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Vascular Mechanics in Decellularized Aortas and Coronary Resistance Microvessels in Type 2 Diabetic db/db Mice

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Abstract

We previously reported differences in stiffness between macro- and micro-vessels in type 2 diabetes (T2DM). The aim of this study was to define the mechanical properties of the ECM independent of vascular cells in coronary resistance micro-vessels (CRMs) and macro-vessels (aorta) in control Db/db and T2DM db/db mice. Passive vascular remodeling and mechanics were measured in both intact and decellularized CRMs and aortas from 0–125 mmHg. We observed no differences in intact control and diabetic aortic diameters, wall thicknesses, or stiffnesses ($p > 0.05$). Aortic decellularization caused a significant increase in internal and external diameters and incremental modulus over a range of pressures that occurred to a similar degree in T2DM. Differences in aortic diameters due to decellularization occurred at lower pressures (0–75 mmHg) and converged with intact aortas at higher, physiological pressures (100–125 mmHg). In contrast, CRM decellularization caused increased internal diameter and incremental modulus only in the db/db mice, but unlike the aorta, the intact and decellularized CRM curves were more parallel. These data suggest that (1) micro-vessels may be more sensitive to early adverse consequences of diabetes than macro-vessels and (2) the ECM is a structural limit in aortas, but not CRMs.

Keywords

type 2 diabetes; vascular biomechanics; extracellular matrix; pressure myography

INTRODUCTION

Type 2 diabetes mellitus (T2DM) is a metabolic disorder characterized by hyperglycemia in the presence of insulin resistance. It is typically considered an adult disease, although the prevalence of T2DM is becoming more common in childhood.^{5, 12} T2DM is considered a cardiovascular disease by the American Heart Association¹¹ in part because 2/3 of diabetes related deaths are directly due to heart disease. Furthermore, diabetic patients are 2–4 times more likely to experience myocardial infarction than non-diabetic patients, which has been linked to coronary artery disease (CAD).²⁰ CAD in diabetic patients has also been linked to atherosclerosis and endothelial dysfunction.²³

Recent studies from our laboratory demonstrated that inward hypertrophic remodeling of coronary resistance microvessels (CRMs) also contributed to coronary artery disease (CAD) associated with reduced coronary flow in both a mouse model of type 2 diabetes mellitus (T2DM)¹³ and a porcine model of metabolic syndrome (MetS).²⁹ Surprisingly in both the T2DM db/db mouse and in Ossabaw miniature pigs with MetS, CRMs were *less* stiff than their non-diabetic controls, in contrast to macrovessels that were stiffer^{13, 29} as evidenced by higher aortic pulse wave velocity and beta stiffness index. To our knowledge, this was the first evidence of a divergent stiffness profile between macro-vessels and micro-vessels in these metabolic disorders, suggesting the existence of vascular bed-specific remodeling and mechanics.

Several factors influence vessel wall stiffness, including the composition, turnover and quality of the extracellular matrix (ECM),^{10, 36} protein crosslinking,^{32, 33} vascular cell stiffness,^{4, 18, 19, 22, 25} cell-ECM interactions (e.g. integrins/focal adhesions),^{21, 27} the contractile state of vascular smooth muscle cells (VSMCs),²¹ and cell volume.¹ Although the influence of most of these factors on vascular stiffness has been demonstrated in various vessels, how these might influence macro- and micro-vascular stiffness in T2DM is not known. To better understand the mechanisms accounting for the differential changes in CRM and aortic mechanics as the result of T2DM, we decellularized vessels and assessed the mechanical properties of the remaining ECM-rich structure. We tested the hypothesis that the ECM contributes differentially to macro- and micro-vascular stiffness in T2DM utilizing a decellularization technique that removes vascular cells and leaves ECM.

