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EPIGENETIC BASIS FOR ABDOMINAL AORTIC VASCULAR SMOOTH MUSCLE ADAPTATION TO PREGNANCY

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Abstract

BACKGROUND/AIMS: Ten-Eleven-Translocation (Tet), a DNA demethylase enzyme, has been identified as a master epigenetic regulator of vascular smooth muscle cell plasticity that could alter the biomechanics of the pregnant aorta. We hypothesized that pregnancy will induce significant adaptive changes in aortic caliber and distensibility that correlate to Tet gene expression.

METHODS: Abdominal aortas from pregnant and non-pregnant mice were dissected and cannulated. Intraluminal pressure was adjusted using a pressure-servo system while using a video dimension analyzer to measure lumen diameter. qPCR and immunoblot was used to analyze expression of Tet genes. Global genomic methylation was assessed with the LUMA assay.

RESULTS: Compared to the NP ($706 \pm 8 \mu\text{m}$) control group, aortic luminal diameter was significantly increased in both E18.5 ($836 \pm 14 \mu\text{m}$) and PP30 ($889 \pm 16 \mu\text{m}$) mice. Distensibility was reduced in E18.5 ($90 \pm 4\%$) mice and returned to NP ($108 \pm 2\%$) values in PP30 ($108 \pm 3\%$) mice. Tet2 transcription decreased in the beginning of pregnancy and subsequently increased in late gestation; inversely corresponding to changes in global methylation.

CONCLUSIONS: Physiologic changes in the aorta are accompanied by changes in gene expression and genomic methylation, suggesting an epigenetic component to maternal vascular remodeling during pregnancy.

Keywords

aorta; epigenetics; pregnancy; smooth muscle; tet; vsm

INTRODUCTION

Successful pregnancy requires a highly orchestrated series of changes in maternal vasculature in order to support extensive changes in blood flow that are needed to support the growing fetus(s) and placenta [1]. While changes in the uterine vasculature have been the subject of many studies, the maternal heart and large conduit vessels must adapt as well. Past studies have shown that the aorta undergoes significant remodeling in pregnancy, as

evidenced by the substantial changes in lumen diameter and increased compliance [2,3]. Interestingly, these adaptations are known to be attenuated in preeclamptic women, and abnormalities in maternal cardiovascular adaptation to pregnancy have been implicated in the pathogenesis of hypertensive disorders of pregnancy [4–7].

In keeping with physiologic studies, epidemiologic data indicate that pregnancy history predicts the future cardiovascular health of the mother [8,9]. Women with normotensive pregnancies and normal birth weight babies have a markedly diminished risk of preeclampsia in future pregnancies and a lower long-term risk of developing hypertensive disorders [9,10]. On the other hand, women with a history of preeclampsia have a marked increased risk of developing cardiovascular disease, including coronary artery disease, hypertension and stroke later in life [11]. Together, the physiologic and epidemiologic data suggest that pregnancy induced changes in maternal large conductance vessels such as the aorta may persist for many years and that abnormalities of adaptation that occur during pregnancy may confer subsequent cardiovascular risk. This thinking is supported by the observation that, independent of pregnancy, the compliance or stiffness of the aorta is known to be an important predictor for the development of hypertension and cardiovascular disease [12,13].

Given the fact that both pregnancy outcome and aortic stiffness predict cardiovascular health, as well as the fact that pregnancy is thought to have effects on the elastic properties of the aorta, we set out to characterize the physiologic changes in the abdominal aorta during pregnancy. Although others have assessed the changes in mechanical properties of the aorta during pregnancy, very few studies have attempted to elucidate the molecular mechanisms that underlie such changes. We hypothesized that vascular remodeling in pregnancy is likely to be analogous to the vascular remodeling that occurs in other settings such as injury or hypertension. In general, blood vessel remodeling relies on plasticity of the vascular smooth muscle (VSM), a subject which has been extensively studied over the past 20 years [14]. In order to assess whether aortic remodeling in pregnancy involves plasticity of the VSM, we assessed the expression of several well-characterized markers of VSM differentiation. Because recent data have indicated that VSM remodeling is fundamentally an epigenetic process that involves alterations in expression of members of the ten-eleven translocation (Tet) gene family [15,16], we sought to test whether epigenetic mechanisms are involved in pregnancy related vascular changes. To this end, we assessed the expression of Tet gene family members, and we looked for changes in genomic methylation during pregnancy.

