# Prenatal Arginase Changes and Fetal Oxygenation

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**Abstract:** We have previously reported that developmentally, lung arginase expression and activity are highest during fetal life. Prenatally oxygenation and gas exchange is dependent on uterine blood flow and placental function. As gestation advances, the systemic vascular resistance decreases and uterine blood flow progressively increases to accommodate the fetal needs. The factors accounting for the pregnancy-associated vasodilation are not fully understood. Arginases compete with nitric oxide synthases for L-arginine as a substrate and their tissue expression and activity are known to modulate nitric oxide-dependent vascular tone. Hypothesizing that the pregnancy-induced vasodilation is mediated by a decrease in arginase activity, we evaluated mesenteric arterial and uterine tissue from nonpregnant and late gestation rats. A statistically significant ($P<0.01$) decrease in arginase activity was documented in both tissues from pregnant animals, when compared with nonpregnant rats. The mesenteric arterial endothelium-dependent vasodilation of pregnant rats was significantly increased ($P<0.01$) when compared with vessels from nonpregnant animals. Arginase inhibition abrogated this difference. Distinct changes in arginases activity were present in the mother and fetus. Whereas in the fetal lung vascular tissue arginase activity was upregulated to maintain a high pulmonary vascular resistance, the opposite occurred in the maternal systemic circulation where a decrease in vascular tissue arginase activity enhanced uterine blood flow.

**Keywords:** Fetus, mesenteric arteries, nitric oxide, uterus.

## INTRODUCTION

There are two known isoforms of arginase, types I and II [1]. Arginase I is located in the cytosol and strongly expressed in liver, whereas arginase II is confined to the mitochondrial matrix and ubiquitously expressed in several organs. The role of arginase I in the liver is well defined, where it catalyzes the deamination of L-arginine to produce ornithine and urea. The role of extrahepatic arginases is not very clear, however, ornithine, the downstream product of arginase activity is known to be further metabolized into polyamines that are involved in tissue repair and growth, as well as to proline, the precursor of collagen formation [2].

The amino acid L-arginine is a substrate for both nitric oxide synthases (NOSs) and arginases. Arginases compete with NOSs for L-arginine as substrate, such that endothelial NOS (eNOS)-dependent nitric oxide (NO) production is inversely proportional to vascular tissue arginase activity [3-5]. Changes in arginase expression and/or activity have been reported in a number of human systemic vascular diseases. These include increased arginase activity and/or expression in diabetic patients with erectile dysfunction [6], systemic hypertension [4], atherosclerosis [7] and preeclampsia [8]. Yet arginases also appear to modulate a number of physiological conditions by altering their expression and/or activity in a tissue-specific manner.

We have recently shown that lung arginase expression and activity in the rat is developmentally regulated, being expressed at the highest levels in the fetus and newborn [9]. As such, arginases likely play an important role in the pulmonary vasodilation that characterizes the transition from fetal to postnatal life.

The oxygenation and gas exchange of the fetus is dependent on the uterine-placental blood flow and function. Arginase I and II are expressed in human placenta [10]. In rats, myometrium arginase type II protein expression decreases with gestation whereas arginase I is mostly undetectable during pregnancy [11]. In these rats, these changes are associated with a progressive decrease in arginase activity throughout gestation with a rapid rise immediately before delivery [11]. In contrast, pre-eclampsia, a human condition characterized by systemic hypertension, placental growth and vascular functional impairment, is associated with an increase in plasma arginase activity [12].

Hypothesizing that a decrease in arginases activity and enhancement in endothelium-dependent relaxation account for the reduced systemic vascular resistance during gestation, we evaluated mesenteric arteries of nonpregnant and late gestation female rats. In these female rats, pregnancy was associated with a significant decrease in mesenteric arterial arginase activity and enhanced endothelium-dependent vasodilation. A similar reduction in uterine tissue arginase activity was documented in the pregnant rats suggesting that

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uterine vascular tone is also regulated by this enzyme during gestation.

## METHODOLOGY

### Animals

Timed-pregnant and non-pregnant young adult **(**250-300 g) Sprague-Dawley female rats (Charles River, Ontario, Canada) were studied. The animals were sacrificed with pentobarbital sodium overdose (40 mg/kg ip). Immediately following death, the uterine tissue and mesenteric bed were removed. Secondary branches of mesenteric arteries (average diameter 200 μm) were isolated and either frozen with the uterine tissue for subsequent measurement of arginase activity, or mounted in the pressurized vessel system for study of their vasodilatory potential.