MATERIALS AND METHODS

Animals

Experiments were conducted on 16–17 week old male control, non-diabetic heterozygous Db/db and T2DM db/db mice that were obtained from The Jackson Laboratories. These mice are leptin receptor deficient and develop obesity, hyperglycemia, insulin resistance, and dyslipidemia by 4–8 weeks of age. Importantly, they mirror the micro- and macro-vascular remodeling phenotype of the pre-clinical Ossabaw pig model and thus are clinically relevant.^{13, 29} All mice were housed under a 12-hour light/dark cycle at 22°C and 60% humidity and were allowed *ad libitum* access to standard low-fat laboratory chow and water. This study was conducted in accordance with the National Institutes of Health Guidelines,

and it was approved by the Institutional Animal Care and Use Committee at Nationwide Children's Hospital.

Fasting Glucose Measurements

Mice were fasted for 8 hours during the light cycle, after which blood was drawn from the tail vein at 16 weeks of age. Blood glucose concentration was measured using the AlphaTrak veterinary blood glucometer calibrated specifically for rodents (Abbott Laboratories, Abbott Park, IL).

Preparation of Aortas and Septal Coronary Resistance Microvessels

Mice were anesthetized using 2% isoflurane, vaporized with 100% oxygen. Descending thoracic aortas (devoid of branching) and hearts from the same mouse were excised and dissected in 4°C physiologic saline solution (PSS) composed of the following (in mM): 130 NaCl, 4 KCl, 1.2 MgSO₄, 4 NaHCO₃, 10 HEPES, 1.2 KH₂PO₄, 5 glucose, and 2.5 CaCl₂ at pH 7.4. Perivascular fat was carefully dissected away and the intact aortas were mounted onto 2 custom 20 gauge MicroFil cannulas (World Precision Instruments, Sarasota, FL) retrofitted within a pressure myograph chamber (Living Systems, Burlington, VT) and lengthened to the approximate *in vivo* length. Next, the right ventricles of the hearts were removed, and septal coronary resistance microvessels (CRMs, <150 µm internal diameter) were carefully isolated from the ventricular septum at the level of the papillary muscle. CRMs (generally at least 2–3 times longer than the diameter) were excised and mounted onto 2 glass microcannulas within a separate pressure myograph chamber (Living Systems, Burlington, VT). Prior to any measurements, all vessels were equilibrated for 30 minutes under constant intraluminal pressure (50 mmHg) at 37°C in PSS. All experiments were performed in Ca²⁺-free PSS in the presence of 2 mM EGTA and 100 µM sodium nitroprusside. Experiments were conducted under passive conditions to avoid potential confounds from vascular tone. Passive pressure-diameter (P-D) curves were generated by increasing intraluminal pressure from a minimum of 0 mmHg to a maximum of 125 mmHg in both aortas and CRMs. The upper limit of 125 mmHg was chosen because it closely approximates normal systolic blood pressure and normal systolic blood pressure observed in these mice.¹³ Sufficient time was given at each stepwise pressure increase until such time as the diameter stabilized (typically less than one minute).

For aortic studies, external diameters (D_e) were directly measured at each pressure step using a calibrated video dimension analyzer (Living Systems Instrumentation, Burlington, VT; (approximate resolution of 4 µm/pixel for the aortic system). Due to the thickness of the aortic wall, neither internal diameter (D_i) nor wall thickness (WT) could be directly or reliably measured by pressure myography. Aortic D_i , WT, and cross-sectional areas (CSAs) over a range of pressures were calculated indirectly from morphometric measurements made on calibrated images from a 4X objective of unloaded aortic rings cut from tissue adjacent to the section utilized for pressure myography using DP2-BSW software connected to an Olympus IX51 microscope. As an additional internal control, the average variance between unloaded aortic external diameters measured using the calibrated pressure myography video dimension analyzer and calibrated morphometric images was less than 10%. Images were

analyzed for internal and external cross-sectional areas (iCSA and eCSA), and medial cross-sectional areas (mCSA) were calculated by:

