Local Effects of Pregnancy on Connexin Proteins That Mediate Ca$^{2+}$-Associated Uterine Endothelial NO Synthesis

Timothy J. Morschauser, Jayanth Ramadoss, Jill M. Koch, Fu Xian Yi, Gladys E. Lopez, Ian M. Bird, Ronald R. Magness

Abstract—Uterine artery adaptations during gestation facilitate increases in uterine blood flow and fetal growth. Hypothesis: local expression and distribution of uterine artery connexins play roles in mediating in vivo gestational eNOS activation and NO production. We established an ovine model restricting pregnancy to a single uterine horn and measured uterine blood flow, uterine artery shear stress, connexins 37/43, and P635eNOS protein levels in uterine artery and systemic artery (omentum and renal) endothelium and connexins in vascular smooth muscle. Uterine blood flow and shear stress were locally (unilaterally) and substantially elevated by gestation. During pregnancy, uterine artery endothelial gap junction proteins connexins 37/43 were locally regulated in the gravid horn and elevated 10.3- and 25.6-fold; uterine artery endothelial P635eNOS and total eNOS were elevated 3.3- and 2.9-fold; whereas uterine artery vascular smooth muscle connexins 37/43 were locally elevated 12.5- and 5.9-fold, respectively. Less pronounced changes were observed in systemic vasculature except for significant pregnancy-associated increases in omental artery vascular smooth muscle connexin 43 and omental artery endothelial P635eNOS and total eNOS. Gap junction blockade using connexin 43, but not connexin 37–specific Gap peptides, abrogated uterine artery endothelial ATP-induced Ca$^{2+}$-mediated NO production. Thus, uterine artery endothelial connexin 43, but not connexin 37, regulates Ca$^{2+}$-mediated NO production required for the vasodilation to accommodate increases in uterine blood flow and shear stress during healthy pregnancies. (Hypertension. 2014;63:00-00.) • Online Data Supplement

Key Words: ATP • calcium • connexins • endothelium • estrogens • nitric oxide • pregnancy
We hypothesize that associated with physiological rises in UBF and UA shear stress, ex vivo expressions of UAendo and UAvsm Gap junction proteins are elevated during pregnancy versus the nonpregnant luteal and follicular phase, and that restricting pregnancy to a single uterine horn only induces local unilateral ipsilateral increases in UBF, UA shear stress, and UAendo/UAvsm Cx expression. We evaluated (1) UBF, UA shear stress, and expressions of Cx37/Cx43 in UAs (endo versus VSM), as well as UAendo serine P\textsuperscript{385} and total eNOS during the ovarian cycle and pregnancy; (2) if changes in UBF, UA shear stress, and Cx/eNOS expressions are unilaterally and locally specific to the gravid uterine vascular bed or also noted in systemic (omental artery [OA] and renal artery [RA]) arteries; and (3) if Cx37 or Cx43 ex vivo are functionally responsible for modulating Ca\textsuperscript{2+}-induced NO production by UAendo during gestation.

**Materials and Methods**

See the online-only Data Supplement.

**Animal Ethical Approval**

We used 54 multiparous ewes (*Ovis aries*). Protocols were approved by the Animal Care and Use Committee.

**Tissue Collection and Ex Vivo Endothelium Versus VSM Isolation**

Nonsurvival surgery was performed on nonpregnant (luteal, n=8; follicular, n=8), unilateral pregnant (nongravid versus gravid sides, n=15), and control pregnant (n=23) groups. Using transonic flow probes, bilateral UBF was determined,\textsuperscript{16,25} and arteries were obtained and frozen. Endothelial-isolated proteins from UAendo, OAendo, R Avaendo, and adjacent VSM\textsuperscript{11–15} were prepared. Western analysis was performed using Cx37, Cx43, P\textsuperscript{385} eNOS, and total eNOS antibodies.

**Calculation of Shear Stress**

Shear stress was calculated using $\frac{4Q}{\pi r^2}$, where $Q$ is UBF, $\eta$ is viscosity, and $r$ is radius.

**Simultaneous In Situ Measurement of [Ca\textsuperscript{2+}] and NO in Intact UAendo**

[Ca\textsuperscript{2+}]\textsuperscript{i} and NO imaging were performed using fura-2-acetoxymethyl ester (AM) and diaminofluorescein (DAF)-2 diacetate\textsuperscript{26} comparing control versus ATP (100 μmol/L) treatments with or without (43, 37) Gap27 (300 μmol/L) to inhibit Cx37 Gap junctions. Additional control (40, 37)Gap26 and scrambled Gap27 were evaluated.

**Statistical Analysis**

ANOVA was performed (luteal, follicular, nongravid unilateral, gravid unilateral, and pregnant) followed by comparisons using protected Fisher least significant difference. Regression analyses were performed to determine the highest order (linear, quadratic, etc; Table S1 in the online-only Data Supplement) regression models among UBF, Cxs, and eNOS. Data are reported as mean±SEM ($P<0.05$).

**Results**

**UBF and Shear Stress**

When compared with luteal UBF (21.8±1.4 mL/min), perfusion was elevated to 51.1±5.4 and 42.4±8.9 mL/min during the follicular phase and to the nongravid side of the unilateral pregnant ewes. UBF to unilateral gravid and control pregnant horns were equal (850±74 and 838±68 mL/min) and greater than nonpregnant and unilateral nongravid groups. When compared with luteal (4.1±0.35 dynes/cm\textsuperscript{2}), follicular phase (8.0±0.85 dynes/cm\textsuperscript{2}) and unilateral nongravid (6.4±1.76 dynes/cm\textsuperscript{2}) shear stresses were elevated. Shear stress in both the unilateral gravid and the control pregnant groups was equal and significantly elevated (21.5±3.77 and 25.2±1.96 dynes/cm\textsuperscript{2}; Figure 1).