METHODS

Animals.

Virgin B6 mice (8 to 10 weeks of age) were purchased through Jackson Laboratories (Bar Harbor, ME) and housed at the University of Vermont Animal Facility with feed and water provided *ad libitum*. Virgin, cycling mice in the proestrus stage were used for breeding with B6 males overnight in isolated pairs. Day 0.5 of pregnancy was established when copulatory plugs were observed the next morning. For non-pregnancy mice only, the stage of estrus cycle was determined by vaginal smear, and studied in diestrus to provide the largest time

window to dissect vessels [17]. All experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Vermont.

On gestation day E10.5, E15.5, E18.5 and 30 days post-partum (PP30), mice were euthanized with carbon dioxide and cervical dislocation. Virgin, non-pregnant (NP) cycling mice were euthanized in the same manner. The abdominal aorta extending from the renal arteries to the iliac bifurcation was harvested and placed in a dish containing buffered (pH 7.4) HEPES physiological saline solution (HEPES-PSS).

Vessel Dissection and Pressurization.

Using a dissecting microscope, a 2-mm abdominal aorta segment was dissected just proximal to the inferior mesenteric artery and cleared of connective tissue. Each aortic segment was cannulated on two buffer-filled, pulled-glass pipettes in a custom-built arteriograph using small strands of silk suture [18]. A luminal flush was performed at 10 mmHg to clear intraluminal contents prior to distal end attachment. Intraluminal pressure was adjusted and maintained using a pressure-servo system with an in-line transducer (LSI, St. Albans, VT) under no-flow conditions [18]. Aortic segments were tested for leaks at 100 mmHg for 5 minutes by turning off the servo; any vessels showing a decrease in pressure (leakiness) were discarded from the study.

The arteriograph was placed on the stage of an inverted microscope attached to a video dimension analyzer used to measure inner and outer diameter of each transilluminated aortic segment. Three sequential step-wise pressure cycles were performed starting at 10 mmHg; two with HEPES-PSS, and one with added papaverine (0.1 mM) and diltiazem (10 μ M) for maximal relaxation. A 10 mmHg starting pressure was used to ensure precise measurement of the aortic wall, which diminished in clarity below 10 mmHg. Pressure was increased to 30 mmHg and then by 30 mmHg every 2 minutes up to 150 mmHg with inner and outer vessel diameter being recorded from the monitor at the end of each pressure increment using a calibrated ruler. To minimize hysteresis, two pressure cycles were performed initially on each cannulated segment in HEPES-PSS for comparison.

Distensibility.

Aortic distensibility was calculated for each cannulated vessel for all pressure cycles using the following equation, where D_P and $D_{Min,P}$ are the starting diameter at opening pressure and diameter achieved at each pressure step [19]:

$$Distensibility, \max\% = \left[\frac{D_P - D_{Min, P}}{D_{Min, P}} - 1 \right] \times 100$$

Gene Expression.

RNA was isolated with a Trizol-based protocol at 4°C. Sections of aorta from below the renal arteries to the iliac bifurcation were homogenized three times at 2500 g for 15 sec. by a bead-beating technology (Precellys 24; Bertin Technologies, Montigny-le-Bretonneux,

France) in Trizol reagent. RNA was quantified by NanoDrop (Agilent Technologies, Santa Clara, CA, USA) and a 260/280 ratio of >1.8 was required for further study.

qPCR was used to assess gene expression in all experimental groups. RNA was prepared from portions of the abdominal aorta, stripped of adventitia and analyzed for Tet1, Tet2, MyoD and Myh11 genes. All qPCR reactions were performed in triplicate and results were normalized in comparison with Hprt and Sdha expression. Immunoblots and IHC were used to corroborate qPCR studies. The delta-delta Ct method was used to assess relative levels of expression [20].

