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Hypertension and decreased aortic compliance due to reduced elastin amounts do not increase atherosclerotic plaque accumulation in *Ldlr*−/− mice

Justine A. Maedeker [a], Kellie V. Stoka [a], Siddharth A. Bhayani [b], William S. Gardner [b], Lisa Bennett [b], Jesse D. Procknow [a], Marius C. Staiculescu [a], Tezin A. Walji [c], Clarissa S. Craft [c], Jessica E. Wagenseil [a, *]

[a] *Department of Mechanical Engineering and Materials Science, Washington University, St. Louis, MO, USA*
[b] *Department of Biomedical Engineering, Saint Louis University, St. Louis, MO, USA*
[c] *Department of Cell Biology and Physiology, Washington University, St. Louis, MO, USA*

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ABSTRACT

Background and aims: High blood pressure and reduced aortic compliance are associated with increased atherosclerotic plaque accumulation in humans. Animal studies support these associations, but additional factors, such as fragmented elastic fibers, are present in most previous animal studies. Elastin heterozygous (*Eln*+/−) mice have high blood pressure and reduced aortic compliance, with no evidence of elastic fiber fragmentation and represent an appropriate model to directly investigate the effects of these factors on atherosclerosis.

Methods and results: Eln+/− and *Eln*+/+ mice were crossed with low density lipoprotein receptor knockout (*Ldlr*−/−) and wild-type (*Ldlr*+/+) mice and fed normal or Western diet (WD) for 16 weeks. We hypothesized that on WD, *Eln*+/−*Ldlr*−/− mice with high blood pressure and reduced aortic compliance would have increased atherosclerotic plaque accumulation compared to *Eln*+/+*Ldlr*−/− mice. We measured serum cholesterol and cytokine levels, blood pressure, aortic compliance, and plaque accumulation. Contrary to our hypothesis, we found that on WD, *Eln*+/−*Ldlr*−/− mice do not have increased plaque accumulation compared to *Eln*+/+*Ldlr*−/− mice. At the aortic root, there are no significant differences in plaque area between *Eln*+/−*Ldlr*−/− and *Eln*+/+*Ldlr*−/− mice on WD ($p = 0.89$), while in the ascending aorta, *Eln*+/−*Ldlr*−/− mice on WD have 29% less normalized plaque area than *Eln*+/+*Ldlr*−/− mice on WD ($p = 0.009$).

Conclusion: Using an atherogenic mouse model, we conclude that increased blood pressure and reduced aortic compliance are not direct causes of increased aortic plaque accumulation. We propose that additional insults, such as fragmentation of elastic fibers, are necessary to alter plaque accumulation.

1. Introduction

Atherosclerosis is an inflammatory disease with both environmental and genetic components [1,2]. High systolic blood pressure [3–6] and decreased aortic compliance [7,8] are associated with increased atherosclerotic plaque accumulation, although the relationships are complicated and often contradictory. Better understanding of the risk factors in atherosclerosis would help inform preventative and palliative care. Although they have limitations, mouse models of atherosclerosis, including apolipoprotein-E (*Apoe*−/−) [9,10] and low density lipoprotein receptor (*Ldlr*−/−) [11] deficient animals, have become invaluable tools for studying mechanisms of atherosclerosis [12]. *Apoe*−/− and *Ldlr*−/− mice fed a Western diet (WD, 20% fat, 0.15% cholesterol) develop hyperlipidemia and atherosclerosis.

Previously, Van Herck et al. [13] investigated the relationship between atherosclerosis and aortic compliance by crossing *Apoe*−/− mice with mice haploinsufficient for a mutation in the fibrillin-1 gene (*C1039G*+/−). The authors found that on WD *C1039G*+/

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* Corresponding author. Department of Mechanical Engineering and Materials Science, Washington University, One Brookings Dr., CB 1185, St. Louis, MO 63130, USA.

E-mail address: jessica.wagenseil@wustl.edu (J.E. Wagenseil).

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–*Apoe*–/– mice had decreased aortic compliance, which led to increased accumulation of atherosclerotic plaque, and promoted plaque instability compared to *C1039G*+/+*Apoe*–/– mice. Fibrillin-1 is a major component of microfibrils in the extracellular matrix that associate with developing elastic fibers. Mutations in fibrillin-1 cause Marfan Syndrome, an autosomal dominant disorder with skeletal, ocular, and cardiovascular manifestations. Cardiovascular symptoms include dilation and dissection of the ascending aorta [14], and have been associated with dysregulated transforming growth factor beta (TGF-β) signaling [15]. *C1039G*–/– mice die soon after birth due to failure of the arterial wall, while *C1039G*+/– mice show defects in elastic fiber structure and local elastolysis, but have a normal lifespan [16,17]. Elastic fiber fragmentation and injection of elastin-derived peptides have been shown to potentiate atherosclerosis [18]. It is possible that TGF-β signaling and elastic fiber fragmentation contribute to the increased plaque accumulation and instability in *C1039G*+/–*Apoe*–/– mice, and that decreased aortic compliance is not a major factor.

