Augmented dilation to nitric oxide in uterine arteries from rats with type 2 diabetes: implications for vascular adaptations to pregnancy

Styliani Goulopoulou,1 Johanna L. Hannan,1 Takayuki Matsumoto,1,2 Adviye Ergul,1 and R. Clinton Webb1

1Department of Physiology, Georgia Regents University, Augusta, Georgia; and 2Department of Physiology and Morphology, Institute of Medicinal Chemistry, Hoshi University, Shinagawa-ku, Tokyo, Japan

Submitted 5 August 2013; accepted in final form 10 December 2013

Goulopoulou S, Hannan JL, Matsumoto T, Ergul A, Webb RC. Augmented dilation to nitric oxide in uterine arteries from rats with type 2 diabetes: implications for vascular adaptations to pregnancy. Am J Physiol Heart Circ Physiol 306: H610–H618, 2014. First published December 13, 2013; doi:10.1152/ajpheart.00588.2013.—Pre-existing diabetes increases the risk of maternal and fetal complications during pregnancy, which may be due to underlying maternal vascular dysfunction and impaired blood supply to the uteroplacental unit. Endothelial dysfunction and reduced vascular smooth muscle responsiveness to nitric oxide (NO) are common vascular impairments in type 2 diabetes (T2D). We hypothesized that uterine arteries from diabetic rats would have reduced vascular smooth muscle sensitivity to NO compared with nondiabetic rats due to impairment in the NO/soluble guanylate cyclase (sGC)/cGMP signaling pathway. Uterine arteries from pregnant Goto-Kakizaki (GK) and Wistar (nondiabetic) rats were studied in a wire myograph. GK nonpregnant uterine arteries had responses to Ach and sodium nitroprusside (SNP) but increased responses to propylamine NONOate and greater sensitivity to sildenafil compared with Wistar nonpregnant arteries. In late pregnancy, Wistar rats had reduced uterine vascular smooth muscle responsiveness to SNP, but GK rats failed to show this adaptation and had reduced expression of sGC compared with the nonpregnant state. GK rats had a smaller litter size (13.9 ± 0.48 vs. 9.8 ± 0.75; P < 0.05) and a greater number of resorptions compared with Wistar controls (0.8 ± 0.76% vs. 19.9 ± 6.06%; P < 0.05). These results suggest that uterine arteries from rats with T2D show reduced sensitivity of uterine vascular smooth muscle to NO. During pregnancy, the GK uterine vascular smooth muscle fails to show relaxation responses similar to those of arteries from nondiabetic rats.

uterine artery; soluble guanylate cyclase; vascular smooth muscle; vasorelaxation; gestation

PREGESTATIONAL DIABETES CARRIES a high risk of mortality and morbidity for the mother and the fetus and comprises a hostile environment for implantation, and embryonic and fetal development (6, 25). Macrosomia, intrauterine growth restriction, shoulder dystocia, congenital abnormalities, and stillbirth are only a few of the complications seen in pregnancies with maternal diabetes (3, 24). The majority of diabetic pregnancies are complicated by gestational diabetes, but in recent years, the prevalence of pregnancies associated with type 2 diabetes (T2D) has increased steadily as it parallels the increasing rates of obesity and is influenced by the occurrence of childbearing in older ages (2, 7).

During healthy pregnancy, uterine blood flow increases to facilitate adequate nutrition and oxygen supply to the fetus. To accommodate this increase in blood flow, the uterine vasculature undergoes structural and functional changes including hypertrophy and hyperplasia of vascular smooth muscle cells (4, 31), increase in vasodilatory capacity (5, 49), and refractoriness to potent vasoconstrictors (46), respectively. Previous studies have reported that women with pregestational diabetes have increased uterine artery impedance (33). It was proposed that uterine endothelial dysfunction due to hyperglycemia may contribute to increased uterine resistance (41). Animal studies have primarily focused on uterine artery function in models of experimental diabetes that resemble characteristics of type 1 (10, 32, 40) or gestational diabetes (41, 42). These studies showed that a hyperglycemic environment during pregnancy promotes vascular dysfunction and adverse pregnancy outcomes. To the best of our knowledge, no data are currently available on uterine artery dilatory function in animal models of T2D, since the occurrence of T2D during pregnancy is a relatively recent phenomenon.

Endothelial dysfunction is well documented in T2D (13, 23, 38). Emerging clinical and experimental data, however, demonstrate that vascular smooth muscle cells may also be functionally impaired and thus contributing to vascular dysfunction in T2D (29, 35). Endothelium-derived factors [i.e., nitric oxide (NO)] or exogenous nitrates can induce vascular smooth muscle relaxation via activation of the soluble guanylate cyclase (sGC)/cGMP pathway. In humans, relaxation responses of the umbilical vascular smooth muscle change throughout pregnancy (18, 19), showing an increase in early pregnancy and a reduction in late pregnancy back to levels seen in the nonpregnant state (20). Humans and rodents with T2D show attenuated responsiveness of vascular smooth muscle to exogenous NO (30, 34, 47) and persistent reduction in vascular NO-sensitive sGC (48). One could speculate, therefore, that women with T2D enter pregnancy with preexisting deficiency of the NO/sGC/cGMP pathway; yet whether such impairment is evident in uterine vascular smooth muscle and if it compromises uterine artery adaptations to pregnancy is unknown.

