Previous gestational diabetes impairs long-term endothelial function in a mouse model of complicated pregnancy

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GESTATIONAL DIABETES MELLITIS (GDM) is an acknowledged risk factor for the development of type 2 diabetes and cardiovascular disease (CVD). GDM, which is defined by the American Diabetes Association as glucose intolerance of any degree with onset or first recognition during pregnancy (2), is one of the most commonly observed obstetrical complications, affecting between 5–8% of all pregnancies worldwide (18). Depending on the presence of other risk factors, women with GDM have around a 60% risk of developing type 2 diabetes within 5–16 yr (9). One of the earliest consequences of diabetes, and a risk factor for CVD, is the development of endothelial dysfunction. Studies of women with previous GDM have been carried out up to 1 yr after pregnancy, following a return to normal glucose tolerance. These studies indicate the presence of endothelial dysfunction (3, 17). It is not known, however, whether these women had developed endothelial dysfunction prior to the GDM pregnancy. The dysfunction observed may have been unmasked by GDM; alternatively the endothelial dysfunction may have developed as a consequence of GDM. If GDM induces permanent endothelial dysfunction, this will be an additional risk factor for developing CVD in women with a history of GDM.

There are a number of different mechanisms by which GDM may mediate endothelial dysfunction, including the production of advanced glycation end products, altered glucose metabolism, and the activation of protein kinase C. These pathways, however, do have a common link: they may all be activated by hyperglycemia-induced overproduction of superoxide. An increase in superoxide production has been observed in endothelial cells exposed to high glucose concentrations (6), as well as in animal models of diabetes (10). Furthermore, clinical studies have observed impaired forearm vasodilation and increased oxygen-derived free radicals following acute glucose loading (12). Superoxide may induce endothelial dysfunction via reduced bioavailability of nitric oxide (NO); superoxide will preferentially react with NO to form peroxynitrite. Peroxynitrite may further reduce NO bioavailability via the oxidation of tetrahydrobiopterin, an essential cofactor of NO synthase (NOS) (18). The resulting uncoupling of NOS causes a reduction in NO production, and a further increase in superoxide production (21). Peroxynitrite may also mediate endothelial dysfunction via the impairment of voltage-gated (15) or calcium-activated (16) K+ channels, as well as through nitrosylation of proteins and DNA damage.

The aim of this study was to utilize a mouse model of GDM to assess the effect of previous GDM on later-life maternal endothelial function. The mouse model used was the db/db mouse, which contains a loss of function mutation in the leptin receptor. Mice which are heterozygous for this mutation (Lepr

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observed (11, 23); these differences parallel the signs observed in women with GDM, and the db/db model was thus used in the studies described herein. By using this model, we will be able to assess the long-term effects of previous GDM on endothelial function in small systemic arteries. Mice with a previously healthy pregnancy (Lepr+/+) and virgin control mice will also be assessed to confirm the impact of previous GDM on endothelial function i.e., the impact of a pregnancy complicated by GDM may be separated from that of a healthy pregnancy and the genetic differences. We hypothesize that GDM on endothelial function i.e., the impact of a pregnancy

MATERIALS AND METHODS

All protocols were approved by the University of Alberta Health Sciences Animal Policy and Welfare committee in accordance with the Canadian Council on Animal Care and conform to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Animal Model of GDM

Twelve-week-old virgin female mice, heterozygous for a leptin receptor mutation (Lepr<sup>dm/+</sup>) or WT littermate controls (Lepr<sup>+/+</sup>), were used (24 mice total, acquired from the Jackson Laboratory). WT and He females (n = 6) were housed overnight with control males; the presence of a vaginal plug the following morning was deemed day 0.5 of gestation. Mice were weighed at day 18.5 of gestation, and a blood sample was collected via venopuncture to measure blood glucose concentration (to confirm development of GDM). Mice were allowed to nurse and their pups, which were weaned at 3 wk of age. The dams were then group housed according to genotype. At 9–10 mo of age (6–7 mo after the index pregnancy) mesenteric artery function was assessed. Arteries obtained from age-matched virgin control mice (both He and WT, n = 6) were also investigated.

Blood Glucose Concentration

Fasting blood glucose concentration was measured at day 18.5 of gestation, then again at 9 mo of age. Mice were fasted for 3 h before testing. A blood sample was collected following tail venopuncture, and blood glucose concentration was measured using a glucometer (Accu-Check Advantage; Roche Diagnostics, Mannheim, Germany).