$$mCSA = eCSA - iCSA$$

We^{13, 28, 29} and others^{3, 7} have shown that vascular mCSA remains unchanged over a range of pressures from 0–125 mmHg. Therefore, assuming a constant mCSA (i.e. constant mass) across a range of pressures, and using direct aortic D_e pressure myography measurements, the internal diameter (D_i) was calculated at each pressure step:

$$Internal\ Diameter\ (D_i) = \sqrt{(D_e^2 - (4 \times mCSA)/\pi)}$$

CRM D_i and WT were measured directly using a calibrated pressure myography video dimension analyzer (approximate resolution of 0.56 $\mu\text{m}/\text{pixel}$ for the coronary system), and the following structural and mechanical parameters were then calculated for both aortas and CRMs as previously described:^{13, 28, 29}

$$\begin{aligned} External\ Diameter\ (D_e) &= D_i + 2(WT) \\ Wall/Lumen\ Ratio &= (WT/D_i) \times 100 \\ Circumferential\ Stress\ (\sigma) &= (P \times D_i)/(2WT), \end{aligned}$$

where P is pressure in dynes/cm².

$$Circumferential\ Strain\ (\varepsilon) = (D_i - D_0)/D_0,$$

where D_i is the internal diameter for a given intraluminal pressure and D_0 is the original diameter measured at 0 mmHg of intraluminal pressure.

$$Incremental\ elastic\ modulus\ (E_{inc}) = \Delta\sigma / \Delta\varepsilon$$

Vascular Decellularization Procedure

Following the measurements of intact aortic and CRM dimensions and mechanics, the same vessels were immediately decellularized using 1% sodium dodecyl sulfide (SDS) with slight modifications to a protocol used to decellularize the heart.¹⁷ Others have shown that SDS is an effective detergent with which to remove cells from various tissues,² having minimal effect on major ECM disruption over other methods,³⁵ although care must be taken to minimize exposure to avoid ECM degradation. A pilot study was performed to optimize the conditions for decellularization for aorta and CRMs. Aortas were bathed in and perfused with 1% SDS at a constant flow (~0.1 mL/min) for 4 hours, and CRMs were bathed in and perfused with 1% SDS at a constant pressure (100 mmHg to approximate mean arterial pressure) for 22 hours (overnight). These conditions were the *minimum* exposure times at which cell nuclei stained with DAPI and F-actin were absent in both aorta and CRMs,

respectively (Figures 1 and 2). The use of two separate decellularization conditions was necessary because the CRMs could not be perfused at a constant flow without compromising the integrity of the vessel wall by increased pressures that resulted during the pilot studies. Histochemical and electron microscopic evaluation revealed moderate perturbations of collagen in decellularized aortas and CRMs that were not different between normal and diabetic vessels, while elastin remained unaltered as a result of the process (Figures 1 and 2). Reductions in collagen content (by picrosirius red staining) as a result of decellularization were not different between normal and diabetic aortas (Figure 1; $46.6 \pm 7.2\%$ vs. $45.7 \pm 7.8\%$, respectively, $p > 0.05$).

Immediately after decellularization, the vessels were washed 3 times with PSS, re-equilibrated in PSS for 30 minutes at 37°C, and the passive remodeling and mechanical measurements were repeated. Calculations were repeated as above except that strain for each vessel was normalized to its intact D_0 .

Histochemistry (HC) and Transmission Electron Microscopy (TEM)

For DAPI staining, intact and decellularized aortas and CRMs were placed on slides and mounted using Vectashield mounting medium with DAPI (Vector Laboratories, Inc, Burlingame, CA). Vessels were imaged *en face* to determine the presence or absence of vascular cells (punctate DAPI staining).

For HC, intact and decellularized aortas were fixed in 4% paraformaldehyde for 24–48 hours and stored in 70% ethanol until embedding. Paraffin-embedded sections (5 µm thick) were deparaffanized in a graded series from xylenes to alcohol-water. Elastin was stained using the Accustain Elastic Stain (HTA25, Sigma Aldrich, St. Louis, MO) according to the manufacturer's instructions. F-actin was assessed using the CytoPainter F-actin Staining Kit (ab112127, Abcam, Cambridge, MA) according to the manufacturer's instructions. Briefly, slides were stained at room temperature for 60 min. Slides were then rinsed in PBS-T and mounted with Vectashield Hard Set Mount with DAPI counterstain for nuclei (catalog number H1400, Vector Labs, Burlingame, CA). Images were captured using DP2-BSW software connected to a microscope (model IX51, Olympus America, Center Valley, PA).