Immunoblot.

Protein lysates were prepared in RIPA buffer with protease inhibitor from sections of the abdominal aorta extending from the renal arteries to the iliac bifurcation using a FastPrep-24 homogenizer (MP Biomedicals, LLC, Santa Ana, CA). Protein was quantitated using a Pierce BCA protein assay kit and 40 µg aliquots were electrophoresed on 4–15% polyacrylamide reducing gels using standard methods (Thermo Fisher, Waltham, MA). Protein was then electroblotted to nitrocellulose membranes and stained with Ponceau red to assess quality. Membranes were blocked and incubated with primary antibodies. MYHII (ab53219) and beta-tubulin (ab6046) were obtained from Abcam (Abcam, Cambridge, MA). Tet2 (21207-AP) was obtained from Proteintech (Proteintech, Rosemont, IL). Appropriate HRP-conjugated secondary antibodies were obtained (Abcam, Cambridge, MA). Proteins were visualized using the upperSignal WestPico Chemiluminescent Substrate kit (Thermo Fisher, Waltham, MA). The relative quantity of proteins of interest was determined after normalization with beta-tubulin using ImageJ Version 1.5 (U. S. National Institutes of Health, Bethesda, Maryland, USA).

Global methylation using LUMA—Genomic methylation was assessed using the Luminometric Methylation Assay (LUMA) method, as described [21]. Briefly, genomic DNA was prepared from sections of abdominal aorta by proteinase K digestion followed by organic extraction and ethanol precipitation. DNA quality and quantity was assessed by spectrophotometer and gel electrophoresis. DNAs were then digested to completion with a combination of EcoRI/HpaII or EcoRI/MspI, and a Qiagen pyrosequencing instrument was used to assess the relative amounts of digestion with HpaII and MspI, with EcoRI serving to assure normalization of DNA quantity. Methylation percentage was calculated as follows:

$$\text{Methylation, \%} = \left[1 - \frac{\text{EcoRI, HpaII}}{\text{EcoRI, MspI}} \right] \times 100$$

Each sample was run in duplicate and three independent animals were tested for each gestational age and for post partum.

Statistical Analysis.

Analysis of structural differences in NP, E8.5, E12.5, E15.5, E18.5 and PP30 groups of animal utilized a 2-way ANOVA with physiological pressure and gestational age as factors. At each pressure Fisher's Least Significant Difference method was used to compared the

mean value for each gestational age with the mean for non-pregnant mice. A comparable analysis of relative gene expression was performed using 1-way ANOVA. Data were analyzed using GraphPad Prism Version 7 (GraphPad Software, La Jolla, CA) and SAS Version 9.2 software (SAS Institute Inc, Cary, SC).

RESULTS

Inner Aortic Diameter

At 90 mmHg, which approximates physiologic blood pressure, relaxed aortic lumen diameters increased significantly by E15.5, and continued to increase thereafter, with largest values measured in the PP30 group of animals (Fig. 1A). This difference was maintained over a broad range of transmural pressures (10–150 mmHg, Figure 1B).

Wall Thickness and cross-sectional area.

Aortic wall thickness tended to increase with increasing gestational age; however, this did not reach statistical significance. At PP30 wall thickness diminished, but, again, the change was not statistically significant. As with lumen diameter, wall thickness is shown at a physiological pressure (Fig. 2A), and across a broad range of transmural pressures (Figure 2B).

