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Localized micro- and nano-scale remodelling in the diabetic aorta

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ABSTRACT

Diabetes is strongly associated with cardiovascular disease, but the mechanisms, structural and biomechanical consequences of aberrant blood vessel remodelling remain poorly defined. Using an experimental (streptozotocin, STZ) rat model of diabetes, we hypothesized that diabetes enhances extracellular protease activity in the aorta and induces morphological, compositional and localized micromechanical tissue remodelling. We found that the medial aortic layer underwent significant thickening in diabetic animals but without significant changes in collagen or elastin (abundance). Scanning acoustic microscopy demonstrated that such tissue remodelling was associated with a significant decrease in acoustic wave speed (an indicator of reduced material stiffness) in the inter-lamellar spaces of the vessel wall. This index of decreased stiffness was also linked to increased extracellular protease activity (assessed by semi-quantitative in situ gelatin zymography). Such a proteolytically active environment may affect the macromolecular structure of long-lived extracellular matrix molecules. To test this hypothesis, we also characterized the effects of diabetes on the ultrastructure of an important elastic fibre component: the fibrillin microfibril. Using size exclusion chromatography and atomic force microscopy, we isolated and imaged microfibrils from both healthy and diabetic aortas. Microfibrils derived from diabetic tissues were fragmented, morphologically disrupted and weakened (as assessed following molecular combing). These structural and functional abnormalities were not replicated by in vitro glycation. Our data suggest that proteolysis may be a key driver of localized mechanical change in the inter-lamellar space of diabetic rat aortas and that structural proteins (such as fibrillin microfbrils) may be biomarkers of diabetes induced damage.

1. Introduction

Diabetes is one of the most common non-communicable diseases in the world, with an estimated 382 million people affected worldwide in 2013 [1]. Whether as type 1 or type 2, its major outcomes, or health-related events leading to illness or death, are cardiovascular [2], resulting in reduced life expectancy and greatly increased healthcare costs. In both types, vascular dysfunction occurs early in the disease process [3–5]. The structural and biomechanical alterations in diabetic macro- and micro-vasculature are complex and the mechanisms remain poorly understood [2,6,7]. A better understanding of the processes driving vascular remodelling in diabetes should help develop new therapies [2].

Impaired biomechanical function of the diabetic aorta is generally attributed to changes in the extracellular matrix (ECM), notably in collagen abundance. Most studies suggest that collagen fibrosis causes increased vessel stiffness in the diabetic aorta [6,8,9] but there is a lack of consensus in the literature. For example, not all studies have reported increased collagen content in diabetes [10,11]. Potential mechanisms which underpin this ECM remodelling and hence vessel stiffening include matrix metalloproteinase (MMP) driven catabolic pathways [12,13], the accumulation of advanced glycation end-product (AGE) cross-links [6,8] and aberrant transforming growth factor-β (TGF-β) signalling [14]. This latter mechanism may be initiated by disruption of fibrillin microfibril based TGF-β sequestration, as is evident in the

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profound aortic remodelling which characterizes the vessel prior to rupture in Marfan syndrome, a congenital disease that compromises the mechanical integrity of connective tissues, particularly the aorta [15,16].

The contribution that micromechanical mapping can make to identifying local vessel stiffening within the vessel wall was highlighted in an earlier review [17] Using scanning acoustic microscopy (SAM), we have previously demonstrated that increased tissue acoustic wave speed (and hence increased stiffness) was localized to medial inter-lamellar regions in both ageing sheep [18] and Cardiotrophin-1 (CT-1) treated rat aortas [19]. In this study, we have used SAM with conventional histology and semi-quantitative in situ zymography to test our first hypothesis that experimental type 1 diabetes would induce morphological, compositional and localized micromechanical remodelling in the aorta associated with increased protease activity.

The lack of consensus regarding structural changes in the diabetic aorta may be due, in part, to the inability of conventional light microscopy to characterize the changing composition and/or macromolecular structure of long-lived ECM proteins [20–22]. Fibrillar collagens and elastic fibres are complex macromolecular assemblies whose function may be impaired, without affecting their global charge distribution or epitope availability and hence their detection, by histological or immunohistochemical techniques. Fibrillin microfibrils, as key elastic fibre components, play a central role in the pathogenesis of Marfan syndrome. The longevity and well-characterized structure of these microfibrils make them potential structural biomarkers of aberrant tissue remodelling and their role in Marfan syndrome suggests that in situ microfibril damage may be a key trigger for further inflammatory events [16,23]. In this study therefore we have also employed atomic force microscopy (AFM) and molecular combing [24] to test a second hypothesis that acute diabetes will compromise the ultrastructure and hence extensibility of isolated aortic fibrillin microfibrils [25,26].