All procedures were conducted according to criteria established by the Canadian Council on Animal Care and were approved by the Sunnybrook and The Hospital for Sick Children Research Institute Animal Care Committees.

### Measurement of Arginase Activity

Arginase activity was measured according to the method described by Corraliza *et al.* [13]. In brief, the extracts were incubated with equal volumes of 10 mM manganese chloride in 25 mM Tris-HCl, pH 7.4 at 56$^{o}$C for 10 minutes to activate the enzyme. Then 50 μl of the activated extracts were transferred to boil-proof tubes containing 50 μl of 20-250 mM of L-arginine, pH 9.7 with or without nor-NOHA ($N^{w}$-Hydroxy-nor-L-arginine, Calbiochem), a competitive inhibitor of arginase activity. These assay mixtures were incubated at 37$^{o}$C in a shaking water bath for one hour. The reaction was stopped by adding 800 μl of the acid mix comprised of sulphuric acid, phosphoric acid and water in a ratio of 1:3:7. 50 μl of 9% ISPF (α-isonitrosopropiophenone) dissolved in ethanol was added, the tubes were incubated in a boiling water bath for 45 minutes. The colour was developed by keeping the tubes in the dark at room temperature for 10 minutes. Aliquots of 200 μl were transferred to a 96-well plate and absorbance at 540 nm was measured using an Ersa Max micro plate reader (Plate Technologies, Sunnyvale, California USA). Each assay was run in triplicate and the activity was completely inhibitable with nor-NOHA, confirming that the urea produced was the result of arginase activity.

### Pressurized Vessel System

The mesenteric bed was extracted and placed in ice-cold Krebs-Henseleit phosphate buffered solution. Mesenteric artery second order branches were dissected free and mounted on glass cannulas in an arteriograph chamber (Danish Myo Technology A/S, Denmark) and maintained immersed in Krebs-Henseleit solution at 37$^{o}$ C without flow. The intraluminal pressure was maintained at a constant at 60 mmHg. The vessel diameter changes were continuously monitored and recorded (VediView Software; Danish Myo Technology A/S, Denmark).

Following a 30 minutes equilibration period, a phenylephrine dose response curve was obtained to determine the molar concentration leading to 50% of maximum constriction ($EC_{50}$). For the determination of endothelium-dependent vasodilation, the mesenteric arteries were preconstricted to their $EC_{50}$ and progressively stimulated with acetylcholine in the absence or presence of Nor-NOHA ($10^{-5}$ M).

### Drugs

All drugs were obtained from Sigma Aldrich, Oakville, Ontario Canada.

### Data Analysis

Data were evaluated by two-way analysis of variance (ANOVA) with multiple comparisons obtained by the Tukey-Krammer test, or unpaired Student's t-test when appropriate. Statistical significance was accepted at $P<0.05$. All statistical analyses were performed with the Number Cruncher Statistical System (NCSS, Kaysville, Utah, USA). Data are presented as means±SEM.

## RESULTS

The mesenteric arterial tissue arginase activity of nonpregnant adult female was 70±3 mUnit/mg protein (N=4) and not significantly different than documented in tissue derived from comparable age male rats (58±6; N=4). Following pregnancy, the female rat mesenteric arterial tissue arginase activity decreased and at 21 days gestation was significantly lower, when compared with the nonpregnant state (Fig. **1**). A similar pregnancy-associated decrease in arginase activity was observed in uterine tissue (Fig. **1**).

In order to determine whether the reduced arginase activity of the mesenteric arteries had any functional significance, we compared the endothelium-dependent response in vessels from pregnant and nonpregnant adult rats. In response to a similar concentration of phenylephrine ($6x10^{-7}$ M) to achieve the $EC_{50}$, pregnant rats' mesenteric arteries showed a 77±15 μm diameter reduction (vasoconstriction). In contrast, the vessels from nonpregnant animals showed significantly greater ($P<0.05$) magnitude of diameter change (117±13 μm; N=6).

In the phenylephrine precontracted vessels the magnitude of acetylcholine-induced vasodilation response was left-shifted and statistically greater ($P<0.01$) in pregnant, when compared with mesenteric arteries from nonpregnant animals (Fig. **2**, Panel A). Preincubation with an arginase inhibitor (Nor-NOHA $10^{-5}$ M) abolished the difference in acetylcholine dose-dependent vasodilatory response (Fig. **2**, Panel B).