Distensibility

Aortic distensibility, defined as the relative change in diameter per change in pressure, provides an assessment of vessel elasticity. Distensibility was maximal in NP animals and diminished with increasing gestational age (Fig. 3). By PP30, distensibility had returned to baseline (NP values), despite the continued increase in vessel diameters, as already discussed.

qPCR

In order to assess whether the VSM of the aorta undergoes phenotype switching during pregnancy, we assessed the level of expression of VSM differentiation markers, myocardin (MyoCD) and myosin heavy chain II (MyhII) [22] using quantitative PCR. In comparison with NP levels, significantly decreased expression of both markers was present at e15.5, then decreased postpartum (Fig 4 A and B).

The same RNA samples were used to assess the expression of the ten eleven translocation genes, Tet1 and Tet2 (Fig. 4 C and D). Compared to NP, significantly diminished expression was seen at e10.5 and at PP30, while non-significant increases in expression were seen at e15.5 and 18.5. The profile of Tet1 and Tet2 expression was generally consistent with the changes in expression of both differentiation markers.

Immunoblot showing changes in MyhII and Tet2—Immunoblot using an antibody to MyhII shows that pregnancy related changes in MyhII protein mirrored the qPCR results, although the changes in protein level were not as pronounced as the changes in mRNA (Fig 5A.). This result supports the idea that the aorta undergoes a remodeling process during pregnancy and in the post partum period.

The same protein lysates used to assess MyhII were used to assess the levels Tet2 protein (Fig 5B.). This analysis shows that protein levels generally mirrored mRNA as assessed by qPCR, although, again, the changes in protein expression were more modest than was seen in mRNA.

Global methylation.

The previously described LUMA assay was used to assess pregnancy related changes in global methylation in the aorta. This analysis shows that changes were modest, with statistically significant decreases in methylation at E10.5 and E18.5. Interestingly, overall methylation at 30 days post partum was not different from the non-pregnant aorta (Fig 6).

DISCUSSION

Aortic diameter and distensibility

The measurable increase in abdominal aortic diameter during gestation is in keeping with published studies that have mostly focused on the thoracic aorta [23,24]. Somewhat surprisingly, diameter aortic continued to increase in the PP period, when cardiac output has presumably returned to prepregnancy conditions.

Our data also indicate that the increased lumen diameter was not due to altered distensibility, since the differences persisted across the entire range of intraluminal pressures and were near maximal at physiologic pressure. Rather, it reflects a true increase in wall mass (outward hypertrophic remodeling) since there were no significant changes in wall thickness. Although its determination was beyond the scope of this study, the diameter increase may be due to VSM hypertrophy (particularly cellular elongation) and/or hyperplasia.

Prior studies of aortic elasticity/distensibility during pregnancy have been performed in both rat and human although we are unaware of any such studies in mouse. Both rat and human studies have shown increasing compliance/distensibility during pregnancy it was therefore somewhat surprising to see that distensibility was maximal in NP animals and diminished with increasing gestational age (Fig. 5) [2,3,23,24]. There are several possible explanations for the observation that the mouse aorta becomes less, rather than more distensible during pregnancy: First, vascular responses to pregnancy may be somewhat different in mice than in rats or humans. Second, other assessments of the aorta in pregnancy have focused on the thoracic aorta, while our studies pertained to the abdominal aorta, which may respond differently than the thoracic aorta. Indeed, there are some data to suggest that different parts of the arterial tree respond differently to pregnancy [25] and other data show that the structure of the aorta varies regionally [26]. Finally, others have assessed compliance using non-invasive methods in living humans or rats, whereas our measurements were made on isolated sections of aorta using a pressure arteriograph. Thus, differences could be due to differences inherent in the two methods.

Vessel remodeling and epigenetic change

Although remodeling of large vessels in response to environmental cues has been the subject of earlier research, the mechanisms that underlie structural alterations in large maternal vessels during pregnancy have seldom been investigated [14]. In order to test the hypothesis that large vessel remodeling in pregnancy occurs through a process similar to other vessel remodeling, we examined the expression of well known markers of VSM differentiation, and our data support the idea that changes in aortic diameter and distensibility are accompanied by changes in the differentiation state of VSM.