Due to methodological and ethical considerations, studies in pregnant women with T2D are limited to measurements of uterine blood flow and uterine artery resistance using ultrasound techniques. Thus appropriate rodent models are important tools for the characterization of signaling mechanisms associated with uterine artery function. The Goto-Kakizaki (GK) rat model of T2D was produced by selective inbreeding of nondiabetic Wistar rats that had glucose intolerance (12). GK rats have glucose intolerance, reduced glucose-stimulated insulin release, mild hyperglycemia, decreased functional...
β-cell mass, and changes in islet microarchitecture (36). Most importantly, the GK rat is a nonobese model of T2D, allowing the investigation of the effect of diabetes in the absence of the confounding influences of obesity. In this study, we used the GK model to examine the effects of pregestational diabetes on uterine artery dilatory adaptations to pregnancy. We hypothesized that uterine arteries from GK rats would have reduced vascular smooth muscle sensitivity to NO compared with those from nondiabetic rats due to an impairment in NO/sGC/cGMP signaling pathway.

MATERIALS AND METHODS

Reagents. Phenylephrine (PE), ACh, sodium nitroprusside (SNP), 1H-[1,2,4]oxadiazolo[4,3-a]quinolin-1-one (ODQ), sildenafil citrate salt, N6-nitro-L-arginine methyl ester hydrochloride (l-NAME), 8-bromoguanosine 3′,5′-cyclic monophosphate sodium salt (8-Br-cGMP), and antibody against β-actin were obtained from Sigma Chemical (St. Louis, MO). Propylamine propylamine NONOate (PAPA NONOate) and antibodies against sGCε and sGCβ1 were purchased from Cayman Chemical (Ann Arbor, MI). Antibody against phosphodiesterase 5A (PDE5A) was obtained from Abcam (Cambridge, MA). Stock solutions were prepared in deionized water or dimethyl sulfoxide (DMSO).

Animals. Female, 20-wk-old, virgin GK rats (inhouse bred, derived from the Tampa colony) and age-matched Wistar rats (Charles River Laboratories International) were used in this study. The animals were housed in a temperature- and humidity-controlled environment under 12-h:12-h light/dark cycles and had free access to tap water and standard laboratory rodent chow. Half of the female rats were paired with a fertile male (Wistar, 12–16 wk old) and the morning on which spermatozoa were found in vaginal smears was considered day 1 of pregnancy. In all experiments, rats were anesthetized with isoflurane with a nose cone for surgical procedures (initially with 5% and then maintained at 2.5% in 100% oxygen) and euthanized with isoflurane via a nose cone for surgical procedures (initially with 5% and then maintained at 2.5% in 100% oxygen) and euthanized with isoflurane overdose followed by cutting their diaphragm on gestational days 19 and 20 (term = 21 to 22 days). Fetuses were euthanized immediately following removal from the dam via decapitation. All procedures were performed in accordance with the Guiding Principles in the Care and Use of Animals and approved by the Georgia Regents University Committee on the Use of Animals in Research and Education.

Metabolic parameters. Tail blood samples were used for measurements of nonfasted whole blood glucose (FreeStyle Lite, Alemeda, CA) before vascular reactivity studies. Blood was also collected from the inferior vena cava for measurement of serum insulin (Rat Insulin ELISA; ALPCO, Salem, NH).

In vitro assessment of uterine artery reactivity. Uterine artery reactivity was measured using a wire myograph (Danish Myo Technology A/S, Aarhus, Denmark). After euthanization, the uterus with attached vasculature was excised and placed in ice-cold physiological solution (PSS) of the following composition (in mM): 130 NaCl, 4.7 KCl, 14.9 NaHCO3, 5.5 dextrose, 1.18 KH2PO4, 1.17 MgSO4, 1.6 CaCl2, and 0.026 EDTA. The main uterine arteries were carefully isolated by dissection of fat and connective tissue. One of the main uterine arteries was frozen immediately in liquid nitrogen and stored at −80°C for subsequent Western blot experiments. The midpoints of the contralateral uterine artery (2 mm in length) were mounted in an isometric wire myograph system using two 40-μm wires and allowed to equilibrate for 30–45 min before resting tension was applied. Optimum resting tension was determined via a length-tension curve. Arterial rings were allowed to equilibrate for 45 min in a tissue bath filled with 5 ml PSS, continuously gassed with 95% O2:5% CO2 at 37°C. Vascular integrity was assessed by contracting uterine arterial segments with a depolarizing concentration of potassium chloride (KCl, 120 mM). Vascular endothelium viability was examined by assessing relaxation responses to ACh (3 × 10−6 M) in uterine arteries preconstricted with PE (3 × 10−6 M). Endothelium-dependent relaxation was assessed by concentration-response curves to ACh (10−9 to 10−4 M) in the presence or absence of a NO synthase (NOS) inhibitor (l-NAME; 10−4 M, 30 min incubation). Endothelium-independent relaxation was assessed by concentration-response curves to two NO donors—SNP (10−10 to 3 × 10−6 M) and PAPA NONOate (10−9 to 3 × 10−4 M) in the presence and absence of a specific inhibitor of sGC (ODQ, 10−6 M, 30 min incubation)—and a cGMP analog (8-Br-cGMP, 10−9 to 3 × 10−4 M). Concentration-response curves to a PDE5 inhibitor (sildenafil; 10−10 to 10−6 M) were also performed. All concentration-response curves to various reagents were performed in endothelium-intact arteries preconstricted with PE in a concentration that elicited isometric force corresponding to 80% of maximum response to KCl.

