Consumption of a Western-style diet during pregnancy impairs offspring islet vascularization in a Japanese macaque model

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The prevalence of childhood obesity has risen dramatically in the US, almost tripling in the past 30 years (48) and leading to an increased risk for a range of metabolic complications, including type 2 diabetes, cardiovascular disease, and stroke (24, 58). Although diet and lack of physical activity play a key role in this epidemic, a suboptimal in utero environment poses significant risks to the developing fetus. Specifically, maternal consumption of an obesogenic diet and increased maternal adiposity prior to gestation contribute to an increased predisposition for the development of obesity and type 2 diabetes in the offspring (7, 37, 57). The relationship between poor maternal nutrition and excessive weight gain with type 2 diabetes predisposition in the offspring is poorly understood but likely involves a number of detrimental developmental changes in key fetal metabolic organs, such as the pancreatic islet (reviewed in Ref. 17).

Our group has developed a model of maternal Western-style diet (WSD) consumption during pregnancy in the Japanese macaque (Macaca fuscata), a nonhuman primate (NHP) model whose islet closely resembles the human islet in development, structure, and function (12, 35). Previous data from our group have indicated that maternal WSD consumption during pregnancy leads to a broad range of complications in the fetal and juvenile offspring (21, 25–29, 43, 44, 48, 52–54). Specifically, maternal exposure to a WSD is associated with placental insufficiency (25), a reduction in islet α-cell mass, and an increase in the β-cell/α-cell ratio (21), which may have a negative impact on islet paracrine interactions and thus function. Vascularization and innervation also play key roles in islet development and function during the development of the offspring (reviewed in Ref. 18). The pancreatic islets are highly vascularized micro-organs, and this dense capillary network plays a critical role in its ability to detect changes in glycemia and respond accordingly (10, 41, 47). Furthermore, intraislet capillaries promote exposure to metabolic stimuli and critical growth factors that stimulate normal islet development (20). Similarly, angiogenic markers such as vascular endothelial growth factor A (VEGF-A), angiopoietins, and ephrins are produced by the islet and serve to enhance islet angiogenesis (9, 49, 59). Islet innervation, like vascularization, plays a key role in communication and function. Whereas sympathetic fibers and their neurotransmitters, mediated primarily by noradrenaline, inhibit insulin secretion and stimulate glucagon secretion, parasympathetic neurotransmitters stimulate insulin secretion (reviewed in Ref. 3).

To date, however, the role of vascularization and innervation in altered fetal islet development following maternal WSD consumption has not been investigated. Extensive remodeling of the islet and its vascularization occurs during the second half of gestation, during which time islets become innervated (23). Thus, we investigated the effect of WSD consumption during pregnancy on islet vascularization and innervation in both the fetus during the early third trimester and the 1-yr-old juvenile offspring. We hypothesized that exposure to WSD in utero would result in altered vascularization and innervation, mirroring defects in islet development, that will persist into the juvenile offspring.

Because the second half of gestation is a critical developmental period for the islet, therapeutic interventions during this stage may mitigate some or all of the adverse effects of in utero WSD exposure. Importantly, we have tested both lifestyle modification as well as dietary supplementation with resveratrol to determine whether these might provide novel and...
clinically relevant methods to manage the detrimental developmental outcomes. In particular, we hypothesized that improved dietary intake during pregnancy by women who have been chronically consuming a WSD could ameliorate the harmful consequences on fetal development independently of prevailing obesity and adiposity in the mother. Furthermore, resveratrol, a small naturally occurring polyphenol, has garnered significant attention recently for its anti-aging, anti-inflammatory, and pro-vascular properties (5, 38) and thus is an appealing option for women consuming a WSD. We hypothesized that the vasodilatory actions of resveratrol would improve WSD-induced impairments in the development of the islet vasculature.

MATERIALS AND METHODS

Animal care. All animal procedures were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) of the Oregon National Primate Research Center (ONPRC) and Oregon Health and Science University and were approved by the ONPRC IACUC. The ONPRC abides by the Animal Welfare Act and Regulations enforced by the USDA and the Public Health Service Policy on Humane Care and Use of Laboratory Animals in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health.

