05.

## RESULTS

### Hemodynamics

Measurements of LV end systolic diameter did not reveal any significant differences between control vs. pregnant hearts (Table 1). There was a significant increase in LV end diastolic diameters. There was no significant change in LV end systolic volume (Table 1); however, we did find a large increase in the LV end diastolic volumes during pregnancy (*P* < 0.05 vs. control). LV free wall (LVFW) weights (as measured by direct removal of the LVFW postdeath) increased significantly during pregnancy (Table 1).

LV dP/dt<sub>max</sub>, an index of cardiac contractility (mmHg/s), increased during pregnancy (Table 1). The dP/dt<sub>max</sub> did not change significantly during L-NAME administration compared with pregnant 60 days. The TP, (10,000 units) was used as an index of LV mechanical function (Table 1). The TP increased during pregnancy (*P* < 0.05). Administration of L-NAME reduced the TP back to control levels due to bradycardia.

MV̇O<sub>2</sub> was slightly elevated during pregnancy compared with controls (Table 1). Administration of L-NAME increased MV̇O<sub>2</sub> during pregnancy. The change in MV̇O<sub>2</sub> following L-NAME administration was significantly greater in pregnancy compared with controls (*P* < 0.05).

The efficiency (TP/MV̇O<sub>2</sub>) increased by 20% during pregnancy (*P* < 0.05) (Table 1). The administration of L-NAME caused the oxygen efficiency to decrease nearly twofold.

### Effects of Veratrine on the Coronary Circulation

Figure 1A shows the percent changes in CBF due to veratrine-induced, NO-dependent, coronary vasodilation (Bezold-Jarisch reflex). Veratrine administration at a dose of 5 μg/kg caused significant increases in CBF (*n* = 7) from 50.9 ± 5% to 70.9 ± 6 when comparing controls with animals pregnant

for 60 days (P60). L-NAME entirely abolished the increase in mean CBF due to veratrine injection.

Diastolic CBF increased in control hearts in response to veratrine injection (Fig. 1B). In hearts in pregnant canines, the response to veratrine injection increased the diastolic CBF by 40%.

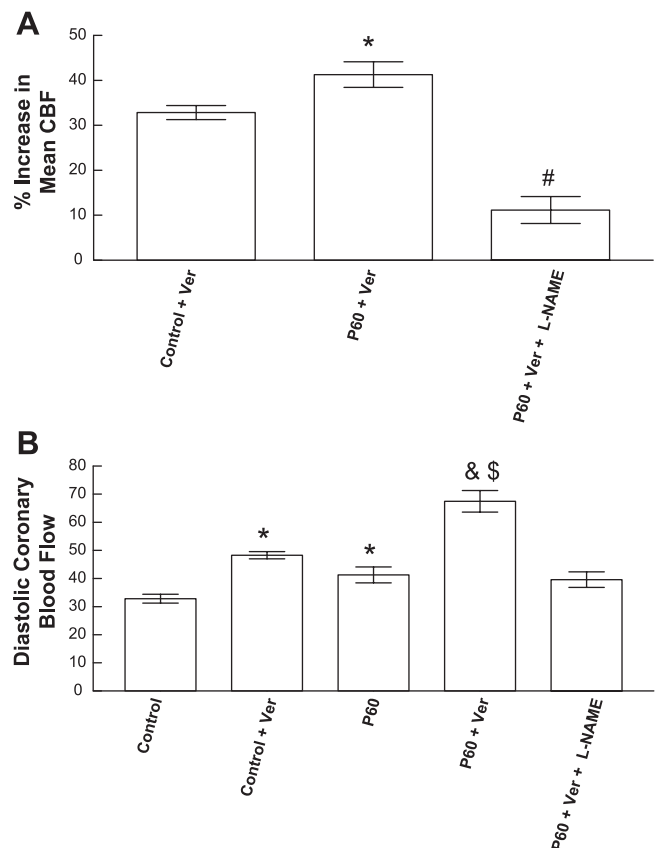


Fig. 1. A: coronary blood flow (CBF) is increased during veratrine injection (\**P* < 0.05 vs. control) and further during day 60 of pregnancy (P60) (#*P* < 0.05 vs. control and P60), which was eliminated by N<sup>G</sup>-nitro-L-arginine methyl ester (L-NAME). B: diastolic CBF is increased during veratrine injection (\**P* < 0.05 vs. control) and even more during pregnancy (&*P* < 0.05 vs. P60, \$*P* < 0.05 vs. control veratrine).