Western blot analysis. Uterine arteries were homogenized in ice-cold lysis buffer containing T-Per tissue protein extraction solution (Thermo Scientific, Rockford, IL), 100 mM sodium orthovanadate (Na3VO4), 100 mM PMSF, and 1% protease inhibitor cocktail (Sigma). Homogenates were centrifuged at 10,000 g for 15 min at 4°C, the supernatant was collected, and the proteins were solubilized in Laemmli’s buffer containing mercaptoethanol. Protein concentration in the supernatant was measured by bicinchoninic acid assay (Thermo Scientific). Samples (10 μg protein/lane) were resolved by electrophoresis on 10% SDS-PAGE gels and then transferred to nitrocellulose membranes. Membranes were blocked in blocking solution (Tris-buffered saline-Tween 20 with 5% skim dry milk or 5% bovine serum albumin) and subsequently incubated with primary antibodies (overnight at 4°C). The immunobinding was detected using horseradish peroxidase-conjugated anti-rabbit immunoglobulin G (IgG) (GE Healthcare, Buckinghamshire, UK) or anti-mouse IgG (GE Healthcare) for 1 h at room temperature. Results were normalized by β-actin expression. Primary antibodies were as follows: rabbit anti-sGCε1 (77–82 kDa; 1:1,000), rabbit anti-sGCβ1 (70 kDa; 1:1,000), rabbit anti-PDE5A (105 kDa; 1:500), mouse anti-β-actin (42 kDa; 1:15,000). Immunoreactive bands were visualized with an enhanced chemiluminescence detection system and quantified using UN-SCAN-IT gel analysis software (v. 6.1; Silk Scientific, Orem, UT).

Data analysis. Sigmoidal curve fitting was performed on wire myography concentration-response curve data using GraphPad Prism software (v. 5.0; GraphPad Software, San Diego, CA). Two pharmacological parameters were obtained from these curves: the maximal effect generated by the agonist (E(max)) and the EC50 (molar concentration of agonist producing 50% of the maximum response). EC50 was calculated only for those responses that showed a sigmoidal curve. Maximal vasodilatory responses were calculated and expressed relative to the maximal changes from the contraction produced by PE in each segment, which was determined as 0% relaxation. The baseline tension before addition of PE was considered as 100% relaxation.

In addition to E(max) and EC50 calculations, the area under the curve (AUC) of the concentration-response curves to SNP, in the presence and absence of ODQ, was calculated (GraphPad Prism) to determine total relaxation. The contribution of sGC-dependent mechanisms to SNP- and PAPA NONOate-induced responses was calculated as the difference (%Δ) between AUC corresponding to the agonist alone and AUC corresponding to the agonist in the presence of ODQ.

Statistical analysis. Values are presented as means ± SEM, and n represents the number of animals used in the experiments. Before statistical analysis, all data sets were tested for normality using the Kolmogorov-Smirnov test. Statistical differences were calculated by Student’s t-test (fetal physical parameters), two-way ANOVA followed by Bonferroni post hoc test (body weight, blood glucose, insulin, E(max), EC50, AUC) and two-way ANOVA with repeated measures (concentration-response curves). Group differences in protein levels were determined using Kruskal-Wallis one-way ANOVA. All statistical tests were performed with GraphPad Prism (v. 5.0;
pregnancy caused a rightward shift in the uterine SNP concentration-response curve in Wistar nonpregnant uterine arteries (AUC; Wistar nonpregnant: 144.6 ± 20.04 vs. pregnant: 179.3 ± 18.10; \( P > 0.05 \); Fig. 2A and Table 2). Wistar pregnant rats had reduced responses (maximum and total relaxation) to SNP compared with pregnant Wistar rats (\( P < 0.05 \); Fig. 2A and Table 2).

In contrast with SNP responses, GK nonpregnant uterine arteries had greater relaxation responses to PAPA NONOate compared with Wistar nonpregnant arteries (\(-\log EC_{50}\) GK nonpregnant: 6.36 ± 0.061 vs. Wistar nonpregnant: 6.00 ± 0.119; \( P < 0.05 \); Fig. 2B). Pregnant GK and Wistar rats had greater relaxation responses to ACh compared with pregnant Goto-Kakizaki (GK) rats (\(-\log EC_{50}\) GK pregnant: 7.99 ± 0.09 vs. pregnant: 6.00 ± 0.119; \( P < 0.05 \); Fig. 2B). The presence of the NOS inhibitor L-NAME abolished uterine artery responses to ACh in all groups (Fig. 1, A and B).

Body weight and metabolic parameters. Body weight was lower in pregnant and nonpregnant GK rats compared with pregnant and nonpregnant Wistar rats, respectively (\( P < 0.05 \); Table 1). GK nonpregnant rats had increased blood glucose levels compared with Wistar controls and pregnancy reduced blood glucose levels only in GK rats (\( P < 0.05 \); Table 1). Furthermore, GK animals had reduced serum insulin levels compared with those from Wistar controls and this difference was unchanged at the late pregnancy stage (\( P < 0.05 \); Table 1).

Maternal and fetal parameters. There were no significant differences in either fetal weights (Wistar, \( n = 7 \): 1.68 ± 0.142 g vs. GK, \( n = 5 \): 1.96 ± 0.184 g; \( P > 0.05 \)) or placental weights (Wistar, \( n = 7 \): 0.43 ± 0.001 g vs. GK, \( n = 5 \): 0.45 ± 0.001 g; \( P > 0.05 \)). GK rats (\( n = 11 \)) had a smaller litter size (9.8 ± 0.75 vs. 13.9 ± 0.48; \( P < 0.05 \)) and a greater number of resorptions compared with Wistar controls (\( n = 10 \)) (19.9 ± 6.06% vs. 0.8 ± 0.76%; \( P < 0.05 \)).