For TEM, intact and decellularized aortas and CRMs were fixed for 24 hours in 2.5% glutaraldehyde made in Millonig's phosphate buffer. 80 nm sections on 1 µm grids were incubated in 5% tannic acid for 30 mins at room temperature. After rinsing well with DDH_2O , sections were incubated in uranyl acetate for 10 mins, rinsed, and incubated in lead citrate for 10 mins. Stained sections were again rinsed, dried, and stored until imaging. Sections were imaged using a JEOL JEM-1400 TEM (JEOL Ltd. Tokyo, Japan) equipped with a Veleta digital camera (Olympus Soft Imaging Solutions GmbH, Münster, Germany).

Statistical Analysis

All data are expressed as mean $\pm$ SEM with a probability of $p < 0.05$ used to denote statistical significance using GraphPad Prism 6.0 (GraphPad Software, LaJolla, CA). Two-way repeated measures ANOVA followed by a Bonferroni's post-hoc test was performed on pressure myography data.

RESULTS

Body Weights and Glucose

In agreement with what we have previously reported,^{13, 24, 28} T2DM db/db mice exhibited significantly higher body weights and fasting blood glucose than non-diabetic Db/db mice (Body weight: Db/db 29.6 ± 1.0 vs. db/db 43.6 ± 3.6 grams, $p < 0.01$; Fasting Glucose: Db/db 153 ± 13 vs. db/db 508 ± 44 mg/dL, $p < 0.00001$).

Diameters: Intact and Decellularized Aortas and CRMs

Neither internal nor external diameters were different between intact normal and diabetic aortas (Figure 3A–B). Aortic decellularization resulted in a significant increase in both internal and external diameters over a range of pressures, and the percent increase in internal diameter as a result of decellularization was similar in normal and diabetic aortas (Figure 3C). The increases in aortic diameter due to decellularization generally occurred at lower pressures (0–75 mmHg), and the decellularized pressure-diameter curves converged with intact pressure-diameter curves at higher, physiological pressures (100–125 mmHg). As we have previously shown,¹³ internal diameter was reduced ($p < 0.05$), while external diameter was unchanged in db/db CRMs relative to normal CRMs (Figure 3D–E). Decellularization caused a significant increase only in the db/db CRM internal diameter ($p < 0.05$ vs. db/db – Intact), which contributed to the greater % increase in internal diameter in those decellularized CRMs (Figure 3F). In contrast to the intact and decellularized pressure-diameter curves for the aorta that converge at physiological pressures (100–125 mmHg), the decellularized CRM pressure-diameter curves were more parallel to the intact CRMs. If one restricts their considerations to normal systolic pressures (100–125 mmHg) in the aorta, there was no difference between internal (Db/db-Intact: 1369 ± 19 μ m vs. db/db-Intact: 1428 ± 18 μ m, $p > 0.05$ at 125 mmHg) and external (Db/db-Intact: 1452 ± 18 μ m vs. db/db-Intact: 1503 ± 17 μ m, $p > 0.05$ at 125 mmHg) diameters between normal and diabetic mice (Figure 3A–C), and decellularization did not change internal aortic diameter (Figure 3A and 3C, $p > 0.05$). In contrast, for the CRM at 125 mmHg, there was a marked reduction in internal diameter between intact normal and diabetic CRMs (Db/db-Intact: 133 ± 6 μ m vs. db/db-Intact: 104 ± 7 μ m, $p < 0.05$), and decellularization only caused a significant increase in internal diameter in db/db CRMs (db/db-Intact: 104 ± 7 μ m vs. db/db-Decellularized: 129 ± 8 μ m, $p < 0.05$, Figure 3D and 3F).