L-NAME entirely blocked the increase in diastolic CBF due to veratrine. There was also a significant increase in basal diastolic CBF during pregnancy compared with control ( $P < 0.05$ ).

#### Cardiac NOx

There was a statistically significant increase in transcardiac NOx production during pregnancy (Fig. 2A), an increase in the arterial ( $0.67 \pm 0.06$  vs.  $0.84 \pm 0.03$  nmol; control vs. P60) and venous ( $0.82 \pm 0.07$  vs.  $1.12 \pm 0.04$  nmol; control vs. P60) content of NOx, as well as a significant increase in the total A-V difference ( $0.16 \pm 0.03$  vs.  $0.28 \pm 0.03$  nmol; control vs. P60) in plasma NOx.

#### Immunohistochemistry

Immunohistochemistry of the left ventricle showed an increase in eNOS staining in the LV from 60 day pregnant dogs (Fig. 2B).

#### Substrate Metabolism During Pregnancy

**Glucose. UPTAKE.** There was no glucose uptake at control or during pregnancy ( $P < 0.05$  from zero; Fig. 3A). Administration of L-NAME increased the glucose uptake ( $P < 0.05$ ).

**OXIDATION.** Glucose oxidation was not significantly different between control and pregnancy. Glucose oxidation during pregnancy is greatly increased ( $>200\%$ ) in response to L-NAME injection (Fig. 3B).

**mRNA.** mRNA levels of genes associated with glucose and glycogen metabolism such as glycogen phosphorylase (GP), pyruvate dehydrogenase, and 6-phosphofructokinase, among others, were downregulated (Fig. 3C).

**FFA. UPTAKE.** FFA uptake was significantly increased (Fig. 4A) during pregnancy. Following L-NAME administration, the increased uptake of FFA during pregnancy was completely abolished.

**OXIDATION.** FFA oxidation is increased during pregnancy vs. control hearts (by 100%). FFA oxidation decreased by 50% in response to L-NAME injection during pregnancy (Fig. 4B).

**mRNA.** Those genes involved in FFA oxidation such as isocitrate dehydrogenase (IDH), 3-ketoacyl-CoA thiolase (3KCT), and acetyl-coenzyme A synthetase (ACA Syn) were elevated (Fig. 4C).

**Lactate.** Lactate uptake was not significantly different between control and pregnant hearts ( $11.3 \pm 3.2$  vs.  $11.4 \pm 2.0$   $\mu\text{mol/min}$ ). Administration of L-NAME resulted in a large increase of  $\sim 90\%$ .

**RQ.** The RQ decreased slightly during pregnancy, but the difference was not significant compared with control (Fig. 5A). This indicates that the heart is still preferentially metabolizing FFA as opposed to glucose in pregnancy. Administration of L-NAME shifted the RQ upward ( $P < 0.05$ ) as the heart began metabolizing more glucose and less FFA.

**Total ATP.** We calculated the moles of ATP generated from metabolism of each substrate (Fig. 5B). Even though there is a preferential uptake and oxidation of FFA by the heart during pregnancy, administration of L-NAME can still switch the heart from FFA to glucose.

#### DISCUSSION

The major findings of this study are as follows. Pregnancy results in an increase in veratrine-induced, NO-dependent,