Endothelium-dependent relaxation in rat uterine arteries. In the nonpregnant state, GK uterine arteries had reduced responses to ACh at submaximal concentrations (i.e., \( 3 \times 10^{-7} \) M, \( 10^{-6} \) M, and \( 3 \times 10^{-5} \) M) compared with Wistar arteries (\( P < 0.05 \); Fig. 1A). Uterine arteries from Wistar nonpregnant rats had increased sensitivity to ACh compared with those from Wistar pregnant rats (\(-\log EC_{50}\) Wistar nonpregnant: 7.43 ± 0.127 vs. Wistar pregnant: 6.83 ± 0.134; \( P < 0.05 \)), but there were no differences between GK pregnant and nonpregnant uterine arteries (GK nonpregnant: 6.71 ± 0.176 vs. GK pregnant: 6.67 ± 0.204; \( P > 0.05 \)). In late pregnancy, there were no differences in uterine artery sensitivity to ACh between GK and Wistar rats (\( P > 0.05 \); Fig. 1B). The presence of the NOS inhibitor L-NAME abolished uterine artery responses to ACh in all groups (Fig. 1, A and B).

Endothelium-independent uterine artery relaxations. Uterine arteries from GK nonpregnant rats had diminished total relaxation responses to SNP compared with those from Wistar nonpregnant rats (AUC; Wistar nonpregnant: 195.0 ± 6.06 vs. GK nonpregnant: 144.6 ± 20.04; \( P < 0.05 \); Fig. 2A). Late pregnancy caused a rightward shift in the uterine SNP concentration-response curve in Wistar (\(-\log EC_{50}\) nonpregnant: 8.67 ± 0.07 vs. pregnant: 7.99 ± 0.09; \( P < 0.05 \); Fig. 2A) but not in GK rats (\(-\log EC_{50}\) nonpregnant: 8.48 ± 0.19 vs. pregnant: 8.65 ± 0.18; \( P > 0.05 \); Fig. 2A). In addition, Wistar pregnant uterine arteries had reduced maximum (Table 2 and Fig. 2A) and total relaxation responses to SNP (AUC) compared with Wistar nonpregnant arteries (AUC; Wistar nonpregnant: 195.0 ± 6.06 vs. Wistar pregnant: 108.8 ± 17.18; \( P < 0.05 \); Fig. 2A), and there were no differences in responses to SNP between GK nonpregnant uterine arteries and pregnant arteries (AUC; GK nonpregnant: 144.6 ± 20.04 vs. pregnant: 179.3 ± 18.10; \( P > 0.05 \); Fig. 2A and Table 2). Wistar pregnant rats had reduced responses (maximum and total relaxation) to SNP compared with pregnant Wistar rats (\( P < 0.05 \); Fig. 2A and Table 2).

GraphPad Software). The significance level of all tests was set at \( \alpha = 0.05 \).

Table 1 Body weight, serum glucose, and insulin levels

<table>
<thead>
<tr>
<th></th>
<th>Wistar Nonpregnant</th>
<th>Wistar Pregnant</th>
<th>GK Nonpregnant</th>
<th>GK Pregnant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g(^s)</td>
<td>269.8 ± 3.77(^a)</td>
<td>362.0 ± 8.58(^b)</td>
<td>228.2 ± 3.56</td>
<td>269.1 ± 5.21(^cd)</td>
</tr>
<tr>
<td>Nonfasting glucose, mg/dL(^s)</td>
<td>89.5 ± 6.33(^a)</td>
<td>87.2 ± 6.95</td>
<td>210.9 ± 10.14</td>
<td>116.8 ± 7.03(^cd)</td>
</tr>
<tr>
<td>Insulin, ng/mL(^s)</td>
<td>1.92 ± 0.388(^a)</td>
<td>3.14 ± 0.632</td>
<td>0.35 ± 0.053</td>
<td>0.41 ± 0.076(^d)</td>
</tr>
</tbody>
</table>

Values are means ± SE; \( n = 7–11 \) for Wistar nonpregnant, \( n = 6–10 \) for Wistar pregnant, \( n = 7–10 \) for Goto-Kakizaki (GK) nonpregnant, and \( n = 5–10 \) for GK pregnant. *\( P < 0.05 \): main effect, Wistar vs. GK; \(^{a}\)\(^{b}\)P < 0.05: main effect, nonpregnant vs. pregnant; \(^{c}\)\(^{d}\)P < 0.05: Wistar nonpregnant vs. GK nonpregnant; \(^{e}\)\(^{f}\)P < 0.05: Wistar nonpregnant vs. Wistar pregnant; \(^{g}\)\(^{h}\)P < 0.05: GK pregnant vs. Wistar pregnant.

In contrast with SNP responses, GK nonpregnant uterine arteries had greater relaxation responses to PAPA NONOate compared with Wistar nonpregnant arteries (\(-\log EC_{50}\) GK nonpregnant: 6.36 ± 0.061 vs. Wistar nonpregnant: 6.00 ± 0.119; \( P < 0.05 \); Fig. 2B). Pregnant GK and Wistar rats had greater relaxation responses to ACh compared with pregnant Goto-Kakizaki (GK) rats (\(-\log EC_{50}\) GK pregnant: 7.99 ± 0.09 vs. pregnant: 6.00 ± 0.119; \( P < 0.05 \); Fig. 2B). The presence of the NOS inhibitor L-NAME abolished uterine artery responses to ACh in all groups (Fig. 1, A and B).