## DISCUSSION

In the adult female rat, we showed that pregnancy induces a decrease in tissue arginase activity of near resistance mesenteric arteries. This was associated with a lower vasoconstrictive response to phenylephrine, suggestive of enhanced endothelium-derived basal nitric oxide generation. The mesenteric arteries' endothelium-dependent vasodilatory response was significantly greater in pregnant animals, when compared with nonpregnant rats. Further supporting the evidence that this increased basal and acetylcholine-stimulated vasodilatory response was related to the reduced vascular tissue arginase activity, enzyme inhibition abolished the pregnancy-associated vasodilatory response difference. As a surrogate marker of its vasculature,

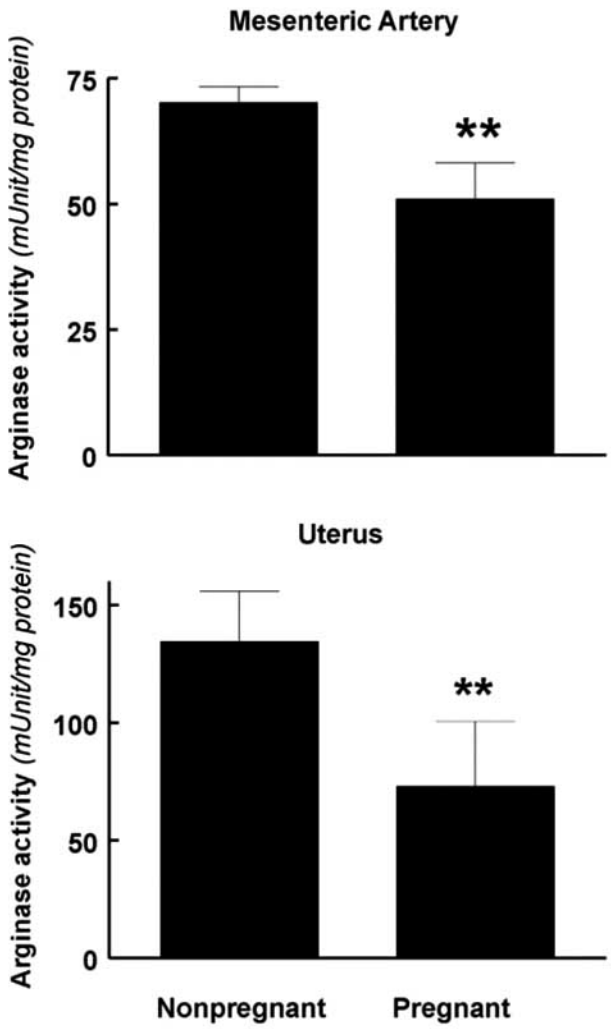


**Fig. (1).** Mesenteric artery (Panel A) and uterine (Panel B) tissue arginase activity of nonpregnant and pregnant rat. N=4 for each tissue and pregnant/nonpregnant group. ** P<0.01 as compared with nonpregnant tissue

the uterine tissue arginase activity was significantly reduced at the end of pregnancy, when compared with nonpregnant values. Together these data provide strong evidence that arginase activity changes modulate the reduction in systemic vascular resistance and enhanced uterine blood flow during pregnancy.

Arginase I and II are both found in human placenta, but the pattern and cell distribution of expression varies according to gestational stage [10]. Placental tissue arginase activity increases with gestation and is highest at term [14]. Arginase type I is only present in cytotrophoblasts, whereas type II is expressed in these, as well as syncytiotrophoblasts. Both types are expressed in the placenta villi with arginase II being more abundant in the first, as compared with last trimester of pregnancy [10]. In pregnant sheep placental arginase activity increased with gestation [15]. In contrast arginase activity was not detected in porcine placenta [16].

Whether changes in arginase activity or even the enzyme itself are responsible for placental growth and polyamine generation is controversial. Arginase activity was not detected in the porcine placenta, but ornithine aminotransferase, ornithine decarboxylase, proline transport, polyamine synthesis from proline and its concentrations increased from early to mid-gestation and declined towards term [16]. Thus the extent to which arginase, as compared with other downstream enzymes, regulate polyamine metabolism in the placenta requires further investigation.

Equally unclear is the cell distribution and role of arginases in the uterus and their potential role in pregnancy. The role of arginases in uterine contractions during pregnancy is poorly understood. In the present study, we showed that when pregnant rats' uterine tissue was compared with nonpregnant rats' uterine tissue, arginase activity was reduced in late gestation. Similar results were reported by Hirata *et al* in the rat [11]. Arginase activity of non-decidualized tissue was higher when compared with placental implantation sites in the pregnant rat [17], further supporting the evidence that uterine arginase activity decreases with pregnancy. Arginase gene expression was found to be altered in COX-1 knockout mice implicating this enzyme in the delayed parturition observed in this genetically modified mammal [18]. In contrast, the guinea pig myometrium arginase activity was found to be higher during pregnancy, when compared with nonpregnant female animals and increased with gestation until term [19]. A distinct method of measuring arginase activity was employed in the latter study possibly accounting for the conflicting results. However, species differences in the pattern of arginase activity changes during pregnancy cannot be discarded.