Adult NHP model. Five adult male rhesus macaques (Macaca mulatta) aged 9–13 yr were fed ad libitum with a CTR diet (14% calories from fat; Test Diet). Tissue was collected at necropsy by a Veterinary Pathologist, as described previously (46). The pancreas was isolated and divided into sections from head to tail to be fixed in zinc formalin for immunohistochemical analysis.

Maternal WSD NHP model. This animal model has been described previously (43). Briefly, Japanese macaques were provided ad libitum with a control (CTR) diet (14% calories from fat) or WSD (32% calories from fat; Test Diet; SLOP, Purina Mills) supplemented with calorically dense treats for 4–7 yr. Metabolizable energy from CTR and WSD diets was 2.87 and 3.80 kcal/g, respectively. The diet reversal (REV) cohort was maintained on the WSD for 4 yr before being switched onto the CTR diet during the breeding season of the 5th yr. The WSD/resveratrol (RESV) cohort received the WSD for 7 yr prior to being switched onto a WSD containing resveratrol (High-purity trans-resveratrol, Resvida; DSM Nutritional Products) at a final concentration of 0.37% 3 mo prior to the breeding season. Animals were housed in indoor/outdoor pens with one to two males and three to 11 females and were allowed to breed naturally. Gestational age was estimated by ultrasound technology, as described previously (25), and pregnancies were terminated by Cesarean section (C-section) at GD130 in the Surgical Services Unit. Both male and female offspring were used. Maternal body weight and composition and fetal body weights can be found in Refs. 21, 43, and 52.

Juvenile studies were performed as described previously (21). Briefly, male and female offspring were born naturally and maintained on the dam’s diet until weaning. At ~8 mo, offspring were either weaned onto their in utero diet or switched onto the opposing diet to generate four treatment groups: CTR/CTR, CTR/WSD, WSD/CTR, and WSD/WSD.

Figure 2, A and B, provides an overview of fetal and juvenile study design, respectively.

Maternal intravenous glucose tolerance tests. Intravenous glucose tolerance tests were performed on overnight-fasted dams at 1 wk prior to C-section, as described previously (43). Briefly, animals were sedated and administered a glucose bolus of 0.6 g/kg body wt via the saphenous vein. Maternal insulin secretion was calculated as area under the curve from fasting value at t = 0.

Tissue collection. Following C-section, fetuses were taken to the Pathology Unit for full necropsy. Juvenile animals were sent to necropsy at ~13 mo of age. Fetal and juvenile organs were dissected by the veterinary pathologist, weights and measures were documented, and individual tissues were processed according to protocol requirements. The pancreas was isolated and divided into sections from head to tail to be fixed in zinc formalin for immunohistochemical analysis or flash-frozen in liquid nitrogen for RNA isolation, as described previously (21).

Pancreas RNA isolation and quantitative RT-PCR. Total RNA isolation, reverse transcription, and quantitative PCR were performed as described previously (21). RNA polymerase II was used to normalize real-time expression using the Pfaffl method (51).

Islet immunohistochemistry and quantitative analysis. Five-micrometer paraffin-embedded sections from the pancreatic tail were deparaffinized, and standard immunohistochemical methods were used for subsequent analyses (21). Primary antibodies were applied for 48 h at 4°C. The following primary antibodies were used to detect pancreatic antigens: guinea pig anti-insulin (1:2,500; Dako), rabbit anti-glucagon (1:100; Cell Signaling Technology), sheep anti-platelet endothelial cell adhesion molecule 1 (PECAM1; 1:250; R & D Systems), goat anti-VEGF-A (1:100; R & D Systems), mouse anti-Ki67 (1:25; Dako) and mouse anti-TH (1:100; Millipore). Secondary antibodies were applied for 1 h at room temperature (1:1,500; Jackson Immunoresearch Laboratories).