Wire Myography

Mice were euthanized at 9–10 mo. The mesentery and the surrounding tissue were removed and placed in ice-cold physiological salt solution (PSS) (in mmol/l: 10 HEPES, 1.56 CaCl<sub>2</sub>, 142 NaCl, 4.7 KCl, 1.17 MgSO<sub>4</sub>, 1.18 KH<sub>2</sub>PO<sub>4</sub>, 5.5 glucose; pH 7.4). Vascular function was assessed in second-order mesenteric arteries; these are small systemic arteries from mice with previous GDM will pregnancy and the genetic differences. We hypothesize that GDM on endothelial function i.e., the impact of a pregnancy

RESULTS

Body Weight and Fasting Blood Glucose Concentration

The effect of previous GDM on both body weight and fasting blood glucose concentration was assessed. At 9 mo of age, He mice that were previously pregnant were heavier than all other mice, but their fasting blood glucose concentration was not significantly different (Fig. 1). The increase in body weight following GDM reflects the larger increase in weight gain observed during pregnancy. He mice gained significantly more weight during pregnancy than their WT controls (19.3 vs. 13.2 g; P < 0.01). Pregnant He mice also displayed a significant increase in blood glucose concentration compared with pregnant WT mice (7.9 ± 0.5 vs. 5.1 ± 0.1 mmol/l; P < 0.01).

Superoxide Production and Peroxynitrite Formation

Superoxide production was assessed using dihydroethidium according to a previously developed protocol (19). Peroxynitrite formation was measured using dihydrorhodamine. Twenty-micrometer second-order mesenteric artery sections were prepared and stored at −80°C. Before use, sections were equilibrated for 10 min at 37°C using HBSS (GIBCO). Sections were then incubated with 10 μmol/l dihydroethidium (Molecular Probes, Mississauga, ON, Canada) or 25 μM dihydrorhodamine (Sigma) in HBSS for 20 or 25 min, respectively, at 37°C. Sections were then washed with HBSS ×3, and coverslips applied. Slides were visualized immediately, and images were taken using a fluorescence microscope (Olympus IX 81) using the CY3 filter. Images were analyzed using Adobe Photoshop to determine mean fluorescence intensity/pixel in each vessel. Two arterial sections per animal were used, and the mean value was determined; arteries from three animals per group were studied.

Statistical Analysis

The distribution of all data was tested for normality using the Kolmogorov-Smirnov test prior to statistical analysis. All normally distributed data are expressed as means ± SE and were compared using a two-way ANOVA followed by Bonferroni post hoc test. Sigmoidal curve fitting was performed on wire myography concentration-response curve data using Graphpad Prism software; these curves were then used to determine EC<sub>50</sub> and EC<sub>90</sub> values.

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Effect of Previous Gestational Diabetes on Mesenteric Artery Function

Agonist-induced vasoconstriction. Previous GDM had a significant effect on the vasoconstrictor response, and a significant interaction between genotype and previous pregnancy was observed ($P < 0.01$). Mesenteric arteries taken from mice with previous GDM were significantly more sensitive to U46619 when compared with those from previously pregnant WT mice ($P < 0.01$, Fig. 2B). There was, however, no difference in maximum constriction of arteries from mice with previous GDM or WT controls (123 ± 16 vs. 111 ± 19% maximum response to KPSS; Fig. 2A).

In comparison, there was no effect of genotype on maximum constriction in response to U46619 in mesenteric arteries obtained from virgin WT and He mice (136 ± 12 and 151 ± 18% maximum response to KPSS; Fig. 2A). Furthermore, there was also no difference in sensitivity between arteries from virgin WT and He mice (Fig. 2B).

Agonist-induced vasodilation. Previous GDM also significantly affected endothelium-dependent vasodilation, and a significant interaction between genotype and previous pregnancy was observed ($P < 0.01$). MCh-induced maximum relaxation of arteries from mice with previous GDM was significantly reduced compared with those from previously pregnant WT mice (29 ± 8% vs. 58 ± 16% relaxation; $P < 0.05$, Fig. 3A). The maximum relaxation response of arteries from virgin mice, or those with a previously healthy pregnancy, was in line with that previously observed by our laboratory (5). Similarly, sensitivity of arteries from mice with previous GDM to MCh was significantly decreased compared with that of arteries from previously pregnant WT mice ($P < 0.01$, Fig. 3A). In comparison, there was no effect of genotype in virgin animals. Maximal relaxation of arteries from virgin WT and He mice (54 ± 5 and 57 ± 12% relaxation, respectively; Fig. 3A) was comparable to that seen in arteries from previously pregnant WT mice. There was also no difference in sensitivity between vessels from never-pregnant WT and He mice (Fig. 3B).