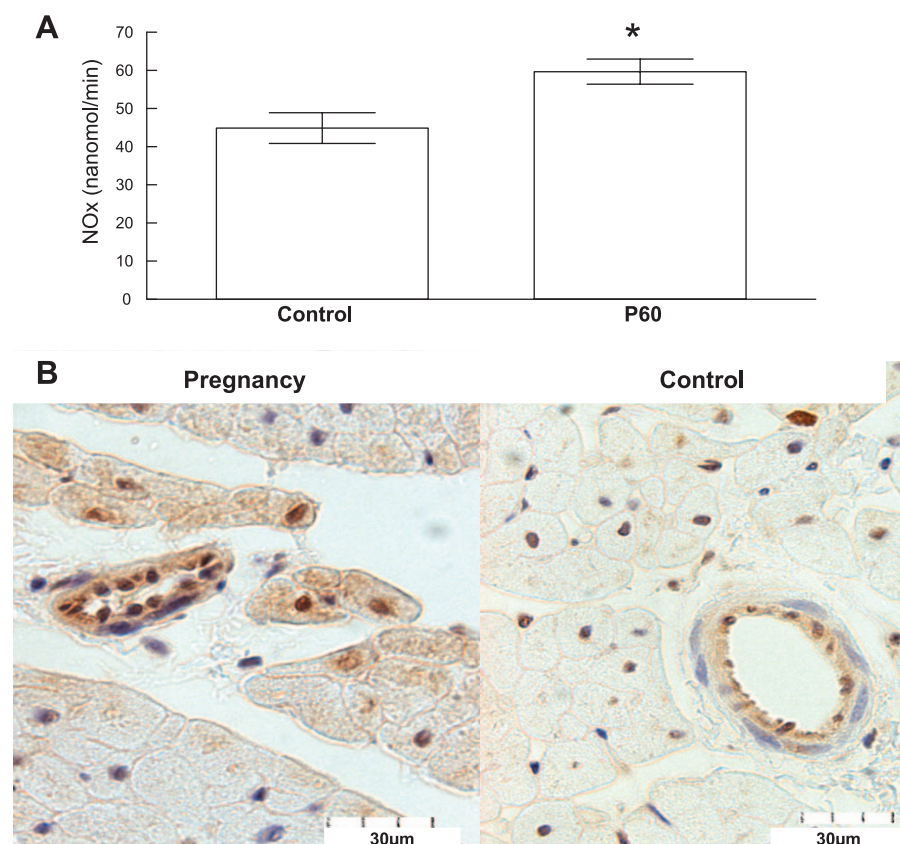


Fig. 2. A: Cardiac nitric oxide (NO) production increased during pregnancy ( $*P < 0.05$  vs. control). B: endothelial nitric oxide synthase (eNOS) staining is enhanced in endothelial cells of P60 left ventricles.

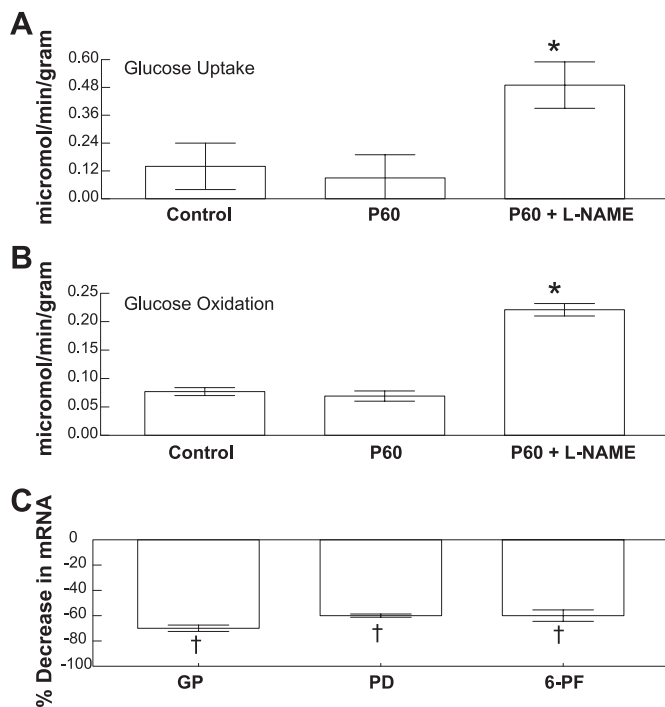


Fig. 3. Changes in carbohydrate oxidative pathway. A: there is no glucose uptake during pregnancy. L-NAME administration causes a significant increase in glucose uptake (\* $P < 0.05$  vs. pregnant). B: there is no change in glucose oxidation during pregnancy. L-NAME administration causes a significant increase in glucose oxidation (\* $P < 0.05$  vs. control and pregnant). C: mRNA levels of glycogen phosphorylase (GP), pyruvate dehydrogenase (PD), and 6-phosphofructokinase (6-PF) are decreased during pregnancy († $P < 0.05$  from 0).