Fig. 1. Concentration-response curves to ACh in the presence and absence of a nitric oxide synthase inhibitor (N\(^\text{\textsuperscript{N}}\)-nitro-L-arginine methyl ester hydrochloride, or L-NAME; \( 10^{-4} \) M) in uterine arteries from nonpregnant (A) and pregnant (B) control (Wistar) and diabetic (Goto-Kakizaki, GK) rats. ACh-induced uterine relaxation was reduced in GK nonpregnant (\( n = 8 \)) and Wistar pregnant (\( n = 8 \)) rats compared with Wistar nonpregnant rats (\( n = 8 \)), but there were no differences between GK nonpregnant and GK pregnant (\( n = 6 \)) rats.

Values are means ± SE. *\( P < 0.05 \), Wistar vs. GK ACh alone; \#P < 0.05, ACh alone vs. ACh plus L-NAME for each group. PE, phenylephrine.
in W-Preg compared with W-NP rats. Values are means ± SE; n = 9) rats and in GK-Preg compared with W-Preg (n = 8) compared with W-NP. PAPA NONOate-induced relaxation was increased in GK-NP (n = 8) compared with W-NP (n = 9) rats and in GK-Preg compared with W-Preg (n = 7) rats, and was reduced in W-Preg compared with W-NP rats. Values are means ± SE. *P < 0.05, GK-NP vs. W-NP; †P < 0.05, W-NP vs. W-Preg; ‡P < 0.05, GK-Preg vs. W-Preg.

Reduced uterine artery sensitivity to PAPA NONOate compared with their nonpregnant counterparts (−logEC50, GK nonpregnant: 6.46 ± 0.08 vs. GK pregnant: 5.71 ± 0.11; Wistar nonpregnant: 6.10 ± 0.21 vs. Wistar pregnant: 5.84 ± 0.15; P < 0.05; Fig. 2B).

Inhibition of sGC by ODQ significantly reduced SNP-induced uterine artery relaxation in all groups (P < 0.05; Fig. 3, A and B). ODQ abolished the group differences in SNP-induced responses in pregnant but not in nonpregnant uterine arteries (Fig. 3, A and B). The contribution of sGC-dependent mechanisms to SNP-induced responses was greater in uterine arteries from GK rats compared with arteries from Wistar rats regardless of pregnancy status (main effect of diabetes, P < 0.05; Fig. 3C).

Inhibition of sGC caused a rightward shift in the PAPA NONOate concentration-response curves in all groups but did not affect maximum responses to PAPA NONOate (Fig. 4, A and B). ODQ abolished the group differences in pregnant uterine arteries (Fig. 4B). The contribution of sGC to PAPA NONOate-induced relaxation (%ΔAUC; Fig. 4C) was smaller in GK nonpregnant uterine arteries compared with Wistar nonpregnant arteries (P < 0.05; Fig. 4C). Pregnancy reduced this contribution in uterine arteries from Wistar rats (%ΔAUC, Wistar nonpregnant: 69.0 ± 5.15 vs. Wistar pregnant: 46.0 ± 9.03; P < 0.05), whereas the opposite was found for the GK uterine arteries (%ΔAUC, GK nonpregnant: 33.8 ± 6.04 vs. GK pregnant: 59.6 ± 9.44; P < 0.05).

Neither diabetes nor pregnancy had any effects on 8-Br-cGMP-induced uterine artery relaxation (concentration-response curves not shown; Emax at Table 2).

Effects of PDE5 inhibition on rat uterine artery function. Sildenafil induced greater maximum relaxation responses in uterine arteries from Wistar compared with GK nonpregnant rats (P < 0.05; Fig. 5 and Table 2). However, GK nonpregnant uterine arteries had greater sensitivity to sildenafil compared with Wistar controls (−logEC50, Wistar: 7.10 ± 0.052 vs. GK: 7.96 ± 0.282; P < 0.05; Fig. 5). Pregnancy diminished the maximum responses to sildenafil in uterine arteries from Wistar rats (P < 0.05; Fig. 5 and Table 4) and reduced sensitivity (−logEC50), GK nonpregnant: 7.94 ± 0.143 vs. GK pregnant: 6.90 ± 0.118; P < 0.05; Fig. 5) but did not alter maximum responses to PDE5 inhibition in arteries from GK rats (P > 0.05; Fig. 5 and Table 2). There were no differences in uterine artery responses to sildenafil between pregnant uterine arteries from Wistar and GK rats (P > 0.05; Fig. 5).

Protein expression of components of the sGC/cGMP pathway. No differences existed between GK and Wistar nonpregnant uterine arteries in basal protein expression levels of the sGC isoforms α1 and β1 (P > 0.05; Fig. 6) and PDE5 (P > 0.05; Fig. 7). GK pregnant uterine arteries had reduced protein levels of sGC (both isoforms) compared with nonpregnant GK arteries (P < 0.05), but pregnancy had no effect on these proteins in Wistar uterine arteries (Fig. 6). Pregnancy did not alter the expression of PDE5 in uterine arteries from any of the groups.