Images were acquired with the Marinas imaging workstation (Intelligent Imaging Innovations, Denver, CO), as described previously (21). Cell vascularization and innervation were assessed using the stereology module within the tail region of each pancreata. Vessels and nerves were counted manually, where any fluorescently labeled vessel or nerve fiber within the islet or making contact with an islet was included. PECAM1+ area was calculated by intensity segmentation, using Slidebook 5.0 imaging software. Vascularization is expressed as either number of capillaries or PECAM1+ area per islet area. Area per capillary is calculated by dividing the total PECAM1+ area by the number of PECAM1+ fibers. Vascular proliferation was measured by colocalization of PECAM1 with the proliferation marker Ki67. Sympathetic innervation is expressed as number of TH+ fibers per islet area. Representative images were acquired with the Leica SP5 confocal microscope using the ×40 objective at 1,024 × 1,024 pixel resolution. Focal planes were 1 μm apart.

Data analysis. Fetal data were analyzed using a one-way ANOVA with Tukey’s multiple comparison post hoc analysis (CTR vs. WSD vs. REV vs. WSD/RESV). Juvenile data were analyzed using a two-way ANOVA to test for maternal or postweaning diet effect. Correlation analyses were performed using SPSS Statistics 19 software (Armonk, NY).

RESULTS

The adult NHP islet is highly vascularized and expresses VEGF-A in the pancreatic β-cell. Although rodent and human islet vasculature and its angiogenic signals have been relatively well characterized, the vasculature in the NHP islet has not been investigated previously. We sought to define and quantify vascularization in the healthy NHP islet. Consistent with data in both the rodent (10) and the human (reviewed in Ref. 31), the NHP adult islet is highly vascularized compared with the surrounding exocrine tissue, possessing an approximately sixfold greater capillary density (Fig. 1, A and B). In addition, we investigated the expression pattern of VEGF-A, which in the rodent is highly expressed and secreted by the pancreatic β-cell, where it recruits endothelial cells and promotes capillary growth and angiogenesis (6, 19, 32). Similarly, we demonstrated that the NHP β-cell expresses VEGF-A, indicating overlapping angiogenic mechanisms between mammalian species (Fig. 1C).
Maternal WSD consumption impairs the development of the islet vasculature in the fetal offspring. We have demonstrated previously that WSD consumption during pregnancy results in a significant reduction in α-cell mass and an increase in the β-cell/α-cell ratio by the early third trimester (21). Because islet vascularization is critical for normal islet development and defects in vascularization often precede changes in islet morphology (30), we hypothesized that exposure to a WSD in utero would result in impaired islet vascularity. We used PECAM1 to visualize islet capillaries in the fetal pancreas at gestational day (GD) 130, where term is ~170 days. WSD offspring display a significant reduction in islet capillary area (Fig. 3, A and B). This effect is due to both a decrease in the number of islet-associated capillaries (Fig. 3C) and a smaller average size of the remaining vessels (Fig. 3D), possibly indicative of impaired capillary dilation. Interestingly, increased maternal insulin secretion during a glucose tolerance test was weakly associated with reduced islet vascularization (Fig. 3E), an effect that was influenced but not entirely dependent on three high-responding dams. These data indicate that maternal insulin resistance may increase the risk of maladaptive islet development.

Improved maternal nutrition and maternal resveratrol supplementation increase islet vascularization. To investigate whether the detrimental effects of maternal WSD consumption on islet vascularization could be mitigated, we characterized two models of dietary intervention. First, dams that had been maintained on a WSD were switched onto a healthy CTR diet prior to pregnancy (REV) (Fig. 24). REV dams do not lose a substantial amount of body weight, nor do they display significant metabolic improvements during this time (43). However, diet reversal normalized islet vascular area (Fig. 3B), capillary density (Fig. 3C), and average area per capillary (Fig. 3D) in the fetal offspring at GD130 at a time when α-cell mass was increased by 28% (6.525 vs. 5.086 mg in WSD) and the β-cell/α-cell ratio decreased by 28% (1.889 vs. 2.611 in WSD) with no change in islet mass compared with WSD offspring. Similarly, islet size distribution did not differ between the cohorts (data not shown).