**Cx37 and Cx43 Expression in UAendo and UAvsm**

UAendo Cx37 remained unchanged and relatively low in follicular and unilateral nongravid groups (2.1±0.8- and 1.5±0.5-fold of luteal; Figure 2A). UAendo Cx37 in the unilateral gravid and control pregnant groups was, respectively, 6.4±2.0- and 10.3±1.6-fold higher than luteal and greater than follicular and unilateral nongravid groups. UAendo Cx43 levels remained unchanged and were relatively low in nonpregnant follicular and unilateral nongravid groups (3.2±0.9- and 5.1±1.0-fold). Unlike Cx37, UAendo Cx43 levels were elevated similarly in the unilateral gravid (21.3±4.1-fold) and control pregnant groups (25.8±4.7-fold). UAendo Cx43 was correlated ($P<0.001$) more so than Cx37 with UBF (Figure S1A and S1B).

Follicular UAendo Cx37 had intermediary values (2.2±1.8-fold) when compared with either nonpregnant luteal or unilateral nongravid groups (Figure 2B). The unilateral nongravid group was elevated 6.8±2.1-fold. UAendo Cx37 was elevated similarly in the unilateral gravid and control pregnant animals 13.4±2.5- and 12.5±2.2-fold, UAvsm Cx37 was elevated in the unilateral nongravid, gravid, and pregnant groups 4.7±1.3-, 5.7±1.1-, and 5.8±1.3-fold, respectively. Follicular UAendo Cx37 had intermediary values (3.2±1.1-fold) similar to the other 4 groups. UAvsm Cx37 was correlated ($P<0.001$) more so than Cx37 with UBF (Figure S1C and S1D).

**Cx37 and Cx43 Expression in OAvsm and R Avaendo**

OAvsm Cx37, OAendo Cx43, and RAendo Cx37 remained unchanged (Figure S2). RAendo Cx37 was lower in the unilateral group ($P<0.012$).

**Cx37 and Cx43 Expression in OAvsm and R Avaendo**

OAvsm Cx37 and Cx43 were unchanged except Cx43 was elevated in pregnant controls (Figure S3). R Avaendo Cx37 was
decreased in follicular, unilateral, and pregnant. RAvsm Cx43 levels were similar among the 4 groups.

**P**<sup>635</sup> eNOS and Total eNOS Expression in UAendo, OAendo, and RAendo

Levels of the UAendo excitatory eNOS phosphorylation site P<sup>635</sup> were relatively low and remained unchanged in the follicular and unilateral nongravids (2.4±0.8- and 1.1±0.4-fold; Figure 3A). UAendo P<sup>635</sup>eNOS levels were elevated 3.8±0.7- and 3.3±0.6-fold in the unilateral gravid and pregnant groups, respectively, which were greater than unilateral nongravid but not the folliculars (0.1>P<0.05). UAendo total eNOS for follicular and unilateral nongravids averaged 1.9±0.8- and 0.3±0.1-fold and was equally elevated in unilateral gravid (4.6±1.0-fold) and pregnant (2.9±0.6-fold). UAendo Cx37 and Cx43 were highly correlated with either UAendo P<sup>635</sup>eNOS or total eNOS more so in the unilateral gravid (<i>P</i>&lt;0.007) than in the pregnant controls (Figure S4). OAendo P<sup>635</sup>eNOS remained unchanged in follicular and unilateral groups (1.4±0.4- and 1.1±0.15-fold; Figure 3B) but was elevated 3.0±0.6-fold in pregnant controls. OAendo total eNOS levels remained low in the follicular and unilateral groups (1.9±0.8- and 0.7±0.1-fold) and was elevated 5.8±1.5-fold in pregnant controls. RAendo P<sup>635</sup>eNOS and total eNOS levels did not change among the 4 groups (Figure 3C).

**Simultaneous Ex Vivo Imaging of [Ca]<sup>2+</sup> and NO in UAendo**

To determine the role of Gap junctions on ATP-induced periodic [Ca]<sup>2+</sup> bursts and rapid oscillations in UAendo (Figure 4), (43, 37)Gap27<sup>29</sup> was used to interrupt Gap junction communication. Pretreatment (3 hours) with (43, 37)Gap27 had little effect on the ATP-induced maximal [Ca]<sup>2+</sup> height or incidence of the initial [Ca]<sup>2+</sup> peak but abrogated long-term [Ca]<sup>2+</sup> bursts and oscillation responses. Because (43, 37) Gap27 (sequence SRPTEKTIFII) inhibits Cx43 and Cx37, we established peptide specificity using (40, 37)Gap26 (specific to Cx40 and Cx37; n=5; Figure 4) or (43, 37 scramble peptide)Gap27 (n=2).<sup>29</sup> UAendo (43, 37)Gap27 treatment, not the other peptides, specifically blunted both overall NO production and maximum UAendo NO production rate by 49%.