The effect of the endothelium-independent vasodilator SNP was also assessed. There was no effect of previous GDM on this response (Fig. 4).
relaxation compared with that seen with MCh alone (44 ± 10 vs. 51 ± 6% relaxation; Fig. 6A). When arteries from mice with previous GDM were investigated, however, l-NAME significantly reduced maximal relaxation compared with that seen in response to MCh alone (7 ± 4% vs. 29 ± 8% relaxation; Fig. 6D). Similar effects were seen when vessels were incubated with l-NAME and meclofenamate in combination. There was no effect on relaxation in arteries from previously pregnant WT animals (38 ± 3% relaxation; Fig. 6E). The maximal response seen in arteries from mice with previous GDM was significantly impaired (10 ± 5% relaxation; Fig. 6F). Again there was no effect of meclofenamate on arteries from either previously pregnant WT mice or mice with previous GDM (50 ± 3% and 27 ± 10% relaxation; Fig. 6, B and E).

**Superoxide Production and Peroxynitrite Formation**

Similarly, previous GDM had a significant effect on the production of superoxide in mesenteric arteries, and a significant interaction between genotype and previous pregnancy was observed (P < 0.01). When superoxide production was measured in arteries taken from WT mice, there was a significant reduction in vessels from previously pregnant mice (Fig. 7C) compared with those from virgin controls (Fig. 7A, 64.6 ± 1.6 vs. 84.1 ± 2.1, mean fluorescence intensity; P < 0.01; Fig. 7E). In comparison, superoxide production in arteries taken from mice with previous GDM (Fig. 7D) was significantly increased compared with that seen in arteries from virgin He mice (Fig. 7B, 93.3 ± 2.3 vs. 59.6 ± 1.7, mean fluorescence intensity; P < 0.01, Fig. 7E). There was also a significant reduction in superoxide production in arteries from virgin He mice compared with virgin WT mice (P < 0.01, Fig. 7E).

Previous GDM had a significant effect on peroxynitrite formation, and a significant interaction between genotype and pregnancy was demonstrated (P < 0.01). Peroxynitrite formation was significantly increased in arteries from mice with previous GDM compared with their virgin controls (173.5 ± 11.0 vs. 57.4 ± 16.2, mean fluorescence intensity; P < 0.01; Fig. 8). There was also a significant increase in peroxynitrite formation in arteries from mice with previous GDM compared with arteries from previously pregnant WT mice (133.7 ± 11.3, mean fluorescence intensity; P < 0.05; Fig. 8). Similar to

**Mechanisms of Endothelium-Dependent Vasodilation**

To determine the effect of previous GDM on mechanisms of endothelial-dependent relaxation, the response to MCh was assessed in the presence of inhibitors of NOS (l-NAME) and COX (meclofenamate). This demonstrated a significant effect of previous GDM on the mechanisms of endothelial-dependent relaxation. When arteries from virgin WT and He mice were investigated, maximal relaxation was significantly reduced in the presence of l-NAME compared with that seen in response to MCh alone (16 ± 7 vs. 51 ± 6% relaxation and 14 ± 10 vs. 50 ± 12% relaxation, respectively, Fig. 5, A and D). A similar reduction of maximal relaxation was observed in arteries from virgin WT and He mice in the presence of l-NAME and meclofenamate (23 ± 11% and 24 ± 7% relaxation, Fig. 5, C and F). There was, however, no effect of meclofenamate alone in vessels from either virgin WT or He mice (39 ± 8 and 52 ± 11% relaxation, respectively, Fig. 5, B and E).

In comparison, when arteries from previously pregnant WT mice were used, there was no effect of l-NAME on maximal

![Fig. 3. Maximal relaxation and sensitivity to methacholine (MCh) was significantly reduced following GDM. A: relaxation in response to MCh was significantly impaired in mesenteric arteries of mice with previous GDM compared with their virgin controls and both groups of WT mice. B: sensitivity to MCh was significantly decreased in arteries from mice with previous GDM compared with all other groups. Means ± SE, n = 6; two-way ANOVA, *P < 0.05, **P < 0.01.](image-url)

![Fig. 4. Endothelium-independent relaxation was not impaired by previous GDM. There was no effect of genotype or PP on endothelium-independent relaxation elicited by sodium nitroprusside (SNP).](image-url)
the superoxide levels, there was a significant reduction in peroxynitrite formation in arteries from virgin He mice compared with the WT mice ($P < 0.05$, Fig. 8).