Wall Remodeling: Intact and Decellularized Aortas and CRMs

Wall thickness and wall/lumen ratios remained unchanged between normal and diabetic intact aortas, while decellularization significantly decreased wall thickness and wall/lumen ratio to similar degrees in both normal and diabetic aortas (Figure 4A–B), primarily at low pressure (0–50 mmHg). In contrast, there was a trend for increased wall thickness and a significant increase in wall/lumen ratio ($p < 0.01$) in intact CRMs isolated from diabetic mice (Figure 4C–D). Decellularization significantly decreased the wall/lumen ratio only in diabetic CRMs ($p < 0.001$; Figure 4D).

Vascular Mechanics: Intact and Decellularized Aortas and CRMs

The stress-strain curves for intact normal and diabetic aortas were similar, as was the incremental modulus of elasticity (Figure 5A–B; $p>0.05$). Interestingly, decellularization of normal and diabetic aortas resulted in a similar increase in the stress-strain slope, which increased the incremental elastic modulus (E_{inc} ; Db/db-Intact: $4.6 \times 10^6 \pm 0.3 \times 10^6$ vs. Db/db-Decellularized: $9.2 \times 10^6 \pm 0.8 \times 10^6$, $p<0.0001$ at 125 mmHg; db/db-Intact: $6.3 \times 10^6 \pm 0.6 \times 10^6$ vs. db/db-Decellularized: $9.9 \times 10^6 \pm 0.7 \times 10^6$, $p<0.001$ at 125 mmHg, respectively), and therefore, stiffness of the macrovessels (Figure 5A–B). As we have previously shown,^{13, 28, 29} the slope of the intact db/db CRM stress-strain curves appeared less than normal CRMs (Figure 5C), which corresponded to reduced incremental elastic modulus (Db/db-Intact: $7.4 \times 10^6 \pm 1.2 \times 10^6$ vs. db/db-Intact: $3.9 \times 10^6 \pm 0.6 \times 10^6$, $p<0.0001$ at 125 mmHg; Figure 5D). Interestingly, decellularization shifted the normal and diabetic CRM stress-strain curves to the right, but only resulted in an increase in incremental elastic modulus in db/db CRMs (db/db-Intact: $3.9 \times 10^6 \pm 0.6 \times 10^6$ vs. db/db-Decellularized: $7.4 \times 10^6 \pm 1.7 \times 10^6$, $p<0.001$ at 125 mmHg; Figure 5C–D).

DISCUSSION

This study examined the influence of the ECM on macro- and coronary micro-vascular remodeling and mechanics in type 2 diabetes. To accomplish this goal, we measured passive macro- and coronary micro-vascular remodeling and mechanics before and after decellularization of the vessels. Our data reveal differences in macro- and micro-vascular mechanics in the presence and absence of T2DM. We show that vascular decellularization causes significant increases in lumen diameter and decreases in wall/lumen ratios of normal and T2DM aortas and T2DM CRMs, but not in normal CRMs. Decellularization was associated with increased incremental elastic modulus, except in the normal CRMs. Moreover, our data reveal a novel and intriguing pattern in the macro- and coronary micro-vascular pressure-diameter curves in response to decellularization. The intact and decellularized aortic P-D curves converged at higher, physiological pressure, which was not affected by T2DM, while the intact and decellularized P-D curves of the CRMs appeared more parallel to one another.