**Discussion**

Consistent with previous reports,<sup>2,10,16,17</sup> follicular phase and late gestation UBFs were elevated. Restricting pregnancy to a single uterine horn<sup>9,20</sup> only showed increased ipsilateral UBF possibly because of pregnancy-specific uterine adaptations to local endogenous hormone secretion (eg, estrogen, VEGF). Although there was a 25% to 50% reduction in total number of placentomes,<sup>19</sup> UBF to the gravid unilateral and control pregnant horns was equal as reported in another study<sup>30</sup> in which 1 horn was ligated on day 5 of pregnancy; however, they did not measure nongravid UBF. The mechanism(s) how UBF equilibrates...
Cx43 is upregulated when endogenous estrogen is elevated, and Cx43 is categorized as a contraction-associated protein increased in labor when the estrogen/progesterone ratio is high. Pregnancy-associated Cx37 was increased equally in UAendo and UAvsm (10.3- and 12.5-fold), suggesting that Cx37 synthesis may be coregulated for forming myoendothelial Gap junctions between UAendo and UAvsm. By contrast, pregnancy-induced Cx43 increases were much greater in UAendo than in UAvsm (25.5- and 5.8-fold).

Consistent with previous reports, we observed that UAendo total eNOS is elevated during the follicular phase and pregnancy reporting for the first time that surgically induced unilateral gravid, but not nongravid UAendo, had increases in eNOS expression. Stimulatory phosphorylation serine 635, an index of enzyme activity in UAendo,22,23 is also elevated unilaterally by pregnancy, suggesting that both expression capacity and activity of eNOS are increased. In addition, we observed pregnancy-associated adaptations in the nonreproductive OAendo, which showed increases in both P635 eNOS and total eNOS, the latter confirming our previous observation.24 This suggests a systemic OAendo adaptation to a vasorelaxation phenotype consistent with studies demonstrating that systemic and uterine resistance vessels show greater endothelium and NO-mediated vasorelaxation in pregnant versus nonpregnant rats and women.26

Using least squares regression analysis, we defined significant relationships between UA Cxs with UBF or eNOS. Cx37 or Cx43 relative to UBF (Figure S1) data points for pregnant controls and the unilateral gravid sides clustered in very similar patterns. Moreover, UBF was maintained in the unilateral model similar to that observed in the pregnant controls even though overall uterine space and tissue mass were reduced. R² values for UAendo Cx37 and Cx43 with UBF were 0.48 and 0.60, respectively, demonstrating a stronger relationship for UAendo Cx43 to regulate UBF. In contrast, UAvsm Cx37 and Cx43, showed a strong relationship with UBF; R² values of 0.43 and 0.18, respectively, suggesting that UAvsm Cx37 may, in part, be regulating vascular growth and remodeling.24,25 Correlations of UAendo Cx43 with P635 eNOS and total eNOS were highly significant and greater than UAendo Cx37 (Figure S4). Within the gravid unilateral, this relationship was greatly improved, demonstrating that Cx43 and eNOS are more correlated with the gravid side of the pregnancy (ie, R² values increased to 0.43 for P635 eNOS and 0.47 for total eNOS independent of the substantially lower control pregnant data points). Thus, UAendo Cx37, but not Cx37, has an intimate positive association with P635 eNOS and total eNOS. This suggests that UBF maintenance, even in the face of greatly reduced uterine space, is adaptive via an eNOS mechanism to maintain UA delivery of nutrients and oxygen for fetal growth.

The current physiological functional ex vivo data showing that Cx43, but not Cx37, was a prerequisite requirement for ATP-mediated Ca²⁺ burst–associated NO production are consistent with our observations in passage 4 P-UAECs, but not NP-UAECs.28 Gap27, a Cx (43, 37)–specific peptide, did not alter the initial peak of calcium but reduced subsequent Ca²⁺ bursts seen on ATP treatment. As recently described,39 the ATP-stimulated [Ca²⁺]i response in individual UAendo cells is composed of an initial peak followed by transient calcium bursts that are required for the simultaneous and robust

Figure 4. Simultaneous in situ measurements of intracellular free Ca²⁺ concentrations ([Ca²⁺]i) and NO levels in individual cells of intact uterine artery (UA) endothelium. A, Representative simultaneous recordings of ATP-induced (100 μmol/L; 25 minutes) increases in [Ca²⁺]i, and diaminofluorescein (DAF)-2 fluorescence (NO) from a single intact UAendo cell from control pregnant ewes. B, Effect of Gap27 (300 μmol/L) pre-exposure on ATP-induced [Ca²⁺]i and NO production. C, Effect of Gap26 (300 μmol/L) pre-exposure on ATP-induced [Ca²⁺]i and NO production. D, Summary: effect of Gap27 and Gap26 (300 μmol/L) pretreatment on means±SEM ATP-induced [Ca²⁺]i and NO production in intact UAendo. Responses: we determined [Ca²⁺]i and NO levels in individual panels. Using least squares regression analysis, we defined significant relationships between UA Cxs with UBF or eNOS. Cx37 or Cx43 relative to UBF (Figure S1) data points for pregnant controls and the unilateral gravid sides clustered in very similar patterns. Moreover, UBF was maintained in the unilateral model similar to that observed in the pregnant controls even though overall uterine space and tissue mass were reduced. R² values for UAendo Cx37 and Cx43 with UBF were 0.48 and 0.60, respectively, demonstrating a stronger relationship for UAendo Cx43 to regulate UBF. In contrast, UAvsm Cx37, not Cx43, showed a strong relationship with UBF; R² values of 0.43 and 0.18, respectively, suggesting that UAvsm Cx37 may, in part, be regulating vascular growth and remodeling.24,25 Correlations of UAendo Cx43 with P635 eNOS and total eNOS were highly significant and greater than UAendo Cx37 (Figure S4). Within the gravid unilateral, this relationship was greatly improved, demonstrating that Cx43 and eNOS are more correlated with the gravid side of the pregnancy (ie, R² values increased to 0.43 for P635 eNOS and 0.47 for total eNOS independent of the substantially lower control pregnant data points). Thus, UAendo Cx37, but not Cx37, has an intimate positive association with P635 eNOS and total eNOS. This suggests that UBF maintenance, even in the face of greatly reduced uterine space, is adaptive via an eNOS mechanism to maintain UA delivery of nutrients and oxygen for fetal growth.