Whether arginases are expressed by the uterine smooth muscle cells or its vasculature merits further discussion. Pregnancy is associated with a decrease in systemic vascular resistance and concomitant increase in cardiac output [20]. A greater than threefold increase in uterine blood flow is observed during pregnancy and this is essential to ensure the fetal nutritional and gas exchange needs. The factors accounting for the pregnancy-associated increase in uterine blood flow are unclear. In the present study, we showed evidence that uterine tissue arginase activity decreased with pregnancy in the rat. These novel data raise the possibility that most of the arginase present in the uterine tissue derives from its vasculature. When compared with nonpregnant animals, the documented decrease in pregnant rat arginase activity may increase L-arginine substrate to uterine arterial eNOS and thereby promoting enhanced NO generation and consequent vasodilation of this vasculature.

In the present study, we showed that a similar pregnancy-associated decrease in vascular tissue arginase activity was present in near resistance mesenteric arteries. This gestation-induced change was associated with a greater basal and endothelium-dependent vasodilatory potential confirming a causal relationship between arginase activity changes and vascular NO generation in the rat systemic vascular bed. A similar pregnancy-induced increase in vasodilatory response to calcitonin gene-related peptide was demonstrated in rat mesenteric arteries [21]. In women, an enhanced endothelium-dependent flow-mediated relaxation was also documented in systemic vessels during pregnancy [22]

Berkowitz *et al.* [23] had shown that both arginase isotypes were expressed in the rat aortic endothelium. Arginase I had been found to be constitutively expressed in

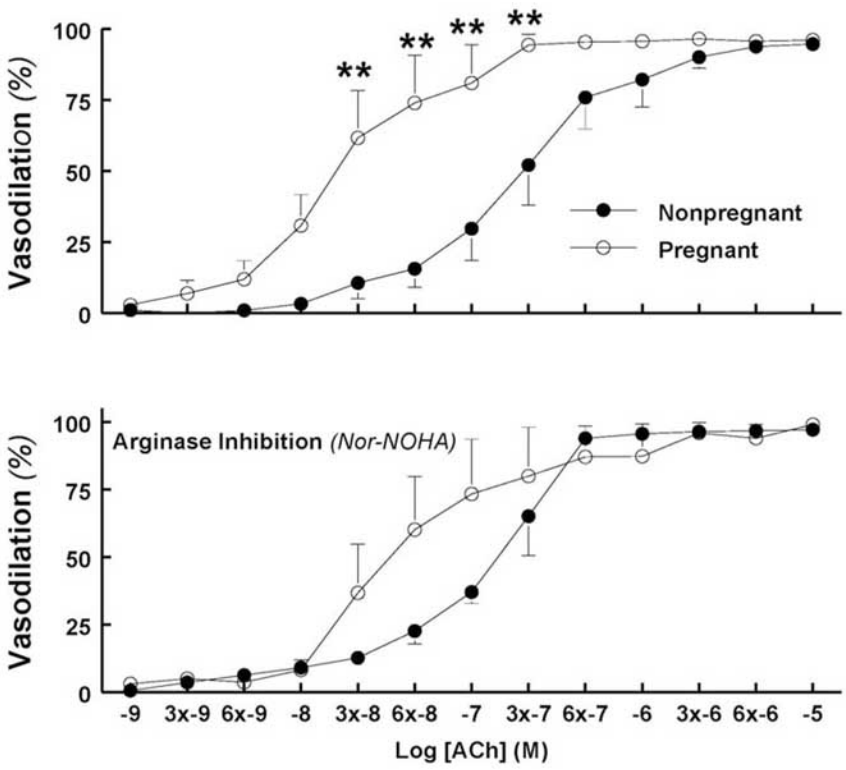


**Fig. (2).** Nonpregnant (N=) and pregnant (N=) phenylephrine (EC 75) preconstricted mesenteric artery acetylcholine (ACH) endothelium-dependent dose response in the absence (Panel A; N=4-6) and presence (Panel B) of an arginase inhibitor (Nor-NOHA $10^{-5}$ M; N=3-4). ** P<0.01 as compared with nonpregnant vessels by two way ANOVA and multiple comparison by Tukey-Kramer test.