Table 2 Maximum uterine artery relaxation responses (percent relaxation from submaximal responses to phenylephrine) to various agonists in Wistar and GK nonpregnant and pregnant rats

<table>
<thead>
<tr>
<th></th>
<th>Wistar Nonpregnant</th>
<th>Wistar Pregnant</th>
<th>GK Nonpregnant</th>
<th>GK Pregnant</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACh</td>
<td>87.2 ± 3.56</td>
<td>80.2 ± 4.71</td>
<td>74.7 ± 6.24</td>
<td>84.3 ± 4.10</td>
</tr>
<tr>
<td>Sodium nitroprusside</td>
<td>92.3 ± 1.31</td>
<td>69.7 ± 7.82b</td>
<td>81.8 ± 2.61</td>
<td>88.3 ± 2.81</td>
</tr>
<tr>
<td>NONOate</td>
<td>97.5 ± 1.27</td>
<td>90.3 ± 2.73a</td>
<td>97.4 ± 0.76</td>
<td>96.1 ± 1.67</td>
</tr>
<tr>
<td>8-Bromoguanosine 3',5'-cyclic monophosphate sodium salt</td>
<td>66.6 ± 6.07</td>
<td>66.5 ± 7.92</td>
<td>65.9 ± 11.73</td>
<td>71.9 ± 3.02</td>
</tr>
<tr>
<td>Sildenafil</td>
<td>85.1 ± 5.91a</td>
<td>46.7 ± 4.18b</td>
<td>53.1 ± 6.05</td>
<td>44.8 ± 5.63</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 8–10 for Wistar nonpregnant, n = 4–9 for Wistar pregnant, n = 4–10 for GK nonpregnant, and n = 4–9 for GK pregnant. *P < 0.05; Wistar nonpregnant vs. Wistar pregnant; †P < 0.05; Wistar pregnant vs. GK pregnant; ‡P < 0.05; Wistar nonpregnant vs. GK nonpregnant.
The main finding of this study is that the uterine vascular smooth muscle from rats with T2D has attenuated responsiveness to NO due to impairment in sGC-dependent mechanisms. These results suggest that rodents with T2D enter pregnancy with preexisting dysfunction of the uterine vasculature. In late pregnancy, uterine arteries from nondiabetic pregnant rats show attenuated vascular smooth muscle relaxation, but uterine arteries from GK rats fail to display this adaptation and exhibit greater vascular smooth muscle sensitivity to NO, despite a substantial reduction in protein levels of sGC.

Our studies showed that endothelium-dependent relaxation was reduced in uterine arteries from nonpregnant rats with T2D, and this impairment was concomitant with a decrease in vascular smooth muscle responsiveness to SNP. These data indicate that in addition to endothelial dysfunction, GK rats exhibit uterine vascular smooth muscle dysfunction. Currently, less is known about the function of the vascular smooth muscle cells in T2D compared with vascular endothelial cells (35). To the best of our knowledge, our study is the first to demonstrate dysfunction of the uterine vascular smooth muscle in the

DISCUSSION

The main finding of this study is that the uterine vascular smooth muscle from rats with T2D has attenuated responsiveness to NO due to impairment in sGC-dependent mechanisms. These results suggest that rodents with T2D enter pregnancy with preexisting dysfunction of the uterine vasculature. In late pregnancy, uterine arteries from nondiabetic pregnant rats show attenuated vascular smooth muscle relaxation, but uterine arteries from GK rats fail to display this adaptation and exhibit greater vascular smooth muscle sensitivity to NO, despite a substantial reduction in protein levels of sGC.

Fig. 3. Concentration-response curves to SNP in the presence and absence of 1H-[1,2,4]oxadiazolo[4,3-a]quinazolin-1-one (ODQ; soluble guanylate cyclase (sGC) inhibitor, 10−6 M) in uterine arteries from nonpregnant (A) and pregnant (B) control (Wistar) and diabetic (GK) rats, and sGC contribution to SNP-induced relaxation (C). ODQ reduced SNP-induced uterine artery relaxations in GK nonpregnant (n = 9), Wistar nonpregnant (n = 9), GK pregnant (n = 9), and Wistar pregnant (n = 7) rats. GK nonpregnant and pregnant rats had increased contribution of sGC to SNP-induced relaxation compared with Wistar nonpregnant and pregnant rats, respectively. Values are means ± SE. *P < 0.05, Wistar vs. GK SNP alone; #P < 0.05, SNP alone vs. SNP plus ODQ for each group; **P < 0.05 vs. Wistar. AUC, area under the curve.

Fig. 4. Concentration-response curves to PAPA NONOate in the presence and absence of ODQ (sGC inhibitor, 10−6 M) in uterine arteries from nonpregnant (A) and pregnant (B) control (Wistar) and diabetic (GK) rats, and sGC contribution to PAPA NONOate-induced relaxation (C). ODQ decreased sensitivity to PAPA NONOate in uterine arteries from GK nonpregnant (n = 8), Wistar nonpregnant (n = 9), GK pregnant (n = 9), and Wistar pregnant (n = 7) rats. Values are means ± SE. *P < 0.05, PAPA NONOate alone vs. PAPA NONOate plus ODQ for GK rats; #P < 0.05, PAPA NONOate alone vs. PAPA NONOate plus ODQ for Wistar rats; *P < 0.05, Wistar nonpregnant rats PAPA NONOate plus ODQ vs. GK nonpregnant PAPA NONOate plus ODQ; **P < 0.05 vs. Wistar nonpregnant; **P < 0.05 vs. GK nonpregnant.
nonpregnant and pregnant states in rats with T2D. Importantly, GK rats have only mild hyperglycemia, which suggests that chronically small elevations in blood glucose may be adequate to induce impairment in smooth muscle function of the reproductive vascular bed.