coronary vasodilation (Bezold-Jarisch reflex). There is an increase in the transcardiac NO<sub>x</sub> production during pregnancy, in response to an increased eNOS expression in coronary endothelial cells. During pregnancy, there is no increase in the amount of glucose uptake, and the heart relies almost exclusively on FFA to support metabolism despite the increased workload. The left ventricle also undergoes a shift in mRNA expression to support enzymes that increase FFA metabolism and decrease enzymes that support glucose metabolism. L-NAME not only blocks the veratrine-induced, NO-dependent vasodilation but also causes a shift in cardiac metabolism from FFA to glucose, indicating the crucial role of NO.

Previous work by us has shown that pregnancy produces a physiological hypertrophy of the left ventricle (28). The current study confirmed those findings using crystals implanted in the left ventricle to measure LV chamber dimensions. There was an increase in diastolic diameter and volume in left ventricles from pregnant canines. This increase in LV end diastolic volume led to an increase in SV. Using the implanted pressure gauge, we were also able to determine  $dp/dt_{max}$  as an index of contractility. There was a small (10%) increase in  $dp/dt_{max}$  in left ventricles from pregnant canines. Because  $dp/dt_{max}$  usually occurs before the opening of the aortic valve, it is mostly afterload-independent; however, Quinones et al. (18) have demonstrated that  $dp/dt_{max}$  is preload dependent, since increases in preload resulted in corresponding increases in  $dp/dt_{max}$ . Thus the increase in LV function is most likely independent of changes in inotropic state.

In response to the increased demand placed on the heart during pregnancy, the work that the heart performs increased. One important finding from this study was the large increase in the TP and calculated work (cardiac output  $\times$  mean arterial pressure) during pregnancy. There was a  $>30\%$  increase in the TP during pregnancy compared with controls.  $M\dot{V}O_2$  increased slightly but significantly during pregnancy. The increase in  $M\dot{V}O_2$  in response to L-NAME administration was much larger in pregnant canines, suggesting that NO plays an important role in reducing basal oxygen consumption during pregnancy. When we examined the oxygen efficiency (TP/ $M\dot{V}O_2$ ) of the left ventricle, we found an overall increase in the amount of work performed compared with the amount of oxygen consumed. This supports our theory that an increase in NO allows for more work to be performed at a lower oxygen cost, i.e., increased efficiency.

The Bezold-Jarisch reflex is activated under some pathophysiological conditions, such as coronary ischemia, myocardial infarction, and aortic stenosis (33). Hornig et al. (8) have found that 4 wk of daily handgrip training significantly enhances endothelium-mediated flow-dependent dilation in the forearm of patients with heart failure, most likely by increased endothelial release of NO. Recent studies in our laboratory have also shown that coronary vasodilation induced by activation of the Bezold-Jarisch reflex is reduced in Type I diabetes (12) and hyperhomocysteinemia (26) and is enhanced after short-term exercise training in proportion to the altered release of NO (31). It is possible that the pregnancy-induced increase in eNOS could result in an increased control of CBF by the Bezold-Jarisch reflex.