endothelial cells [24], whereas arginase II appeared to be inducible in response to cytokines [25]. White *et al* [3] have demonstrated that arginase type I knockdown could enhance NOS activity in adult rat thoracic aortic tissue and Zhang *et al* had shown in the porcine coronary artery that arginase type I was responsible for modulating the endothelial-dependent relaxation [24]. Arginase I expression was found upregulated in older rats and may account for the endothelial dysfunction observed later in life [23]. The present study data suggest that changes in vascular tissue arginase activity play an important role in this process. In contrast, there is evidence that an increase in pregnancy-associated arginase activity results in systemic hypertension and other vasoconstrictive-related disorders, such as seen in preeclampsia.

Preeclampsia is a serious clinical complication of pregnancy. Its clinical manifestations include systemic hypertension and abnormal placental growth. Its etiology is presently unknown, but arginases may play a role in the maternal systemic hypertension and placental dysfunction. When compared with normotensive women, placental villi from pregnancies associated with preeclampsia showed increased arginase II mRNA gene expression and protein tissue content [8], as well as a relative deficiency of nitric oxide bioavailability [26]. Similarly, plasma arginase activity was significantly increased in preeclamptic, as compared with women with normal pregnancies [12].

In the lung, both isoforms of arginase are expressed and increased arginase activity has been reported in obstructive airway diseases such as asthma [27, 28], cystic fibrosis [29-31], as well as in pulmonary hypertension [32-37]. In the bleomycin-induced lung fibrosis mouse model, arginase expression and activity increased [38], suggesting that these enzymes significantly contribute to the lung remodeling process. We have previously shown that expression and activity of arginases in the lung are developmentally regulated [9]. This evidence, together with the present study's data shows that changes in arginase activity modulate fetal oxygenation and gas exchange. Prenatally, this is done by down-regulating the enzyme activity in maternal systemic vessels to maximize uterine blood flow and up-regulating it in the pulmonary vasculature to minimize lung blood flow. Postnatally, pulmonary vascular arginase activity decreases to enhance NO generation and enhance lung blood flow.

Aside from their role in vascular resistance and uterine contractility, arginases may also play an important role in pregnancy-associated immunotolerance since L-arginine depletion leads to impaired lymphocyte immune responses [39]. Conflicting data, however, were reported regarding the human plasma arginase activity changes associated with pregnancy. When compared with activity levels of nonpregnant women, Carpinteiro *et al.* reported a decrease with pregnancy [40], whereas Remesar *et al* [41] and more recently Kropf *et al.* l showed the opposite results [14, 40].


**Fig. (3).** Mother-fetal changes in arginase activity during pregnancy.

Interestingly, these latter authors suggested that the increase in plasma arginase activity during gestation accounts for the relative T cell immunosuppression in pregnant women [14]. Downregulation of CD3 zeta-mediated T cell response via arginase-induced L-arginine depletion is believed to mediate this process [14.] This suggest that not only is L-arginine an important physiological regulator of T cell proliferation potential and responsiveness, but modulation of this substrate activity might be the most important factor accounting for the relative immunosuppression of pregnancy.

What is intriguing about the changes in arginase activity and/or expression associated with pregnancy is its cell-specific pattern and likely purely autocrine physiological role. The evidence that pregnant rats' mesenteric arteries, a widely used vessel to evaluate the regulation of systemic vessel resistance, exhibit an *in vitro* enhanced vasodilatory potential, suggest that autocrine, and not humoral factors account for the gestation-associated hemodynamic changes. As shown in Fig. (**3**), the direction of change in arginase activity was clearly tissue/cell specific. Whereas arginase activity increased in white blood cells (blood) [14], in the systemic vascular tissue, pregnancy was associated with a decrease in this enzyme activity, as shown in the present study. Thus, the L-arginine availability for eNOS in vasculature appears dependent on its tissue, and not blood, arginase activity.

In summary, the expression and/or activity changes of arginase during pregnancy are orchestrated in a cell specific autocrine fashion. The plasma arginase activity is mostly representative of the changes occurring in the blood cells and neither reflects organ-specific changes, nor contributes to the regulation of vascular resistance. Given the essential nature of L-arginine to a number of biological pathways up- and downregulation of its cell-specific availability via arginase activity changes plays an important and previously unrecognized role in pregnancy. Lastly, fetal oxygenation and gas exchange may be maximized by enhanced uterine blood flow and increased pulmonary vascular resistance resulting from vascular tissue arginase activity regulation.

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