In an additional cohort of WSD-fed dams, we investigated whether resveratrol supplementation during pregnancy could improve the WSD-induced impairment in islet vascularization. Maternal resveratrol supplementation led to a significant increase in fetal islet capillary area (Fig. 3B), density (Fig. 3C), and area per capillary (Fig. 3D) compared with WSD fetal islets. However, supplementation also resulted in a 57% increase in capillary density over CTR offspring (Fig. 3C).

Alterations in islet vasculature are not mediated by changes in angiogenesis. The development of the vasculature is tightly regulated through a complex signaling network consisting of both angiogenic growth factors and their respective receptors (1, 4, 15, 50). To promote the dense capillary network within the islet, islet cell types express several angiogenic factors to attract and stimulate vessel growth and proliferation (Fig. 1) (1, 4, 10, 15, 39, 50). Thus, we investigated whether the alterations in islet vascularization observed were associated with changes in angiogenic factors and/or their receptors. Specifically, we assessed changes in a number of key angiogenic families, i.e., VEGF, angiopoietins, fibroblast growth factor, and ephrins, all of which play important roles in stimulating capillary ingrowth. WSD offspring did not exhibit reductions in the gene expression levels of angiogenic factors within the pancreas...
Maternal WSD consumption negatively impacts islet vasculature in the juvenile offspring. We have shown previously that the early defect in α-cell mass observed at GD130 also results in a reduction in α-cell mass in the juvenile offspring (21). Similarly, we hypothesized that the early impairment in the development of the islet vasculature would persist in the juvenile offspring, paralleling the defects in islet morphology. To address this, offspring from CTR or WSD offspring were born naturally, maintained on their respective diets throughout lactation, and weaned at ~8 mo of age. At this time, offspring were either kept on their in utero diet or switched onto the opposing diet (Fig. 2B), yielding four treatment groups. This study design allows for the determination of the relative impact of maternal vs. postweaning diet on islet vascularization and innervation. Following postweaning WSD exposure (CTR/WSD), juvenile animals tended to display an increase in islet capillary area and area per capillary, consistent with an increase in metabolic demand (Fig. 4, A–D). However, there was a significant reduction overall in islet vascularity in offspring born to WSD dams (maternal diet effect: P < 0.01; Fig. 4B). Furthermore, the reduction in capillary area was accompanied by a reduction in the average size of the existing vessels (Fig. 4D). In WSD/CTR offspring, this results in islet vascularization similar to CTR/CTR offspring and is likely appropriate for postweaning metabolic demand (Fig. 4B). In contrast, WSD/ WSD offspring fail to appropriately expand the islet vasculature in response to a postweaning WSD (Fig. 4B). The impact of maternal WSD consumption on islet vascularization is consistent with the significant impairment in islet capillary area and size observed in the early third trimester (Fig. 3B).

Sympathetic islet innervation is impaired following in utero WSD exposure but does not persist into the juvenile stage. Because angiogenesis and neurogenesis are closely related developmental processes, we hypothesized that maternal WSD consumption would similarly impair the normal development of sympathetic islet nerve fibers. To address this, we visualized sympathetic fibers using tyrosine hydroxylase (TH). WSD offspring displayed a 56% reduction in the density of sympathetic islet fibers at GD130 (Fig. 5, A and B). Neither maternal diet reversal nor resveratrol supplementation during gestation improved TH fiber density significantly.

Early developmental changes to both islet morphology (21) and vascularization as a result of maternal WSD consumption persist into childhood in the NHP model. However, significant remodeling of islet nerve fibers occurs during the perinatal period. To investigate whether the impaired innervation we observe in the WSD fetus leads to altered sympathetic innervation in the juvenile offspring, we measured TH fiber density...
in offspring at 1 yr of age (Fig. 6). Surprisingly, we observed no change in sympathetic islet innervation in this age group, suggesting that the offspring are capable of compensating during the perinatal period.