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production of NO. We show herein that the ATP-stimulated pregnancy programmed burst pattern for Ca2+-mediated NO production was specifically abrogated by pretreatment with (43, 37)Gap27, but not (40, 37)Gap26, or scrambled Gap27. Moreover, (43, 37)Gap27 pretreatment of UAendo from pregnancy converted ATP-stimulated Ca2+ and NO responses to ones that were identical to those we previously observed from nonpregnant luteal or follicular UAendo.36

**Perspectives**

Gap junctions have a role in regulating vasodilatory pathways that modulate numerous cardiovascular functions, which when dysfunctional contribute to hypertension. The connection among endothelial dysfunction, reduced NO biosynthesis, and reduced UBF in preeclampsia was previously noted.4,6,7,9 We show the normal physiological rises in UBF and UA shear stress, Cx43, and eNOS are increased via local mechanisms only in the uterine vessels adjacent to the uterine horn that contain a fetoplacental unit. Understanding the mechanisms regulating UA function gives us greater understanding of the specific mechanisms controlling normal UBF during gestation which may function abnormally in preeclampsia. Mechanisms controlling UBF may be modulated by local steroid hormones or growth factors produced by the placenta, locally secreted into the uterine venous blood and reaching the tissues via arterial-venous shunts or the lymphatic drainage to cause unilateral vasodilation and vascular remodeling. We reported that UA endothelial ATP-induced eNOS activation and NO production is Ca2+ mediated and has an obligatory requirement for Cx43. Thus, even in conditions of uterine space limitations and placental insufficiency, uterine perfusion is partly maintained at control levels via the coregulation of Cx43 and eNOS for more robust NO production. The end result is to maintain UBF for nutrient and oxygen delivery and thus fetal growth in an albeit comprised in utero environment.

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**Disclosures**

None.

**References**

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**Novelty and Significance**

**What Is New?**

- The Gap junction protein, Cx43, is crucial for local Ca2+-mediated eNOS activation and NO production by uterine artery endothelium. We demonstrated an important physiological mechanistic role for connexins and elevations in uterine artery shear stress to maintain uterine perfusion via NO during pregnancy.

**What Is Relevant?**

- Preeclampsia with intrauterine growth restriction is a disorder of pregnancy seen in 5% to 13% of gestations and is associated with reduced uterine blood flow causing significant maternal and fetal morbidity and mortality, as well as greater susceptibility and earlier onset of future cardiovascular disease in both the mother and the baby.

**Summary**

In gravid uterine endothelium, we demonstrated that uterine artery shear stress, endothelial Cx43, and eNOS are locally elevated and that the gap junction protein Cx43 is crucial for Ca2+-mediated eNOS activation. These data demonstrate an important physiological mechanistic role for connexins in regulating the increases in ovine uterine blood flow during pregnancy that is needed for fetal development.
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Local Effects of Pregnancy on Connexin Proteins that Mediate Ca\(^{2+}\)-Associated Uterine Endothelial Nitric Oxide Synthesis

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Short Title: Pregnancy Locally Increases UAendo Connexins and NO

Key Words: Connexins, Estrogen, Nitric Oxide, Endothelium, Vascular Smooth Muscle, Shear Stress, Uterine Blood Flow

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Expanded Materials and Methods:

Animal Ethical Approval: Multiparous ewes (*Ovis Aires*) of mixed Western Breeds (Total Animals for all experiments; n=54) were obtained from the University of Wisconsin-Madison Arlington farm. Ewes were group housed and fed identical diets which consisted of a mixture of corn silage and hay that met the Nutritional Research Council requirements for all stages of gestation. Animal protocols were approved by the University of Wisconsin-Madison Research Animal Care and Use Committee of the School of Medicine and Public Health as well as the College of Agriculture and Life Sciences.

Surgical Procedures: Surgical procedures were followed as described\(^1\). Briefly, nonpregnant multiparous ewes were assigned to two surgical groups: Unilateral vs. Control. The unilateral group was subjected to surgical remodelling of the uterus. Ewes were administered atropine (0.02 mg.kg\(^{-1}\) intramuscular, Sigma Aldrich Company, St. Louis, MO) and antibiotics (400,000 units of penicillin G benzathine and gentamicin sulfate; IVX Animal Health, St. Joseph, MO) and anesthetized with ketamine (16 mg.kg\(^{-1}\) intramuscular; IVX Pharmaceuticals, St. Joseph, MO). The jugular vein was cannulated (Tygon tubing; 0.7 mm outer diameter and 0.4 mm inner diameter) for administration of ketamine (100 mg.ml\(^{-1}\)) in 0.9% saline and 5% dextrose with supplemental sodium pentobarbital (50mg.ml\(^{-1}\); Sigma Aldrich Company, St. Louis, MO) as needed for additional anaesthesia. A mid-ventral laparotomy was performed to provide access to the uterus. The uterine horns were completely bisected down its midline by severing all of the intercornual vascular connections using an electrocautery scalpel. Then one horn was double ligated with a silk ligature and cut to the mesometrial boundary and resected. Other than the disconnection of the intercornual vascular connections, all other uterine and ovarian vasculature remained completely intact. In addition, a subset of unilateral ewes had a single oviductal ligation, ipsilateral to the ligated horn, whereas the remaining ewes did not. Similarly control ewes (n=12) either had a single oviductal ligation or had no surgery (n=11) at all. Single oviductal ligations were performed in an attempt to reduce fetal numbers (thus better equalizing fetal numbers between groups), but additionally served as a sham operated control. Following surgery, ewes were administered flunixin meglumine (75 mg intramuscular; IVX Pharmaceuticals, St. Joseph, MO) analgesia and given access to food and water *ad libitum*.