The influence of the ECM on vascular remodeling and mechanics is important for normal function and structural integrity. Direct evidence for this is supported by studies that showed $Col1a1^{-/-}$ and $Col3a1^{-/-}$ are embryonic or perinatal lethal due to vascular rupture.^{15, 16} Furthermore, elastin-null mice die within the perinatal period due to unchecked VSMC proliferation,¹⁴ presumably from lack of organized lamellar elastic layers. Faury et al. showed that $Eln^{+/-}$ mouse arteries are stiffer than normal,⁹ suggesting that normal elastin expression imparts elasticity to arteries. Indeed, elastin is a critical component of major arteries, accommodating cyclic volume loads in the arterial wall and helping propel blood pulse waves down the arterial tree during diastole.⁶ Under physiological pressures, elastin dictates the majority of vascular mechanical characteristics in large arteries, while stiffer collagen fiber engagement increases beginning at physiological and extending to supraphysiological pressures, ultimately increasing vascular stiffness.³¹ In these instances, such as in frank hypertension, there is more collagen fiber engagement leading to increased

stiffness. Based on these studies, one can appreciate the critical influence of the ECM on vascular structure-function relationships.

The above mechanisms have been elucidated in large arteries, but much less is known on how the mechanical behavior of the ECM might be influenced in diabetes or in smaller arterioles and microvessels. It could reason that elastin is a major determinant of vascular mechanics at physiological pressure or below in both micro- and macro-vessels, but how this might be influenced in T2DM is not well understood. Indeed, we previously showed that the diabetic CRM expressed more elastin mRNA relative to control, in contrast to the diabetic aorta, which expressed lower elastin than control.¹³ Collagen expression remained constant. Mechanically, our current data revealed several noticeable differences between macro-vessels and microvessels both in the presence and absence of vascular cells and in the presence and absence of T2DM. The increase in diameters and the decrease in wall thickness and wall/lumen ratios occurred to similar degrees in decellularized aortas isolated from normal and diabetic mice. However, these differences only occurred at lower pressures (below 50–75 mmHg), whereas the intact and decellularized P-D curves converged at higher, physiological pressures. Importantly, these data may suggest that the ECM serves as the structural limit in fully dilated vessels at physiological pressures (75–125 mmHg) in the normal and diabetic macro-vessel, meaning that the ECM provides a maximal capacity for dilation in those vessels. In keeping with this notion, collagen is known to limit arterial distension with increasing load (i.e. pressure),³¹ so our finding of continued increased stiffness of decellularized aortas at higher pressure (Figure 5B) in the absence of further increases in diameter at higher pressures (Figure 3A) may suggest that collagen is limiting aortic distension, which is not different between normal and T2DM aortas.

Our data also reveal several other important concepts about macro-vascular mechanics, both in the presence and absence of cells. In our current study, the intact diabetic aorta did not undergo any overt structural remodeling and exhibited similar mechanical behavior compared with the intact normal aorta. This finding was somewhat surprising for several reasons. First, differences in remodeling and mechanics occurs in other vascular beds in this model at this age,^{13, 24, 28} albeit in micro-vessels. For example, in addition to CRM remodeling, we previously showed that mesenteric resistance arterioles from db/db mice undergo outward hypertrophic remodeling.²⁴ Second, the macro-vasculature is thought to be a prime target for advanced glycation end-product (AGE)-associated protein crosslinking, inferred primarily from data showing improved vascular stiffness in animals treated with an AGE crosslink breaker, alagebrium (ALT-711).^{30, 32, 34} Increased crosslinking is thought to decrease ECM turnover, thus increasing vascular stiffness. Evidence of the biochemical efficacy of crosslink breakers such as alagebrium has relied heavily on tail collagen solubility – collagen not derived from the vasculature. However, whether an increase in this specific type of AGE results in ECM crosslinking that has a mechanical consequence on the vasculature remains to be determined. Third, our finding of no alteration between intact normal and diabetic aortic stiffness by passive pressure myography does not, at first glance, agree with *in vivo* data showing increased pulse wave velocity (a gold standard measure of stiffness) in the diabetic aorta.¹³ This could be explained in several ways. Pressure myography remodeling experiments were conducted under passive conditions, i.e. in the

absence of calcium and in the presence of a vasodilator (SNP). One possible explanation for the discrepant stiffness between *in vivo* PWV and *ex vivo* passive myography could be the due to differences in smooth muscle tone in part because there is evidence that phenylephrine-induced vascular contraction can increase the stiffness of the aorta.²¹ Alternatively, PWV is dependent upon blood pressure depending on the animal model, and although we showed no difference in 24-hr conscious blood pressure in db/db mice,¹³ others have shown a diurnal rise in blood pressure in those animals.²⁶ We have further shown that db/db mice manifest an increase in blood pressure under anesthesia (unpublished observations), the time at which PWV is measured. Future studies are warranted to ascertain these mechanisms as they are beyond the scope of the current study.