In contrast with a reduction in SNP-induced relaxation, uterine arteries from GK rats had increased sensitivity to another NO donor, PAPA NONOate. These differences may be explained by dissimilarities in the metabolism and actions of these two drugs. The vasodilator effects of NO are primarily mediated by activation of sGC to induce the generation of the second messenger, cGMP. Therefore, it was speculated that NO donors act via the sGC/cGMP pathway. Previous studies, however, identified a NO-related sGC-independent component of vasodilation in response to high concentrations of NO (8, 16, 26). It was suggested that this sGC-independent action of NO was exclusively related to agents that generate NO extracellularly.

PAPA NONOate is a large molecule that does not enter vascular smooth muscle cells. It spontaneously dissociates to generate NO, and this process is not catalyzed by either thiols or biological tissue (26). On the other hand, SNP requires intracellular metabolism to generate NO. Using a specific inhibitor of sGC, we demonstrate for first time that in rat uterine arteries SNP-induced relaxation is primarily mediated by sGC-dependent mechanisms, whereas PAPA NONOate has both sGC-dependent and independent actions. Interestingly, the contribution of sGC-associated mechanisms to SNP and PAPA NONOate-induced relaxations differ between Wistar and GK nonpregnant uterine arteries. Furthermore, uterine arteries from GK rats exhibited reduced sGC-dependent vasodilator mechanisms, whereas the sGC-independent component of vasorelaxation was enhanced. Previous work has suggested that the contribution of NO-related sGC-independent pathways was exclusively related to agents that generate NO extracellularly.

PAPA NONOate is a large molecule that does not enter vascular smooth muscle cells. It spontaneously dissociates to generate NO, and this process is not catalyzed by either thiols or biological tissue (26). On the other hand, SNP requires intracellular metabolism to generate NO. Using a specific inhibitor of sGC, we demonstrate for first time that in rat uterine arteries SNP-induced relaxation is primarily mediated by sGC-dependent mechanisms, whereas PAPA NONOate has both sGC-dependent and independent actions. Interestingly, the contribution of sGC-associated mechanisms to SNP and PAPA NONOate-induced relaxations differ between Wistar and GK nonpregnant uterine arteries. Furthermore, uterine arteries from GK rats exhibited reduced sGC-dependent vasodilator mechanisms, whereas the sGC-independent component of vasorelaxation was enhanced. Previous work has suggested that the contribution of NO-related sGC-independent pathways was exclusively related to agents that generate NO extracellularly.

PAPA NONOate is a large molecule that does not enter vascular smooth muscle cells. It spontaneously dissociates to generate NO, and this process is not catalyzed by either thiols or biological tissue (26). On the other hand, SNP requires intracellular metabolism to generate NO. Using a specific inhibitor of sGC, we demonstrate for first time that in rat uterine arteries SNP-induced relaxation is primarily mediated by sGC-dependent mechanisms, whereas PAPA NONOate has both sGC-dependent and independent actions. Interestingly, the contribution of sGC-associated mechanisms to SNP and PAPA NONOate-induced relaxations differ between Wistar and GK nonpregnant uterine arteries. Furthermore, uterine arteries from GK rats exhibited reduced sGC-dependent vasodilator mechanisms, whereas the sGC-independent component of vasorelaxation was enhanced. Previous work has suggested that the contribution of NO-related sGC-independent pathways was exclusively related to agents that generate NO extracellularly.
may be upregulated in cardiovascular diseases or inflammatory conditions, where inducible NOS (iNOS) is expressed (15). In addition, Miller et al. (26) found that the sGC-independent actions of NONOates were significantly reduced by extracellular NO scavengers. These authors suggested that NONOates release sufficient NO extracellularly to react with molecular oxygen and form nitrosating species (26). S-Nitrosation of thiol-containing enzymes and ion channels, therefore, may be a potential sGC-independent mechanism of relaxation in the uterine arteries of GK rats.

According to our findings, diabetic uterine arteries use sGC-independent mechanisms to compensate for a reduction in sGC-dependent relaxation. Because responses to 8-Br-cGMP did not differ between GK and Wistar uterine arteries, we hypothesize that the signaling defect in GK uterine vascular smooth muscle cells lies upstream of cGMP (e.g., sGC-induced cGMP generation) and may act in concert with reduced basal NO bioavailability. Due to the small size of rat uterine arteries, we were unable to measure production of cGMP in response to sGC activation. Previous studies, however, have shown reduced sGC-induced cGMP production in conduit arteries from GK rats (48).

The intracellular levels of cGMP are controlled by the rate of cGMP synthesis (sGC-mediated) and by the rate of cGMP hydrolysis (cyclic nucleotide PDE dependent). PDE5 is a cGMP-binding, cGMP-specific PDE that controls the hydrolysis of cGMP in vascular smooth muscle cells. Basal levels of PDE5 did not differ between GK and Wistar nonpregnant rats but this does not exclude the possibility of group differences in PDE5 activity.