Reproductive Synchronization Protocol: At least two months after surgery, a progesterone controlled internal drug release (CIDR; 0.3 g; Pfizer, Hamilton, New Zealand) was placed in the vagina of nonpregnant ewes \(^2\). After at least 6 days an intramuscular injection of prostaglandin F\(_2\alpha\) (15MG; Pfizer, New York City, NY) was administered to induce luteolysis. Between days 10 and 12, the CIDR was removed and 500 IU of equine chorionic gonadotropin (Sioux Biochemical Inc., Sioux Center IA) was injected intramuscular. Approximately 48 hours following the last injections, ewes exhibited estrus (day 0). A subset of ewes (unilateral and control) were bred and pregnancy was confirmed by ultrasonography around gestation day (GD) 60 (0.4 of gestation; term =145d). Multiparous nonpregnant ewes were experimentally synchronized using the same protocol to be studied during two distinct and controlled points of the ovarian cycle: i.e. the late follicular phase during the peri-ovulatory period (high estrogen/low progesterone) and the late luteal phase 10-11 (low estrogen/high progesterone) days post ovulation. We have previously reported the uterine blood flow (UBF) and ovarian hormone (estrogen and progesterone) levels during these specific time periods \(^2\).
Tissue Collection and ex vivo Endothelium vs. VSM Isolation: Nonsurvival surgery was performed in all experimental groups: Nonpregnant (Luteal, n = 8; Follicular, n = 8), Unilateral Pregnant (Nongravid vs. Gravid sides, n = 15; 120-130 days = 0.8-0.9 gestations), and Control Pregnant (n=23; 120-130 days = 0.8-0.9 gestations). In the Control Pregnant group (Sham and non-Shams combined) there were 11 singles 9 twins and 3 triplets and in the Unilateral Pregnant group there were 12 singleton, 2 twins and 1 triplet. In brief, ewes were kept under a surgical plane of anaesthesia with sodium pentobarbital (50-60 mg.ml⁻¹) throughout the entire procedure. UBF through both the right and left uterine arteries were determined by placing a flow probe (Transonic Systems Inc.; Ithaca, NY) on the primary uterine artery 5-7cm prior to the first bifurcation in the mesometrium. Ewes were then euthanized by exsanguination via ventricular laceration followed by bilateral pneumothorax and ovo-hysterectomy. Uterine and systemic (omentale and renal) arteries were excised and placed in ice cold PBS (8 mM sodium phosphate, 1.5 mM potassium phosphate, 2.7 mM potassium chloride, 137mM NaCl, pH = 7.4; MP Biomedicals, Solon, OH). The uterine vessels that were isolated and used in all of the experiments were primary or secondary vessels and as previously described the diameters averaged 1-2mm for nonpregnant and 3-5mm for the pregnant ewes. Arteries were then dissected free of connective tissue and rinsed free of blood and snap frozen in liquid nitrogen for later endothelial isolation and analysis. Vessel endothelium was isolated as described and fully validated. Each type of artery was cut open longitudinally and the endothelium/tunica intima of the arteries were gently scraped 3-6x and placed into lysis buffer (4mM Na₃P₂O₇.H₂O, 50mM HEPES, 100mM NaCl, 10mM EDTA, 10mM NaF, 2mM Na₃VO₄, 1mM PMSF, 5 µg/ml leupeptin, 5 µg/ml aprotinin and 0.04% Microcystin) using a curved-end spatula. The remaining vessel was rubbed with a wet cotton swab that was soaked in ice cold PBS and any remaining adventitia was extensively removed before the remaining VSM was placed in lysis buffer (same as above) and immediately homogenized/minced using scissors. The endothelial-isolated proteins from uterine (UAendo), omental (OAendo), and renal (RAendo) arteries as well as their respective VSM were snap frozen in liquid nitrogen immediately upon collection and were stored at -80°C for western analysis.

Western Analysis: UAendo, OAendo, and RAendo as well as their VSM protein concentrations were quantified using BCA Protein Assay (Thermo Scientific, Rockford, IL). Proteins (20 µg/lane) were boiled for 3 min, followed by electrophoresis on a 4-20% Tris-HCl gel (BioRad; Hercules, CA) for 90 min at 150V. Separated proteins were then transferred to a PVDF membrane for 60 min at 100V. Non-specific binding was blocked with 5% fat-free milk in TBST (50 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 0.05% Tween-20) for 2 hours at room temperature. Western analyses were performed for total eNOS (1:750; BD Bioscience; San Diego, CA), P^eo5 eNOS (1:1000; Upstate Cell Signaling; Lake Placid, NY) Cx37 (1:5000; Alpha Diagnostic; San Antonio, TX) and Cx43 (1:3000; Sigma Corporation; St. Louis MO). The membrane was incubated overnight at 4 °C. Following five washes (1 x 5, 1 x 15, 3 x 5 min) with TBST, the membrane was then incubated with either anti-rabbit (1:3000; Cell Signaling; Beverly, MA) or anti-mouse (1:3000; GE Healthcare; Piscataway, NJ) HRP conjugated IgG for 1 hour. The membrane was again washed with TBST twice and then 3 times with TBS. Binding was detected with the ECL (Thermo Scientific; Rockford, IL) or ECL+ (GE Healthcare; Piscataway, NJ) reagents according to the manufacturer’s instructions. The levels of protein expression were quantified by scanning densitometry and expressed as fold of the luteal control replicates run on the same blots, thus accounting for protein loading per lane as previously described for VSM using Myosin. In those studies we reported on using UA, OA, and RA VSM lysates from
luteal, follicular, and pregnant sheep and showed no significant alterations in alpha Smooth Muscle Myosin which are considered as a standard loading control. We also have observed from endothelial-isolated proteins that Beta Actin is not significantly different when comparing luteal or follicular nonpregnant as well as unilateral or control pregnant ewes (data not shown). The current studies were performed exclusively in vivo/ex vivo and thus have direct physiologic and functional relevance to normal pregnancy vascular adaptations.