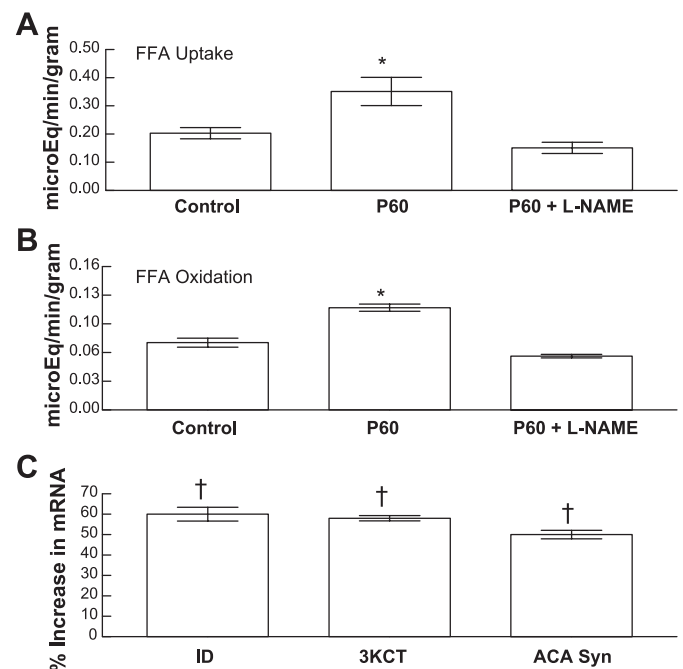


Fig. 4. Changes in free fatty acid (FFA) oxidative pathway. A: FFA uptake is increased during pregnancy (\* $P < 0.05$  vs. control) and is blocked by L-NAME. B: FFA oxidation significantly increases during pregnancy (\* $P < 0.05$  vs. control) and is inhibited by L-NAME administration. C: mRNA levels of isocitrate dehydrogenase (ID), 3-ketoacyl-CoA thiolase (3KCT), and acetyl-coenzyme A synthetase (ACA Syn) are increased during pregnancy († $P < 0.05$  from 0).

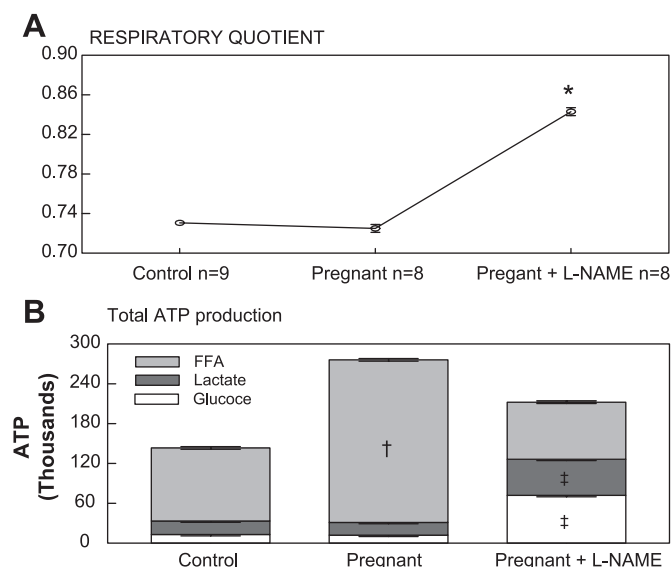


Fig. 5. A: respiratory quotient (RQ) is unchanged between control and pregnant states; L-NAME administration dramatically shifts the RQ toward glucose metabolism ( $*P < 0.05$  vs. control and pregnant). B: there is no change in total ATP production from glucose and lactate between control and P60. FFA ATP generation increases significantly ( $\dagger P < 0.05$  vs. control). L-NAME administration significantly increases ATP generation from glucose and lactate ( $\ddagger P < 0.05$  vs. control and pregnant), whereas FFA ATP generation is reduced to control levels. Error bars are artificially inflated to become visible.

The administration of L-NAME during pregnancy in dogs caused an increase in TPR (from  $0.023 \pm 0.01$  to  $0.056 \pm 0.02$ ,  $P < 0.05$ ) that is similar to an increase in TPR that occurs in preeclamptic patients. Preeclampsia is estimated to affect 7–10% of all pregnancies in the United States. Preeclampsia is typically characterized by hypertension, proteinuria, and edema. Despite being one of the leading causes of maternal death and a major contributor to maternal and perinatal morbidity, the mechanisms responsible for the pathogenesis of preeclampsia have not yet been fully elucidated. Decreases in NO production have been reported in women with preeclampsia (23). Also, NO synthase blockade with L-NAME in animals during mid- to late gestation results in pathological changes similar to those observed in women with preeclampsia, such as proteinuria, thrombocytopenia, and intrauterine growth retardation (14).