**DISCUSSION**

This study investigated the vascular function of small systemic arteries from 9-mo-old mice with previous GDM, as well as age-matched mice with a previously healthy pregnancy and virgin controls. Arteries from mice with previous GDM demonstrated an increased sensitivity to the vasoconstrictor U46619 and reduced endothelium-dependent vasodilation compared with arteries from mice with a previously healthy pregnancy or virgin controls. Within these vessels an increase in oxidative stress (increased superoxide production and increased peroxynitrite formation) was also observed. These results suggest that a pregnancy complicated by GDM has a significant impact on later-life endothelial function and may be an additional risk factor for the development of CVD.

It has been consistently demonstrated that women with GDM do develop endothelial dysfunction during pregnancy, in both systemic (14) and uterine arteries (4). There has, however, been little investigation of whether the GDM pregnancy causes the endothelial dysfunction observed, or whether this endothelial dysfunction was evident prior to pregnancy. A study of women with previous GDM, 3–6 mo after the complicated pregnancy, observed impaired flow-mediated dilation in the brachial artery despite a return to normal glucose tolerance (3). Similar changes were observed by Pleiner et al (17); changes in forearm blood flow in response to vasoactive compounds were investigated in women with previous GDM, but again, normal glucose tolerance, up to 1 yr after the complicated pregnancy. Impaired endothelium-dependent vasodilation was observed that correlated with increased levels of an endogenous NO inhibitor (asymmetric dimethylarginine), indicating the impaired response was associated reduced NO bioavailability.

In the study described here, arteries from mice with previous GDM displayed an increased sensitivity to the vasoconstrictor U46619. An increased pressor response is characteristic of endothelial dysfunction; the increase in sensitivity to U46619 observed suggests the development of vascular dysfunction in these arteries. There was also a significant effect of previous GDM on endothelial-dependent vasodilation; both maximal relaxation and sensitivity to MCh was significantly decreased in arteries from mice with previous GDM compared with all other groups. We observed that endothelium-dependent vasodilation of arteries from the other groups (a previously healthy pregnancy and the virgin controls) was comparable. Thus, it is the impact of a GDM pregnancy, as opposed to previous

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**Fig. 5.** *N*-nitro-L-arginine methyl ester (L-NAME) impaired relaxation in mesenteric arteries of virgin mice. Incubation of mesenteric arteries from virgin WT mice with L-NAME (A) or L-NAME and meclofenamate (C) significantly reduced maximal relaxation. B: incubation with meclofenamate alone had no effect. Maximal relaxation was significantly reduced in arteries from virgin He mice following incubation with L-NAME (D) and L-NAME and meclofenamate (F) but not meclofenamate alone (E). Means ± SE, n = 6; two-way ANOVA, #P < 0.01; *P < 0.05 compared with MCh alone (Bonferroni post hoc test).
pregnancy or the genotype of the mice that results in endothelial dysfunction. Endothelium-independent vasodilation was also assessed in all arteries using SNP; this response did not differ across the groups. This demonstrates that previous GDM does not affect the ability of vascular smooth muscle to relax in response to donated NO and that the reduced vasodilation observed in arteries from mice with previous GDM is due to impaired endothelial function.

To try to understand possible mechanisms by which previous GDM mediates endothelial dysfunction, inhibitors of NOS and COX were used. In virgin animals, prior incubation with L-NAME significantly inhibited the response to MCh, suggesting that endothelium-dependent relaxation in these arteries was largely dependent on NO, regardless of genotype. An interesting observation was made when investigating mechanisms of endothelium-dependent relaxation in arteries from previously pregnant WT animals. When arteries were incubated with L-NAME and meclofenamate, alone or in combination, there was little effect on vasodilation. This suggests that the response to MCh in these arteries was largely mediated by the non-NO/non-COX component and that there may be a lasting impact of pregnancy on mediators of endothelium-dependent vasodilation. These results mirror those seen in arteries from women; a study of myometrial arteries demonstrated that while those from nonpregnant women were primarily dependent on NO, those obtained from women with a healthy pregnancy displayed both NO and EDHF components (13). It is suggested that both pathways may be enhanced during pregnancy to ensure maximal relaxant capacity in the event that one is unable to function. The data presented here suggests that the enhancement of the non-NO/non-COX pathway may be maintained long-term.