In late pregnancy and in the presence of equal amounts of exogenous NO, GK uterine arteries had increased relaxation compared with Wistar rats; however, endothelium-dependent relaxation did not differ between groups. Inhibition of sGC abolished the group differences in uterine responses to NO donors, indicating that these differences were sGC dependent. Our findings are in agreement with previous reports showing reductions in endothelium-independent relaxation to SNP in late pregnant rats (non diabetic) (20, 46). Pregnancy increases endothelium-derived NO production (11, 50) and sGC activity (17) in the uterine vasculature; thus further increases in sGC activity in the presence of exogenous NO to induce vascular smooth muscle relaxation may not be feasible (46). In contrast, GK rats showed a reduction in uterine artery sGC expression and no change in SNP-induced relaxation, whereas PAPA NONOate-induced relaxation (largely dependent on a sGC-independent mechanism) was reduced in response to pregnancy. These data show that the expression of sGC (both subunits) and the sGC-dependent relaxation of smooth muscle in uterine arteries are mediated by the combined effect of pregnancy and T2D, whereas T2D or pregnancy alone have no effect on basal sGC expression and induce relaxation responses opposite from those induced by pregnancy and diabetes together.

The adverse pregnancy outcomes and impairment in uterine vascular smooth muscle responsiveness to NO could be attributed to several factors associated with the pathology of T2D, such as oxidative stress and hyperglycemia. It has been reported that the exposure to a diabetes-associated hyperglycemic environment within the first 7 wk of human pregnancy (equivalent to the first 13.5 gestational days of rat pregnancy) are associated with pre-implantation embryo loss and increased resorption rates (28). Our findings in corroboration with other investigations demonstrated dramatically higher rates of resorptions in rats with T2D compared with nondiabetic pregnant rats (21). Stanley et al. showed endothelial dysfunction and increased production of superoxide in uterine arteries from mice with gestational diabetes, which was attributed to NOS uncoupling (41). These investigators, however, did not investigate the effects of gestational diabetes on vascular smooth muscle sensitivity to NO. Defects in sGC-dependent vascular smooth muscle relaxation have been previously reported in aorta from GK rats and attributed to a reduction in the heme content of the enzyme and/or oxidation of the heme iron (48). Hyperglycemia-induced oxidation of the prosthetic heme group of sGC would lead to reduced uterine vascular smooth muscle sensitivity to NO.

Estrogen and progesterone have been shown to affect vascular smooth muscle reactivity and production of endothelium-derived factors (45). Thus different levels of estrogen and progesterone may be responsible for the differential vascular responses in GK and Wistar rats. However, we had previously demonstrated no significant differences in these hormones between GK and Wistar nonpregnant rats, suggesting that the pregestational vascular dysfunction seen in GK rats cannot be attributed to these hormones (1). Nevertheless, the levels of estrogen and progesterins significantly change during pregnancy (37). It is possible that the alterations in uterine vascular dilatory mechanisms seen in GK pregnant rats are mediated by different estrogen and progesterone levels between GK and Wistar rats.

Pregnant Wistar and GK uterine arteries showed a reduction in sensitivity to sildenafil (PDE5 inhibitor) compared with the nonpregnant state, whereas GK nonpregnant uterine arteries had increased sensitivity to sildenafil compared with Wistar nonpregnant arteries. This is the first report to document such pregnancy- and diabetes-induced adaptations. Sildenafil has been previously used in pregnant women and animal models to increase uterine dilatatory responses and promote an increase in uterine blood flow. Indeed, sildenafil improved endothelial function of isolated myometrial vessels from pregnant women with intrauterine growth restriction and increased uterine blood flow in women with healthy pregnancies (14, 27, 44). In animal models of pregnancy-induced hypertension and preeclampsia, sildenafil also improved endothelial function (9, 43). Moreover, the use of sildenafil has been recommended for pregnancies complicated with pulmonary hypertension because of the dilatary actions of this PDE5 inhibitor in the pulmonary circulation (22). Because sildenafil has the ability to increase uterine blood flow, it could be a reasonable choice of treatment for women with T2D and vascular dysfunction. Nevertheless, high drug concentrations may be necessary in pregnant women and the toxicity of those concentrations should be considered (39). On the contrary, if sildenafil treatment starts before the beginning of pregnancy, where women with T2D may have increased uterine sensitivity to sildenafil, lower concentrations of this drug may be necessary to adequately increase blood flow to the uterine and decidua tissues.

In conclusion, our study demonstrated that uterine arteries from rats with T2D had endothelial dysfunction, which may be partially explained by reduced sensitivity of uterine vascular smooth muscle sGC to NO. During pregnancy, the GK uterine...
vascular smooth muscle fails to show relaxation responses similar to those of arteries from nondiabetic rats of the same gestational age and GK rats have dramatically greater rates of resorptions compared with Wistar rats. We propose that the diabetic uterine vascular smooth muscle may be a novel pharmacological target to induce vascular improvements. Prenatal combination of PDE5 inhibitor treatment with good glycemic control may improve function of the uterine vasculature in women with T2D, increase blood flow to the uteroplacental unit, and improve pregnancy outcomes.

GRANTS
This study was supported in part by the National Institutes of Health Grants R01 HL-071138, R01 DK-083685, and T32 HL-066933-09; American Heart Association Scientist Development Grant 13SDG1705056; the Naito Foundation Japan; the Heart and Stroke Foundation Canada; and the Society for Women’s Research.

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS
Author contributions: S.G., J.L.H., T.M., and R.C.W. conception and design of research; S.G., J.L.H., and T.M. performed experiments; S.G. and

REFERENCES