Another major finding of this study supporting an important role for NO is that NO production across the coronary circulation in the dog heart increases during pregnancy. Previous work in our laboratory has shown that there is a basal level of NO production by the coronary vessels of the dog (29). Bernstein et al. (1) also demonstrated an increase in transcardiac NOx production during exercise in the canine. Sessa et al. (24) showed that the primary stimulus for the production of NO by eNOS found in blood vessels is most likely the shear stress, the frictional force, caused by blood flow. This study demonstrates that, during pregnancy, the upregulation of eNOS occurs mainly in the endothelial cells lining the blood vessels in the heart. The immunohistochemistry staining showed large increases in eNOS staining in the endothelial cells in the LV. There appears to be some slight increase in staining in the myocytes themselves, but the relative increase in staining is small compared with the increase occurring in the endothelial cells.

During this study, plasma NOx from aortic and CS blood was measured in the nonpregnant and pregnant states. The aortic and CS plasma NOx increased during pregnancy. There was also a widening of the difference in plasma NOx across the heart. It is important to note that plasma NOx is measured as a concentration (nmol/l). If molar production were to remain constant in the presence of increased blood flow, the concentration would actually drop. As the concentration of plasma NOx and the level of CBF increased with pregnancy, clearly there is an increase in the molar production (nmol/min) by the coronary circulation. These findings are supported by our increased CBF in response to activation of the Bezold-Jarisch reflex and the ability of L-NAME to block it.

Depre et al. (6) demonstrated that cGMP inhibits cardiac glucose metabolism by decreasing glucose uptake in the isolated rat heart. Tada et al. (27) have shown that glucose uptake is elevated in Langendorff-perfused hearts of eNOS knockout mice, a condition that was mimicked by NO synthase inhibition in hearts from wild-type mice. Furthermore, this increase in glucose uptake was normalized by 8-bromo-cGMP or S-nitroso-N-acetylpenicillamine, which indicates that cardiac NO regulates myocardial glucose uptake via a cGMP-dependent mechanism. Recchia et al. (19) found a significant increase in myocardial glucose uptake and decrease in FFA uptake associated with a fall in cardiac NO production in conscious dogs with pacing-induced heart failure. Moreover, this shift in substrate use was also observed in normal dogs with short-term pharmacological inhibition of NO synthase with nitro-L-arginine, which was completely reversed by a NO donor (20). These previous reports suggest that NO plays an important role in the regulation of myocardial substrate use. This study showed that there was no increase in the uptake of glucose by the heart during pregnancy and an increased uptake of FFA. Despite the increase in work that the heart performs, the amount of glucose taken up from the coronary circulation does not increase, rather FFA uptake and oxidation in the coronary circulation are enhanced.

In a normal metabolic state, FFA is the major substrate for the heart and provides the highest yield of ATP per mole of substrate compared with glucose or lactate. Cardiomyocytes oxidize fatty acids derived from both the plasma and the breakdown of intracellular triacylglycerol stores, whereas pyruvate is derived from either lactate dehydrogenase or glycolysis. The rates of these metabolic pathways are tightly coupled to the rate of contractile work, which is increased significantly in the pregnant state. We demonstrate that there was a significant increase in the amount of FFA oxidized by the heart during pregnancy but that there is no increase in the amount of glucose oxidized.

This study found mRNA levels of genes associated with glucose and glycogen metabolism such as GP, pyruvate dehydrogenase, and 6-phosphofructokinase, among others, were downregulated. Phosphofructokinase-1 is the first enzyme that is common to the many possible starting points for glycolysis and is therefore a pivotal point of regulation. GP catalyzes the phosphorylation of glycogen chains, stopping four residues from an  $\alpha$ -(1 $\rightarrow$ 6) branch point and producing one molecule of glucose 1-phosphate for each glucose metabolized. Further degradation is accomplished by the two activities of the glycogen-debranching enzyme. The downregulation of GP could prevent the utilization of glycogen stores by the myocardium



during pregnancy. The downregulation of genes involved in glucose and glycogen metabolism suggests that the myocardium is shifting away from glucose metabolism.