In comparison, although endothelium-dependent relaxation of arteries obtained from mice with previous GDM was minimal, there was still a significant impairment of relaxation in the presence of L-NAME. This suggests that the upregulation of the non-NO/non-COX pathway that occurred following a healthy pregnancy either did not occur in animals with GDM or was not maintained long-term. These results also mirror those observed in women with previous GDM, up to 1 yr after the pregnancy. In these women, impaired endothelium-dependent vasodilation was also observed (17). There is, however, one important difference between these two studies; it is not known whether the women with previous GDM demonstrated endothelial dysfunction prior to the pregnancy. By using virgin control mice and those with a previously healthy pregnancy it was possible to demonstrate that it was the previous GDM pregnancy that affected endothelial function.
The finding that arteries from the previously GDM group remain primarily dependent on NO could pose further complications with aging due to enhanced vascular oxidative stress. Increased oxidative stress, specifically an increase in superoxide, can lead to an increase in peroxynitrite formation through a reaction with NO. In light of possible scavenging of NO by superoxide to form peroxynitrite, both superoxide production and peroxynitrite formation was measured in these vessels. Previous studies have demonstrated in the homozygous form of this mouse (Lepr<sup>db/db</sup>), which displays type 2 diabetes, that impaired endothelial-dependent relaxation is associated with increased superoxide production (1). Additionally, an increase in superoxide production has also been observed, following exposure to high glucose, in both mouse microvessel endothelial cells and cultured human endothelial cells (8, 10). Superoxide may significantly reduce the bioavailability of NO by scavenging it and forming peroxynitrite. As well as reducing the bioavailability of NO, peroxynitrite may cause long-term endothelial cell dysfunction via protein nitrosylation and DNA damage. Interestingly, in our study, we noted that superoxide production was significantly reduced in arteries from virgin He mice compared with those from virgin WT mice. The mechanism(s) responsible for this change are unclear. The reduced production of superoxide in arteries from virgin He mice, however, only further highlights the dramatic impact of previous GDM on superoxide production in these vessels. Although it was significantly reduced following a healthy pregnancy, there was a significant increase in production following GDM. These data illustrate that there is increased oxidative stress in arteries from mice with previous GDM, despite normal glucose tolerance. Peroxynitrite formation was also significantly increased in arteries from mice with previous GDM, possibly as a result of the increased superoxide formation observed. This is of particular importance as, although endothelium-dependent relaxation of arteries from mice with previous GDM is significantly reduced, it is primarily dependent on NO. The increase in superoxide production and increased scavenging of NO to form peroxynitrite may be one mechanism by which endothelial dysfunction is mediated in arteries from these mice. Again, peroxynitrite formation was significantly reduced in arteries
from virgin He mice compared with virgin WT mice; it is likely this is due to the reduced superoxide production observed in these vessels.

It was observed that, not surprisingly, mice with previous GDM were significantly heavier than either their virgin counterparts or previously pregnant WT mice. It has been demonstrated in other studies that He mice gain more weight during pregnancy than WT mice (11). This was also observed in this study, and the increase in postnatal body weight observed in the animals studied here may reflect this. Indeed, the weight of He mice at term was ~6 g more than that of WT mice; at 9 mo of age, He mice remained ~6 g heavier than WT mice. Pregnant He mice also demonstrated a significant increase in fasting blood glucose compared with all other groups. Despite the changes that occurred during pregnancy, mice with previous GDM demonstrated blood glucose concentrations that were indistinguishable from controls at the time of study (9 mo of age). This reflects the clinical observation that the majority of women with GDM return to euglycemia postpartum (7). It is possible that the endothelial dysfunction observed in mice with previous GDM may be attributable to the small but significant increase in body weight observed. Indeed, a small study of women with previous GDM observed that those who were overweight (body mass index >25 kg/m²) showed reduced forearm blood flow in response to the endothelium-dependent vasodilator acetylcholine compared with those who were not overweight (17). This impaired response was associated with reduced NO bioavailability. Therefore, while it is possible that the small increase in body weight observed in this study may contribute to the endothelial dysfunction seen, it is likely that GDM previously experienced by these mice played an important role in mediating the impairment of endothelium-dependent relaxation.

One potential limitation of this study is that the leptin receptor mutation present in He mice may have effects independent of the rise in blood glucose concentration and increased weight gain. Throughout this study, however, virgin He mice have been used as an additional control, and results suggest specific effects of the pregnancy-associated phenotype.

GDM is currently one of the most commonly observed obstetrical complications. As the incidence of diabetes contin-
ues to rise, it is likely that cases of GDM will also increase. The results presented here highlight the long-lasting effects that GDM may have on vascular function, even in the absence of overt diabetes. Thus our data indicate that a history of GDM, in the absence of other risk factors, should be observed as a risk factor for the development of CVD.

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DISCLOSURES

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

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