In contrast to the aorta, the intact and decellularized CRM pressure-diameter curves appeared more parallel to one another (Figure 3D). Given that the CRM pressure curves do not converge at physiologic pressures, the ECM may not be a limiting factor in the structure of fully-dilated CRMs. CRMs lie within the cardiac wall that has an existing organized structural support, whereas the thoracic aorta does not and requires its own structural support. Therefore, it would reason that CRMs do not physiologically require as much ECM support. VSMCs are known to make more significant contributions to the mechanical support of smaller arterioles relative to larger arteries,^{8, 31} so VSMC mechanics may play a larger role in dictating overall CRM mechanics than does ECM mechanics. Another interesting difference between macro- and micro-vascular mechanical behavior in this study was the lack of significant diameter increase in the decellularized db/db aorta (relative to decellularized Db/db aorta, Figure 3C) despite the significant increase in the decellularized db/db CRM (relative to decellularized Db/db CRM, Figure 3F). The loss of cells in both normal and diabetic aortas resulted in similar % increase in diameter, which may suggest that the status of the ECM, vascular cells, and/or the cell-ECM coupling (e.g. integrins) could be similar. In contrast, the loss of cells in diabetic CRMs caused a greater % increase in diameter relative to normal CRMs, which was associated with a significant increase in stiffness (Figure 5D). We have previously shown that db/db CRMs have increased number of VSMCs relative to normal,¹³ therefore, the greater increase in diameter in the db/db CRMs may be due to the loss of an increased number of cells in those vessels. Alternatively, differential cell-ECM coupling (e.g. integrins) between normal and diabetic CRMs could be another contributing factor, as they exhibit mechanical activity.²⁷ Finally, given that VSMC mechanical behavior is more important in smaller arterioles³¹ relative to large arteries, these data may point to a novel difference in VSMC stiffness that reside in normal and diabetic CRMs. Future studies will address these important possibilities.

Previous work from our group documented differences in microvascular remodeling and mechanics in other vascular beds in this T2DM model.^{13, 24, 28} For example, in addition to CRM remodeling, we previously showed that mesenteric resistance arterioles from db/db mice undergo outward hypertrophic remodeling.²⁴ These data may suggest that microvessels are more influenced by the adverse consequences of type 2 diabetes at earlier stages of disease progression than conduit arteries. At 16 weeks of age, these db/db mice exhibit endothelial dysfunction, and altered vasoreactivity in the presence of overt diabetes, insulin resistance and inflammation. CRM remodeling was associated with decreased coronary flow

reserve, while mesenteric arteriole outward remodeling was associated with increased mesenteric flow. However, there is no manifestation of myocardial infarction, retinopathy or nephropathy at this time point. We postulate that adverse micro-vascular remodeling and mechanics, including the influence of the ECM to micro-vascular stiffness, is an earlier sub-clinical complication that may synergize with later macro-vascular disease to result in clinical complications of T2DM.