In contrast, those genes involved in FFA oxidation such as IDH, 3KCT, and ACA Syn, among others, were elevated. 3KCT and acyl-CoA are both involved in the breakdown of FFA into acyl-CoA, before its transport across the mitochondrial membrane. The IDH step of the tricarboxylic acid cycle, due to its large negative free energy change, is one of the irreversible reactions in the cycle. The increase in mRNA of enzymes that control FFA oxidation during pregnancy compensates, i.e., generates ATP to make up for, the inhibition of glucose metabolism. This allows for increased ATP production in the heart without increased utilization of glucose. All together, this indicates that, during normal pregnancy, the heart adapts with hypertrophy and relies on FFA metabolism over glucose metabolism.

When L-NAME was administered to pregnant canines, we showed a shift in substrate metabolism by the heart away from FFA uptake and oxidation to glucose uptake and oxidation. This occurred despite the increase in expression of genes that regulate FFA metabolism and a decrease in genes that regulate glucose metabolism. Our previous studies have shown that heart failure is characterized by a shift in substrate use (11) and that this is mimicked in the healthy dog simply by inhibiting NO synthase (4).

Peripartum cardiomyopathy (PPCM) is a disorder of unknown etiology in which LV dysfunction and symptoms of heart failure occur between the last trimester and up to the first 5 mo postpartum. The causes of PPCM may include poor nutrition, toxemia, maternal response to fetal antigens, or myocarditis. Following delivery, for unknown reasons, heart failure persists in ~50% of these patients, and, if failure persists, the mortality rate has been estimated to range from 40% to as high as 93% (28). PPCM is the largest cause of heart transplants in women of child-bearing age.

Recently, a model of PPCM was developed in mice overexpressing the  $\alpha$ -subunit of  $G_q$ . These mice exhibit baseline cardiomyopathy and contractile dysfunction (5). Thirty to fifty percent of the mice develop lethal heart failure in the peripartum period, accompanied by cardiac myocyte apoptosis. The frequency of apoptosis in human PPCM specimens is quite low (~0.25%), although it is markedly higher than controls (~0.01%). Hayakawa et al. (7) recently examined the effects of a caspase inhibitor on the mortality of pregnant  $G_q\alpha$  mice. The caspase inhibitor caused an 89% reduction in cardiac myocyte apoptosis, and the 30% mortality characteristic of this model was completely ablated.

NO has been shown to inhibit the activation of caspase-3 (13). By inhibiting caspase activity through S-nitrosylation, NO can inhibit apoptosis in hepatocytes and endothelial cells through the blockage of the proteolytic activation of caspases as well as direct suppression of caspase activity (9). It should be noted that NO has also been implicated in the activation of the apoptotic pathway via the formation of peroxynitrite and subsequent nitrosative stress and mitochondrial damage. This could indicate that NO maintains a balance between inhibiting and activating the apoptotic pathways. Thus a change in the production of NO may result in the same pathophysiology that occurs in the  $G_q\alpha$  mouse during pregnancy. Similarly, women

who manifest PPCM may represent a defect in cardiac NO production.

We calculated the amount of ATP generated from each substrate during pregnancy and noted an increase in ATP generated from FFA with no increase in glucose- or lactate-governed ATP generation. This increase was reversible with L-NAME, indicating that it was NO dependent. LVFW weights (as measured by direct removal of the LVFW postdeath) increased during pregnancy ( $72.4 \pm 2.4$  vs.  $90.5 \pm 4.5$  g, controls vs. pregnant, respectively). This ~25% increase in LVFW mass cannot fully account for the more than doubling of FFA ATP generated in the heart. Indeed, we did not find an increase in glucose or lactate uptake despite the increase in LVFW mass. Thus the increase in NO levels during pregnancy results in a tonic inhibition of glucose and lactate uptake, with the effect of maintaining glucose and lactate uptake at low levels despite the increase in cardiac work.

In conclusion, we have shown that that, during pregnancy, there is an enhanced ability of NO to regulate CBF and to control myocardial substrate utilization. We showed that, during pregnancy, there is no increase in the amount of glucose uptake by the heart despite the increased workload. NO inhibits glucose uptake and promotes FFA metabolism in the heart, especially during pregnancy. This allows the heart to utilize/oxidize primarily FFA to supply the increased energy required. In support of this, there is an increase in the enzymes that metabolize FFA and a downregulation of those that metabolize glucose or lactate.

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