Further, our data suggest the possibility that there may be several phases of remodeling, with an initial decrease in expression of differentiation markers, followed by a return to baseline level in late pregnancy and then a second phase of remodeling in the PP period. It would be interesting to evaluate the effect of sex steroids in this process, as pseudopregnancy – a condition in which sex steroids increase without the presence of a fetus during the first 11–12 days of gestation – stimulated outward remodeling in the uterine circulation of the mouse [27]. Notably, aortic remodeling takes place primarily during the second half of pregnancy as no differences in diameter were yet evident at E10.5. This does not preclude an action of sex steroids, but also increases the likelihood of other mechanisms. For example, since shear stress is a recognized stimulus for arterial growth, aortic enlargement may occur secondary to increased cardiac output, which is well-documented in mammalian pregnancy, and which continues to increase as gestation progresses in the mouse [28].

Given the recent observation that the regulation of VSM differentiation and, hence, vessel remodeling is an epigenetic process, we were interested in determining whether pregnancy was associated with epigenetic alterations [15,16,29]. To this end, we show that the expression of Tet2, which has been shown to play a central role in the epigenetics of blood vessel remodeling, particularly in modulating VSM phenotype changes during pregnancy [15]. Its modulation also followed a two-phase pattern, with low levels in mid-pregnancy (E8.5 and 10.5, followed by increases at later gestational ages E15.5 and 18.5). Our data indicate that, in keeping with the expression of Tet and VSM differentiation genes, modest global changes in the level of genomic methylation occur during pregnancy, with a nadir at E10.5 followed by a rebound later in pregnancy and postpartum. Overall, our data support the idea that remodeling of the aorta during pregnancy may be similar to other types of vascular remodeling (such as in atherosclerosis, for example) and suggest that long-lasting changes that occur in maternal blood vessels as a consequence of pregnancy may have an epigenetic basis. Although examining changes in vessel behavior is difficult in large pressurized vessels, it would be interesting to determine whether maximal contractility of sensitivity to physiological influences (e.g. catecholeamines) is altered during pregnancy, as these would further impact on distensibility and impedance and allow for a better understanding of the actual physiological consequence of phenotypic changes involving VSM, e.g. altered impedance.

In summary, our findings indicate that the mouse abdominal aorta undergoes significant enlargement during pregnancy, and that this process does not appear to be reversed in the immediate postpartum period. We also document an alteration in biomechanical properties (distensibility) of the aorta – an adaptation that is completely reversed following parturition

– along with changes in epigenetic mechanisms that ultimately determine cellular phenotype.

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Fig. 1.

(A) Inner diameter at physiological pressure (90 mmHg). Bar graphs represents means $\pm$ SE.

Unique letters indicate significant differences between groups ($p < .05$). (B) Changes in luminal diameter as a function of pressure. Data are shown as mean $\pm$ SE. (*) indicates significant differences ($p < .05$) between NP and E18.5/PP30. E10.5 and E15.5 values fell between NP and 18.5 but were not significant.

Fig. 2.

(A) Wall thickness at physiological pressure (90 mmHg). Bar graphs represents means $\pm$ SE. No significant difference between groups. (B) Changes in wall thickness as a function of pressure. Data represent means $\pm$ SE. There were no significant differences in corresponding measurements between groups.

Fig. 3. Changes in distensibility as a function of pressure. Data shown as mean $\pm$ SE. (*) denotes significant difference ($p < .05$) between E18.5 and NP/PP30; E 15.5 data were intermediate.

Fig. 4.

Changes in relative expression of mRNA as a function of gestational age as determined by qPCR for (A) MyoCD, (B) MyhII, (C) Tet1 and (D) Tet2. (*) denotes significant difference ($p < .05$) from NP value

Fig. 5.
Relative level of protein expression as determined by immunoblot with antibodies specific for (A) MyH11 and (B) Tet2. (*) indicates significant difference from NP.

Fig. 6.
Relative level of global genomic methylation, as determined by LUMA assay. (*) indicates significant ($p < .05$) difference from NP.