**Calculation of Shear Stress:** Shear stress was calculated as recently described using the formula, \( \frac{4 \, Q \, \eta}{\pi \, r^3} \). Where \( Q \) = uterine blood flow, \( \eta \) = viscosity and \( r \) = radius. The whole blood viscosity was measured using a torque viscometer (Brookfield TC-500; Middleboro, Massachusetts), and were the average across the shear rates (67, 87, 134 sec\(^{-1}\)) in the plateau phase on the viscometer. The vessel diameter was determined at physiologic pressures as we recently described. Specifically intact branching segments of mesometrial uterine arteries were pressurized from 0 to 120 mmHg (in 10 mmHg steps) in, isotonic, calcium-free solution. Digital images were taken at each pressure, and diameter was measured from each image using image analysis software (MetaVue) and in-picture calibration reference. After the mechanical testing reported in the above papers, UA segments were fixed (normal buffered formalin) at physiologic pressure (nonpregnant follicular and luteal~85mmHg; unilateral and control pregnant ~80mmHg) and cut axially to measure inner diameters and wall thickness.

**Simultaneous In situ Measurement of \([\text{Ca}^{2+}]_i\) and NO in Intact UAendo:** Simultaneous \([\text{Ca}^{2+}]_i\) and NO fluorescent imaging analysis upon ATP treatment was performed using a high-speed excitation and emission wavelength switching systems as we recently described. ATP was utilized in our previous and the current studies because it is endogenously released by blood cells as well as by endothelial cells in response to numerous physiologic stimuli including shear stress and produces NO-associated vasodilation. In brief, prior to DAF loading, uterine artery vessels were washed twice and then incubated for 1 hour in Krebs buffer, which was then removed and replaced with fresh buffer or with 300 µM (43,37)Gap 27 or other Gap peptide inhibitors in Krebs buffer for 3 hours. Studies were conducted under basal vs. stimulated (ATP 100µM; 25min) conditions in the absence or presence of (43,37) Gap 27 (300 µM) to specifically inhibit Cx43 Gap junction \([\text{Ca}^{2+}]_i\) -mediated NO responses to ATP. We further established peptide specificity performing parallel UAendo studies using (40, 37)Gap 26 (specific to Cx40 as well as Cx37; n=4) (Figure 4) and (43, 37 scramble)Gap 27 (control scrambled peptide; n=2; data not shown). After loading with both DAF-2 DA (20 µM; Molecular Probes) and fura-2 AM (10 µM) for 90 min, the chamber with the isolated uterine artery was mounted on an inverted microscope (Diaphot 150; Nikon) so that a ×20 phase/fluor objective was focused on the luminal endothelium and individual endothelial cells are visualized (only well-focused endothelial cells had a strong signal, and these cells were randomly selected). The excitation light from a xenon lamp was filtered to provide wavelengths of 340 ± 10 and 380 ± 10 (for fura-2) and 480 ± 20 nm (for DAF-2) with a high-speed wavelength switcher (Lambda 10–2; Sutter, Novato, CA). Emission light from endothelial cells was passed through a dichroic mirror (505 nm) and through an emission filter of 520 ± 20 nm for both fura-2 and DAF-2 with a high-speed rotating filter wheel (Lambda 10–2; Sutter). The fluorescence images were recorded by a digital camera (PixelFly; Cooke). InCyt Im3 imaging and analysis software (Intracellular Imaging) was used to acquire, digitize, and store the images and data for off-line processing and statistical analysis. The relative fluorescence intensities for fura-2 and DAF-2 were quantitatively comparable. When the endothelium was only loaded with fura-2 AM, no signal at 480/535 nm
(for NO imaging) was detected. In contrast, when the endothelium was only loaded with DAF-2 DA, no detectable signal could be recorded at 340/510-nm and 380/510-nm wavelengths (for \([\text{Ca}^{2+}]_i\) imaging; data not shown). To reduce photo-bleaching of these fluorescent dyes, images were acquired at 5-s intervals. Background and autofluorescence were obtained from control-unloaded endothelium. \(F_{340}/F_{380}\), a fluorescence ratio of excitation at 340 nm to that at 380 nm, was determined after background and autofluorescence subtraction, and \([\text{Ca}^{2+}]_i\) was calculated in real time by comparison to a standard curve established for the same settings using buffers of known free \([\text{Ca}^{2+}]_i\). Because there is no significant change in baseline fluorescence during a 60-min experiment, we expressed the intracellular NO production as relative fluorescence (\(f\)), which is the net increment of DAF-2 fluorescence relative to its basal value (\(f = \Delta F/F_0\)), where \(F\) is DAF-2 fluorescence intensity obtained during experiments and \(F_0\) is its basal fluorescence intensity.