**DISCUSSION**

In the current study, we demonstrate that maternal WSD consumption during pregnancy results in alterations in the development of islet vascularization and innervation in the fetal offspring at the beginning of the third trimester. Specifically, WSD fetal offspring display significant reductions in capillary area, density and size (Fig. 3) and fewer sympathetic nerve fibers (Fig. 5). Importantly, the reduction in the size of islet capillaries persists in juvenile offspring born to mothers who consume a WSD (Fig. 4). The observation that vascularization and innervation are impaired is consistent with our previously published data demonstrating altered development of the islet in WSD offspring (21) and likely reflects the close developmental relationship between these factors. Interestingly, recent data also suggest that impaired islet vascularization may in fact precede defects in islet development (30), indicating that the impairments in α-cell mass observed previously in this model may be secondary to defects in the development of the islet vasculature.

The longer-term impact of the observed reduction in vascularization and innervation is unknown but may provide a novel mechanism by which these offspring have an increased predisposition to the development of type 2 diabetes later in life. In rodent models of insulin resistance, intraislet capillaries dilate in response to increased nutrient requirements and secretory demand (22). Similarly, juvenile NHP offspring who receive only a postnatal WSD exposure (CTR/WSD) tend to display an

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**Fig. 3.** Vascularization of the fetal islet is reduced by maternal WSD consumption and restored by dietary intervention. **A**: representative IHC images of the vascularization of fetal islets by platelet endothelial cell adhesion molecule 1 (PECAM1) staining. Scale bar represents 25 µm. **B–D**: quantification of islet PECAM1+ area (B), vascular density (no. of PECAM1+ fibers/islet area; C), and area per vessel in CTR (n = 8; 4 males and 4 females), WSD (n = 9; 3 males and 6 females), REV (n = 6; 2 males and 4 females), and WSD/RESV (n = 6; 4 males and 2 females) offspring (D). E: correlation of maternal insulin secretion during a glucose tolerance test with islet capillary density. F: gene expression of key islet angiogenic factors and their receptors in CTR (n = 13; 6 males and 7 females), WSD (n = 11; 7 males and 4 females), REV (n = 6; 2 males and 4 females), and WSD/RESV (n = 6; 4 males and 2 females) pancreas. Data are expressed as means ± SE. Different superscripted letters indicate a significant difference (P < 0.05). *P < 0.05, **P < 0.01 vs. CTR offspring; †P < 0.05, ‡P < 0.01 vs. WSD offspring.
increase in islet capillary size, which is consistent with increased capillary dilatation. However, WSD exposure in utero significantly reduces islet capillary size compared with offspring who consume the WSD only postnatally (WSD/WSD vs. CTR/WSD). The inability to appropriately expand the islet vasculature likely results in a failure of the islet to respond to metabolic demand and causes accelerated β-cell failure. In fact, islet-specific deletion of VegfA in a murine model results in hypovascularization of the islet and impaired glucose tolerance (10). Furthermore, the decrease in capillary size is consistent with impaired dilatation of the vessels. It has been shown previously that rodent models of type 2 diabetes display reduced vasodilation and impaired eNOS activity (22). Although we did not detect changes in eNOS expression (Fig. 3F), enzymatic activity may be altered without concomitant changes in gene expression.

Interestingly, undernutrition and intrauterine growth restriction in a rodent model have also been associated with impaired islet vascularity (8, 30). Maternal WSD consumption during pregnancy has been linked to intrauterine growth restriction and placental insufficiency (25, 33), suggesting shared attributes between these conditions. However, those authors attributed this deficiency to the significant reduction in expression of VegfA (8, 30), unlike our NHP model of maternal WSD consumption, indicative of distinct mechanisms of impaired vasularization.