During elastic fiber formation, microfibrils interact with tropoelastin, which is then crosslinked into insoluble elastin. Elastin haploinsufficient mice (*Eln*+/–) mice have about 60% of wild-type elastin levels, stable hypertension, and decreased aortic compliance [19]. Elastin haploinsufficiency in humans causes Supravalvular Aortic Stenosis, which can occur as an isolated disease or as a component of Williams-Beuren Syndrome [20]. Mice and humans with elastin haploinsufficiency have thinner, more numerous elastic lamellae across the aortic wall, but they do not appear fragmented [21]. Hence, *Eln*+/– mice represent an appropriate model to isolate the effects of aortic compliance and hypertension on atherosclerosis progression, without complications of elastic fiber fragmentation and dysregulated TGF-β signaling. We bred *Eln*+/–mice to *Ldlr*–/– mice and fed them WD. We measured serum lipid and cytokine levels, blood pressure, aortic compliance, and atherosclerotic plaque accumulation and composition to determine if increased blood pressure and decreased aortic compliance in *Eln*+/–*Ldlr*–/– mice leads to increased plaque accumulation compared to *Eln*+/+*Ldlr*–/– mice.

2. Materials and methods

2.1. Mice

Female B6.129S7-Ldlr$^{tm1Her/J}$ –/– (*Ldlr*–/–) (Jackson Laboratory, stock #002207) were bred with male *Eln* mice [22]. Males at the F3 – F6 generation were used because previous data on blood pressure and aortic stiffness in *Eln*+/– mice were obtained for males [19]. Genotypes included in the study are: *Eln*+/+*Ldlr*–/–, *Eln*+/–*Ldlr*–/–, *Eln*+/+*Ldlr*+/+, *Eln*+/–*Ldlr*+/+. After weaning at three weeks of age, mice were provided with normal diet (ND) or WD (AIN-76A, Purina Test Labs) for 16 weeks. All protocols were approved by the Institutional Animal Care and Use Committee.

2.2. Blood pressure, serum chemistry, and tissue collection

Mice were anesthetized with 2% isoflurane and intra-aortic blood pressure was measured with a 1.2F solid-state catheter (Transonic). Whole blood was collected from a subset of mice via cardiac puncture and serum was separated. Lipid levels were quantified by Advanced Veterinary Laboratory using an automated chemistry analyzer. Additional serum samples for *Eln*+/+*Ldlr*–/– and *Eln*+/–*Ldlr*–/– mice on ND and WD were analyzed for inflammatory cytokines and TGF-β1 using electrochemiluminescence immunoassays from Mesoscale Discovery and read on a MESO Quickplex SQ 120. Only analytes with levels higher than 10 pg/ml on the mouse inflammatory cytokine multiplex assay are presented. These include interleukin 6 (IL6), interleukin 10 (IL10), chemokine (C-X-C motif) ligand 1 (CXCL1), and tumor necrosis factor (TNF).

The heart was removed and the proximal region was frozen at –80 °C in Tissue Tek OCT for sectioning of the aortic root. In about half of the mice, the ascending aorta was removed for mechanical testing and a small piece of the left carotid artery was fixed for wall structure analysis. In the rest of the mice, the entire aorta from the root to the iliac bifurcation was removed for en face plaque analysis.

2.3. Mechanical testing

The ascending aorta was mounted at the approximate in vivo length in a pressure myograph (110P, Danish Myotechnology) in physiologic saline solution at 37 °C, as described previously [23]. Arteries were inflated from 0 to 175 mmHg in steps of 25 mmHg (12 s/step) while pressure, outer diameter, and axial force were recorded at 1 Hz. The diameter compliance was calculated as the change in diameter for each pressure step, and is an inverse measure of aortic stiffness.

2.4. Plaque quantification and characterization

Aortas for en face preparation were fixed in 10% neutral buffered formalin overnight, cut longitudinally, pinned to black dissection wax, stained with Oil Red O in propylene glycol, and imaged [24]. Outlines of the ascending, thoracic, and descending regions of the aorta were defined and positive Oil Red O pixels were traced manually in Image J (NIH). Plaque area for each region was normalized to the aortic surface area. For aortic valve analysis, 5 μm frozen sections of the aortic root were cut with a cryostat. Slides were stained with Oil Red O in 60% isopropanol [25], but were not counterstained. Images were taken of slides at ~10 μm intervals. Three images for each mouse where clear sections of the aortic valves could be seen were analyzed for total Oil Red O positive pixels using Matlab software (Mathworks) and averaged.

Adjacent sections of the aortic valves for a subset of *Eln*+/+*Ldlr*–/– and *Eln*+/–*Ldlr*–/– mice on WD were stained with F4/80 antibody (ab16911, Abcam) followed by DAB and imaged to visualize macrophage content and localization. Adjacent sections of the aortic valves for the same mice were also examined by fluorescence microscopy. Sections were labeled for elastin, collagen, smooth muscle cells (SMCs), and cell nuclei. Alexa Fluor 633 Hydrazide (Life Technologies) was used for elastin [26,27]. CNA35 (kindly provided by Magnus Hook, Texas A&M) labeled with Oregon Green 488 (Life Technologies) was used for collagen [28]. Alpha smooth muscle actin (αSMA) primary antibody (A5228, Sigma) followed by Alexa Fluor 555 goat anti-mouse secondary antibody (Life Technologies) was used for SMCs. The cell nuclei were stained with Hoechst 34580 (Life Technologies). The percentage of the plaque area staining positive for F4/80, αSMA, and collagen was calculated from thresholded images of at least two aortic valves/mouse using Image J software.

2.5. Arterial wall structure

Sections of the left common carotid artery were examined by histology for seven *Eln*+/+*Ldlr*–/– and six *Eln*+/–*Ldlr*–/– mice on WD. Two – three mm long pieces of the artery were fixed in 10% neutral buffered formalin overnight, dehydrated in a graded series of ethanol, embedded in paraffin, sectioned, stained with H&E, Verhoeff Van Gieson (VVG) or picrosirius red (PSR) and imaged.