**Statistical Analysis:** Data were analysed using a one way ANOVA with treatment group (Luteal, Follicular, Nongravid Unilateral, Gravid Unilateral, and Pregnant) as the sole independent factor using SigmaPlot 11.0 software. Upon establishment of overall significance, pair-wise comparisons were performed using protected Fisher’s Least Significant Difference. Least Squares Regression analyses were performed and the highest order (linear, quadratic, cubic, etc.) regression describing the model for the relationship(s) between UBF, connexins, and eNOS are presented (Figure S-1, Figure S-2, and Table S-1). Data are reported as Means ± SEM and the level of significance was established *a priori* at P<0.05.

**Supplemental Results**

**Viscosities and Internal UA Diameters**

In order to calculate unilateral UA shear stress for Figure 1B, viscosities were determined on whole arterial blood at shear rates > 60 sec\(^{-1}\). We observed that blood viscosities did not appear to be substantially altered by the phase of the ovarian cycle in the nonpregnant groups and averaged 4.43 ± 0.012 cP. Viscosity was reduced (P<0.01) equally to 3.68 ± 0.022 cP in the two pregnancy groups. UA diameters for Nonpregnant (Luteal and Follicular) and Nongravid side of the unilateral pregnant sheep averaged 1-2 mm and were similar (P>0.05) between these groups. By contrast, Gravid Unilateral and Control Pregnant groups had similar UA diameters averaging 3-5 mm, which were greater than the nonpregnant or nongravid groups (P<0.01). It is noteworthy that wall thickness was unchanged in all UAs (P>0.05).

**Least Squares Regression Analysis Between Connexins and UBF:** We examined the relationships between connexins and UBF. In all cases either first order linear or second order quadratic associations best described the models (Table S-1). In Figure S-1A and S-1B we noted a significant quadratic regression between UAendo connexin expression (X-Axis; Independent Variable) with UBF (Y-Axis; Dependent Variable) across all five groups (luteal, follicular, unilateral nongravid, unilateral gravid, and control pregnant). Both UAendo Cx37 and Cx43 were significantly correlated (P < 0.001) with UBF, describing 48.4% and 60.1% of the models, respectively. UAendo Cx43 was even more highly correlated with UBF than Cx37. Separate comparisons of these relationships evaluated within Unilateral Gravid vs. Pregnant Controls individually were performed but they yielded similar relationships, demonstrating that relative Cx37 and Cx43 expression UBF in the Unilateral group had fully adapted to a similar Pregnant Control level (data not shown). In Figure S-1C and S-1D we specifically noted a significant
quadratic relationship between UAovsm connexin expression and UBF across all five groups (luteal, follicular, unilateral nongravid, unilateral gravid, and control pregnant). Similar to UAendo, UAovsm Cx37 was significantly correlated (P < 0.001) with UBF describing 43.4% of the model. In contrast to the very strong Cx43-UBF relationship, UAovsm Cx43 was also correlated (P = 0.023) with UBF, but this only described 18.4% of the model, i.e. a much weaker relationship suggesting that Cx43 has less control than Cx37 on UAovsm.

**Connexin 37 and 43 Expression in OAendo and RAendo:** OAendo Cx37 and Cx43 remained unchanged across all groups. Although OAendo Cx37 from pregnancy trended higher (3.4±0.9-fold), this was not significance (0.1>P>0.05) (Figure S-2A). RAendo Cx37 levels also were not statistically different amongst the 4 groups (Figure S-2B). Unexpectedly, RAendo Cx43 was significantly lower in the unilateral group (P < 0.012) versus the other 3 groups.

**Connexin 37 and 43 Expression in OAvsm and RAvsm:** OAvsm Cx37 and Cx43 remained unchanged except in pregnant controls Cx43 was elevated 6.7±1.7-fold (Figure S-3A). Compared to the luteal, RAvsm Cx37 (Figure S-3B) in follicular and pregnant groups was decreased 0.4±0.2 and 0.5±0.2-fold and in unilateral ewes was further decreased (0.0635±0.0508-fold). RAvsm Cx43 levels remained unchanged amongst the 4 groups.

**UAendo Protein Expressions Based on the Number of Fetuses:** In order to determine if the changes eNOS and Cxs observed in the current study were a function of the number of fetuses per uterine horn, we evaluated the eNOS and Cx expressions in single, twin and triplet pregnancies regardless if they came from the unilateral or the control groups. The number of fetuses per horn did not appear to substantially affect the levels of UAendo Cx37, Cx43, P^635, or total eNOS. These data confirm our previous report pertaining to the total UAendo eNOS capacity of the endothelium\(^5,19\). However as we previously suggested in these papers, additional studies are required to more fully address this important issue since the circulating levels of NOx and thus eNOS activation are higher in pregnancies with multiples\(^9\). The current observations implicate the local rises in UA shear stress that could account for the higher NOx levels during gestation.