Limitations

The data presented here represent a novel and intriguing paradigm comparing the remodeling and mechanics of macro-vessels and coronary micro-vessels in type 2 diabetes in the presence and absence of cells. However, there are a few experimental considerations that are worth noting. First, our experiments were conducted at an early time point (16 weeks) when overt cardiovascular complications are not fully manifested. It is possible that the influences of the cells and/or ECM to vascular remodeling and mechanics may be different between earlier and later stages of disease progression in this diabetic model. Future studies will assess this possibility at later time points and in other rodent and large animal models. Second, the removal of vascular cells from both aortas and CRMs required the use of a mild detergent – SDS. In keeping with methods used to decellularize blood vessels and the myocardium for the purposes of tissue engineering, we adapted a protocol whereby macro- and microvessels were exposed to SDS for the minimum amount of time required to remove vascular cells. To determine whether SDS impacted the remaining ECM structure, we undertook electron microscopy and histochemical studies to assess the status of collagen and elastin. In both macro-vessels and coronary micro-vessels, we observed a moderate fraying of collagen that was not different between normal and diabetic vessels. While it is possible that the use of a mild detergent could affect remodeling and mechanics, we contend that observed differences in remodeling and mechanics here are unlikely due to the observed moderate disruption of the ECM.

Conclusions

Our study is the first to comprehensively compare intact vs. ECM contributions to remodeling and mechanics of both macro-vessels and coronary micro-vessels in type 2 diabetes. Based on our data, we postulate that: (1) in contrast to db/db CRMs that undergo inward hypertrophic remodeling associated with reduced stiffness, the db/db aorta (macro-vessel) exhibits similar structural and mechanical behavior to the normal aorta at this age (16 weeks), suggesting that micro-vessels may be more affected by the adverse consequences of early diabetes than macro-vessels; (2) the ECM serves as a similar structural limit for maximal dilation in the normal and diabetic macro-vessel (aorta), whereas this does not appear to be the case for coronary microvessels that reside in the cardiac wall.

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ABBREVIATIONS

T2DM	type 2 diabetes mellitus
CRM	coronary resistance microvessel
ECM	extracellular matrix
VSMC	vascular smooth muscle cell

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Figure 1.

Influence of decellularization on aortas. Top: *en face* punctate DAPI staining and F-actin staining was absent in decellularized aortas. Bottom Left: TEM analysis revealed moderate perturbations in collagen fibers as evidenced by fraying (gray fibers), in contrast to intact collagen (black fibers) that did not appear to be different between aorta or CRMs in the presence or absence of diabetes. Bottom Middle: Picrosirius Red staining showing a similar reduction in collagen content between decellularized normal and diabetic aortas ($46.6 \pm 7.2\%$ and $45.7 \pm 7.8\%$, respectively ($p > 0.05$)). Bottom Right: Aortic elastin remained intact in response to decellularization. Scale bars: Aorta DAPI and Picrosirius Red = 100 μm ; F-Actin and Elastin = 20 μm ; TEM images = 1 μm . Representative images from $n=2-4$ per group.

Figure 2. Influence of decellularization on CRMs. Left: *en face* DAPI staining was absent in decellularized aortas. Right: TEM analysis revealed moderate perturbations in collagen fibers as evidenced by fraying (gray fibers), in contrast to intact collagen (black fibers) that did not appear to be different between normal and diabetic CRMs. Scale bars: DAPI = 50 μm; TEM images = 1 μm. Representative images from n=2–4 per group.

Figure 3. Comparison of internal (top) and external (middle) diameters, as well as % increase in Di (bottom), in both macro-vessels (left) and coronary micro-vessels (right) measured by passive pressure myography. $^{\dagger}p<0.05$ vs. Db/db – Intact; $*p<0.05$ and $****p<0.0001$ vs. Intact; $^{\ddagger}p<0.001$ vs. Db/db – Decellularized. n=9–14 per group.

Figure 4. Comparison of wall thickness (top) and wall/lumen ratio (bottom) in both macro-vessels (left) and coronary micro-vessels (right) measured by passive pressure myography. $^{\dagger}p<0.05$ vs. Db/db – Intact; $*p<0.05$, $**p<0.01$, $***p<0.001$, and $****p<0.0001$ vs. Intact. $n=9-14$ per group.

Figure 5. Aortic (left) and CRM (right) stress-strain curves (top) and incremental modulus of elasticity (bottom). [†] $p < 0.05$ vs. Db/db – Intact; $**p < 0.01$, $***p < 0.001$, and $****p < 0.0001$ vs. Intact. $n = 9-14$ per group.