Previously, changes in islet innervation following maternal WSD consumption had not been investigated in any species. Although we demonstrate a significant reduction in sympathetic fiber density in WSD offspring, surprisingly, this effect did not persist with juvenile offspring. In rodents, islet innervation undergoes significant remodeling in the postnatal period, resulting in a decrease in innervation after postnatal day 20 (13). This may explain the significant reduction in sympathetic innervation in the juvenile NHP islet compared with the fetal islet. Interestingly, data from our group have indicated that WSD consumption in adult NHPs results in an increase in islet sympathetic fiber density, suggesting that this adaptation is required for the response to increased metabolic demand (Pound LD and Grove KL, unpublished observation). Similar to impairments in vasculature, failure of the islet neural net work development may also predispose the individual to the development of type 2 diabetes later in life. Importantly, animal models of insulin resistance or type 2 diabetes display altered sympathetic islet innervation (36), likely leading to enhanced α-cell function. Future studies will address whether this observation is specific to sympathetic nerve fibers or is indicative of an overall reduction in innervation.

Although compliance can be a challenge, maternal diet reversal during pregnancy is a straightforward, cost-effective, therapeutic option. Women consuming a WSD may be more motivated to improve their dietary intake during pregnancy to mitigate some of the potentially negative impacts on their fetus than they may be otherwise. Despite no significant short-term improvements in maternal metabolic health, a switch to a CTR diet for the duration of pregnancy normalized islet vascularization in the fetal offspring in the early third trimester. The observation that this occurred independent of improvements to maternal weight, adiposity, and insulin sensitivity suggests that the impairments in vasculature seen in the WSD fetus are
driven primarily by the diet itself and thus are easily reversible with improved dietary intake. However, it was surprising that there was not a similar normalization in H9251-cell mass or sympathetic innervation (Fig. 6). These factors may be more sensitive to the phenotype of the mother than to the diet itself, suggesting that the mechanisms are distinct from those driving the impairment in islet vascularization.

Previous studies have demonstrated that resveratrol supplementation improves vascular function and induces vasodilation (16, 34, 56). Although human studies have not yet assessed the safety or efficacy of resveratrol treatment during pregnancy in women consuming a WSD, our group demonstrated recently in our NHP model of WSD consumption during pregnancy that supplementation improved placental blood flow, consistent with its role in enhancing vascular function (52). However, this was accompanied by a concerning increase in total pancreas mass and proliferation and an elevation in the H9252-cell/H9251-cell ratio (52). In this study, resveratrol supplementation during pregnancy resulted in a significant increase in islet vascularization, consistent with its known vasodilatory effect. However, in particular, resveratrol consumption during pregnancy resulted in hypervascularization in the offspring significantly above CTR offspring. Although the long-term consequences of this observation are unknown, hypervascularization has been shown in rodent models to have a negative impact on islet development, as the vasculature is critical for restricting pancreas growth and differentiation. Specifically, VegfA overexpression leads to reduced pancreatic branching and impaired islet growth (2, 14). Furthermore, this effect may be of particular concern, as hypervascularization is a precursor to malignancy (11). Because resveratrol is a readily available dietary supplement, the potentially harmful consequences in fetal pancreas warrant further investigation.

Interestingly, despite the enhanced islet vascularization observed following maternal WSD/RESV consumption, VegfA expression was paradoxically impaired compared with CTR offspring. Similarly, Kdr, the gene encoding the VEGF receptor-2, and Angpt2, the gene encoding angiopoietin-2, expres-
sion were impaired vs. WSD offspring. A number of studies have reported reduced Vegfa expression following resveratrol supplementation (40, 42, 45, 55); however, this effect was accompanied by a similar reduction in angiogenesis in one study (55), in contrast to the data reported here. The mechanisms underlying this effect remain to be elucidated, but these data indicate a complex role of resveratrol in islet angiogenesis. However, it is important to note that although the observed increase in vascularization was specific to the islet, the alterations in the gene expression of key angiogenic markers were taken from the whole pancreas and thus may at least partially explain these paradoxical observations.

Overall, our data indicate that maternal WSD consumption during pregnancy results in impaired islet vascularization and innervation and, importantly, may present a novel mechanism by which offspring are predisposed to developing type 2 diabetes later in life. Furthermore, an improvement in maternal nutrition during pregnancy restored the loss of islet vascularity. Furthermore, an improvement in maternal nutrition during pregnancy results in impaired islet vascularization and function. Diabetology 55: 2974–2985, 2006.