**Least Squares Regression Analysis UAendo Connexins and UAendo eNOS:** We also performed regression analysis of UAendo connexin expression with UAendo P^635 eNOS (Figure S-4A and S-4B) and total eNOS (Figure S-4 C and S-4D) used as the dependent variables across all five groups, but unlike that observed with UBF, the Unilateral Gravid lines consistently demonstrated significantly better correlations and much higher slopes than the Pregnant Control lines (Table S-1). Specifically UAendo Cx37 was highly correlated with UAendo P^635 eNOS, however the Unilateral Gravid quadratic (R\(^2=0.23\)) relationship was much better (P<0.007) than that for the Pregnant Control group (R\(^2=0.079\)). Regressions for UAendo Cx43 and P^635 eNOS, showed linear relationships that were stronger for Unilateral Gravid (R\(^2 = 0.425\)) compared to Pregnant Controls (R\(^2 = 0.144\)). Similarly, UAendo Cx37 was highly correlated with UAendo total eNOS, with the Unilateral Gravid linear (R\(^2=0.271\)) relationship being somewhat stronger (P<0.001) than that for the Pregnant Control group (R\(^2=0.187\). As seen for the P^635 eNOS, regressions for UAendo Cx43 and total eNOS, showed linear relationships that were also stronger for Unilateral Gravid (R\(^2 = 0.466\)) compared to Pregnant Controls (R\(^2 = 0.26\). These in
in vivo data suggest that eNOS levels and its activation are partly responsible for the UBF adaptation between the Gravid Unilateral being equal to the Pregnant Control levels via Cx43-associated mechanisms.

Supplemental Discussion

We examined OAs and RAs as vessels important for blood pressure regulation (Figures S-2 and S-3). The uterine vasculature is affected to a greater extent by pregnancy and estrogen treatments than nonreproductive vasculatures\(^3,4,19-22\). Both OAendo and RAendo Cx37 and Cx43 levels remained unchanged demonstrating that gestational endothelial adaptations were specific to uterine, not systemic vasculature. Only OAvsm Cx43, not Cx37, was increased during pregnancy which is in contrast to a study\(^23\) in which the authors commented that OA Cx43 expression did not change between nonpregnant premenopausal, normal Pregnant, and preeclamptic women. Discrepancies may be explained because in that study whole OAs were homogenized including both endothelium and VSM and their vessel diameters were much smaller which increases the endothelium/VSM ratio.

References


<table>
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<tr>
<th>X vs. Y</th>
<th>1st order equation</th>
<th>2nd order equation</th>
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<tbody>
<tr>
<td>UAendo Cx37 vs. UBF</td>
<td>NS</td>
<td>UBF = 91.9 + (1108 * Cx37) - (370.5 * (Cx37)^2)</td>
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<td>NS</td>
<td>UBF = 19.1 + (1664.9 * Cx43) - (760.6 * (Cx43)^2)</td>
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<td>UBF = 105.8 + (763.7 * Cx37) - (212.1 * (Cx37)^2)</td>
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<td>UAvsm Cx43 vs. UBF</td>
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<td>UBF = 159.1 + (834.8 * Cx43) - (324.4 * (Cx43)^2)</td>
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<td>P635 eNOS = 0.305 + (0.263 * Cx37) NS</td>
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<td>P635 eNOS = 0.197 + (1.3 * Cx37) - (0.581 * (Cx37)^2) NS</td>
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<td>Tot. eNOS = 0.048 + (1.192 * Cx43) NS</td>
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Shown are the highest order regression analysis equations that describes the model presented (P<0.05). NS - Not significantly improved.
Figure S-1. Relationships between UAendo and UAvm connexin 37/43 (X coordinate) and paired UBF (Y coordinate) values for each individual ewe from luteal, follicular, unilateral nongravid, unilateral gravid, and control pregnant groups. There were significant correlations between (A) UAendo Cx37 and UBF (B) UAendo Cx43 and UBF; (C) UAvm Cx37 and UBF; and (D) UAvm Cx43 and UBF. Each point represents data from an individual animal. Least Squares Regression analysis was performed using highest order correlation describing the model (e.g. linear, quadratic, cubic, etc.). Equations are shown in supplement Table S-1.
Figure S-2. Systemic Artery Connexin 37 and 43 protein expression in (A) Omental and (B) Renal artery endothelium (OAendo/RAendo) from luteal, follicular, unilateral, and pregnant ewes. Western Blot analysis comparing the relative levels of Cx37 and 43 in (A) OAendo (B) RAendo from luteal (L; n=4), follicular (F; n=4), unilateral (Uni; n=8), and pregnant (P; n=7) sheep. Data are expressed as Means±SEM fold of Luteal. Different letters denote differences (P<0.05).
Figure S-3. Systemic Artery Connexin 37 and 43 protein expression in (A) Omental and (B) Renal artery vascular smooth muscle (OAvsm/RAvsm) from luteal, follicular, unilateral, and pregnant ewes. Western Blot analysis comparing the relative levels of Cx37 and 43 in (A) OAvsm and (B) RAvsm from luteal (L;n=4), follicular (F;n=4), unilateral (Uni;n=8), and pregnant (P;n=7) sheep. Data are expressed as Means±SEM fold of Luteal. Different letters denote differences (P<0.05).
Figure S-4. Relationships between UAendo and UAvsm connexin 37/43 (X coordinate) and paired eNOS/P635/Total eNOS (Y coordinate) and values for each individual ewe from luteal, follicular, unilateral nongravid, unilateral gravid, and control pregnant groups. There were significant correlations between (A) UAendo Cx37 and P635 eNOS; (B) UAendo Cx43 and P635 eNOS; (C) UAendo Cx37 and Total eNOS; (D) UAendo Cx43 and Total eNOS. Each point represents data from an individual animal. Least Squares Regression analysis was performed using highest order correlation describing the model (e.g. linear, quadratic, cubic, etc. in order to specifically describe differences in these relationships for both unilateral gravid versus control pregnant groups). Dashed lines represent regression analysis independent of the control pregnant group whereas solid lines represent regression analysis independent of the unilateral gravid group. Equations are shown in supplement Table S-1.