2. Materials and methods

2.1. Animals and tissues

All procedures accorded to the UK Animals (Scientific Procedures) Act 1986 and the University of Manchester ethical review process. Type 1 diabetes was induced in adult male Wistar rats (Charles River, Kent, UK; 250–300 g; $n = 9$) by a single intraperitoneal injection of streptozotocin (STZ: Sigma Aldrich, Poole, Dorset, UK), freshly dissolved in normal saline, at a dose of 55 mg kg^{-1} [27]. Hyperglycaemia was confirmed (>15 mmol l^{-1}) three days following the STZ injection and at the end of the experiment (see Table 1). Experimental and control animals were group housed in Double Decker Rat Housing ICV cages (Tecniplast, Kettering, UK) for 56 ± 0 and 56 ± 1 day, respectively, after which time they were killed by anaesthetic overdose (isoflurane). The mean start and end weights for the controls were 313 ± 11 g and 517 ± 23 g, respectively. The mean start and end weights for the diabetics were 314 ± 13 g and 352 ± 18 g, respectively. The mean blood glucose for the controls was 10.5 ± 0.9 mmol l^{-1}. These values are expressed as means ± standard error of the mean (SEM).

The descending thoracic aorta was dissected and either snap-frozen in liquid nitrogen, or prepared for cryosectioning by freezing in optimal cutting temperature (OCT) resin (Sakura Fintek Europe BV, Alphen aan den Rijn, The Netherlands) in pre-cooled isopentane and stored at −80 °C [18].

2.2. SAM

Localized changes in tissue acoustic wave speed were measured for hydrated, unfixed aortic cryosections (5 μm thickness) with SAM as previously described using the Multi-Layer Phase Analysis (MLPA) method [28]. Briefly, SAM imaging was conducted on a KSI 2000 microscope (PVA TePla Analytical Systems GmbH; Herborn, Germany) modified with a custom data acquisition and control system. Imaging was conducted at 760 MHz in this study, which provided a spatial resolution of ~1.3 μm. The acoustic wave speed (v_L) is related to Young's modulus (stiffness) by the following equation:

$$v_L = \sqrt{\frac{C_{11}}{\rho}} = \sqrt{\frac{E}{\rho}\left(\frac{1-\nu}{(1+\nu)(1-2\nu)}\right)} \quad (1)$$

where ρ is the mass density (kg m^{-3}), and C_{11} (Pa) is a component of the elastic stiffness tensor, which can be expressed a function of Young's modulus (E) and Poisson's ratio (ν) [29]. Hence, a higher acoustic wave speed indicates a stiffer material.

The resulting SAM images contain sufficient structural information to allow the acoustic wave speed of the elastic lamellae and inter-lamellar regions of the aortic wall to be measured independently (Fig. 1).

Table 1
Body weight (start and end weights) and end blood glucose parameters for the Wistar rats. Note all readings for the diabetic rats were higher than the upper limit of detection for the glucose meter, i.e. >27.8 mmol l^{-1}.

Group	Start weight (g)	End weight (g)	Blood glucose (mmol l^{-1})
Control	342	629	7.3
Control	365	598	8.1
Control	331	545	9.7
Control	322	647	6.6
Control	318	610	13.7
Control	261	429	12.4
Control	299	514	11.9
Control	282	517	12.7
Control	301	524	12.5
Diabetic	320	305	>27.8
Diabetic	341	404	>27.8
Diabetic	333	396	>27.8
Diabetic	350	329	>27.8
Diabetic	357	437	>27.8
Diabetic	329	362	>27.8
Diabetic	247	282	>27.8
Diabetic	261	300	>27.8
Diabetic	290	350	>27.8

2.3. Histological and biochemical analysis

2.3.1. Quantification of collagen and elastin content

The relative fibrillar collagen and elastic fibre content (tissue section area) from control and diabetic rat aortas ($n = 6$ per group) was quantified as previously described [18]. Briefly, 5 μm cryosections were taken from the same animals used for SAM. Collagen content was quantified by obtaining both bright field and circular polarized light images of identical contiguous regions around the aortic circumference. The blue channel from the bright field image was thresholded (which enabled us to exclude voids in the tissue) and total tissue area was measured in pixels. The red channel from the polarized light image of the identical region was thresholded to reveal the collagen positive pixels. Collagen content of each image was then expressed as percentage tissue area. For elastin quantification, the blue channel from the bright field images of Millers stained sections were thresholded to measure the total tissue area (and to exclude voids in the tissue). The red channel was then thresholded to exclusively reveal the blue-black stained elastin fibres. The total elastin positive pixels were expressed as a percentage of total tissue area. Medial thickness (intimal to external elastic lamina of the medial layer) was determined from complete circumference montages of bright field images (at ×100 magnification) of Millers elastic stained cryosections. The images were then