Atherosclerosis 249 (2016) 22–29

Contents lists available at ScienceDirect

Atherosclerosis

journal homepage: www.elsevier.com/locate/atherosclerosis

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

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ARTICLE INFO

Article history:
Received 11 November 2015
Received in revised form
17 February 2016
Accepted 16 March 2016
Available online 21 March 2016

Keywords:
Atherosclerosis
Elastin
Arterial compliance
Arterial stiffness
Hypertension

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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http://dx.doi.org/10.1016/j.atherosclerosis.2016.03.022
0021-9150/

–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.

2.6. Statistics

Three-way ANOVA (SPSS) was used to evaluate the effects of diet, *Ldlr* genotype, *Eln* genotype and the interactions between each independent variable on the measured dependent variables. Cytokine levels were only measured in *Ldlr*−/− mice, so two-way ANOVA was used to evaluate the effects of diet and *Eln* genotype. Additionally, two-way student's t-test with unequal variance was used to evaluate differences in plaque amounts and composition between *Eln*+/−*Ldlr*−/− and *Eln*+/+*Ldlr*−/− mice on WD. $P < 0.05$ was considered significant.

3. Results

3.1. Cholesterol increases with WD and Ldlr−/− genotype, but is not affected by Eln genotype

Total cholesterol, triglyceride, and low density lipoprotein (LDL) levels all increase with WD (200–400%), *Ldlr*−/− genotype (400–4000%), and show a significant interaction between diet and *Ldlr* genotype (Table 1). *Eln* genotype does not affect total cholesterol, triglyceride, or LDL levels. High density lipoprotein (HDL) levels are increased about 40% with WD, but are not affected by *Ldlr* or *Eln* genotype (Table 1).

3.2. Systolic and pulse pressure increase with WD, Ldlr−/− genotype and Eln+/− genotype

Systolic pressure is increased 8% with WD, 6% with *Ldlr*−/− genotype, and 10% with *Eln*+/− genotype, with no interactions between the independent variables (Table 1). Diastolic blood pressure is increased 7% with WD, but is not affected by *Ldlr* or *Eln* genotype (Table 1). Pulse pressure is increased 11% with WD, 13% with *Ldlr*−/− genotype, and 20% with *Eln*+/− genotype, with interactions between diet and *Ldlr* genotype and all three independent variables (Table 1). Heart rate is not significantly affected by diet, *Ldlr* or *Eln* genotype (Table 1).

3.3. Aortic compliance is reduced in Eln+/− mice at systolic pressures, but is not affected by diet or Ldlr genotype

Average pressure-diameter curves are shown in Fig. 1A. *Ldlr* genotype and diet do not have significant effects on the diameter for any applied pressure. Diameter is decreased 7–11% at pressures between 0 − 50 and 150–175 mmHg in *Eln*+/− mice ($p = <0.001 - 0.013$). There are significant interactions between *Eln* genotype and diet at 0–50 mmHg ($p = 0.007 - 0.042$) and between all three independent variables at pressures above 0 mmHg ($p = 0.001–0.045$). Average pressure-compliance curves are shown in Fig. 1B. *Ldlr* genotype and diet do not have significant effects on the compliance for any applied pressure. The compliance curves for *Eln*+/− mice are shifted so that the peak occurs at lower pressures than *Eln*+/+, with a steep drop in compliance at higher pressures. At pressures of 50 and 75 mmHg, compliance is 21–43% higher in *Eln*+/− aorta compared to *Eln*+/+ ($p = <0.001$ and 0.004). At pressures between 100 and 175 mmHg (near and above systolic pressure values), compliance is 19–53% lower in *Eln*+/− aorta compared to *Eln*+/+ ($p = <0.001 - 0.004$). There are significant interactions between *Eln* and *Ldlr* genotype at pressures between 0 and 75 mmHg ($p = 0.002 - 0.017$).

3.4. Atherosclerotic plaque is not increased in Eln+/− mice

Cross-sections of the aortic root were stained with Oil Red O to estimate atherosclerotic plaque accumulation. Total plaque at the aortic root is increased with WD ($p < 0.001$) and *Ldlr*−/− genotype ($p < 0.001$), but is not affected by *Eln* genotype ($p = 0.991$). There are significant interactions between diet and *Ldlr* genotype ($p < 0.001$). There are no significant differences for the plaque area at the aortic root between *Eln*+/+*Ldlr*−/− and *Eln*+/−*Ldlr*−/− mice on WD ($p = 0.89$) (Fig. 2A). Representative images of plaque at the aortic root are shown in Fig. 2B and C. En face preparations of the aorta were stained with Oil Red O as an additional estimate of plaque accumulation. As most of the plaque accumulation occurs in the ascending aorta in *Ldlr*−/− mice after 16 weeks of WD, we focused our analyses on that region. The fraction of ascending aortic lumen covered in plaque is increased with WD ($p < 0.001$) and *Ldlr*−/− genotype ($p < 0.001$), and is decreased in *Eln*+/− mice ($p = 0.010$). There are significant interactions between all two-way combinations and the three-way combination of independent variables ($p < 0.001–0.011$). *Eln*+/−*Ldlr*−/− mice on WD have 29% less normalized plaque area in the ascending aorta than *Eln*+/+*Ldlr*−/− mice on WD ($p = 0.009$) (Fig. 2D). Representative images of plaque in the ascending aorta are shown in Fig. 2E and F. In the thoracic and abdominal regions, there are no significant differences in plaque accumulation between *Eln*+/+*Ldlr*−/− and *Eln*+/−*Ldlr*−/− mice on WD, but the plaque amounts in these regions are highly variable (Supplemental Fig. I). When total normalized plaque area

Table 1
Serum cholesterol levels and blood pressures in *Eln*+/+ and *Eln*+/−mice crossed with *Ldlr*+/+ and *Ldlr*−/− mice and fed ND or WD for 16 weeks after weaning. Total cholesterol, triglyceride (Tri), low density lipoprotein (LDL) and high density lipoprotein (HDL) serum concentrations were measured. Diet and *Ldlr* genotype significantly affect most measures of serum cholesterol, while *Eln* genotype does not. Systolic (Sys) and pulse pressures measured with a solid-state catheter are significantly affected by diet, *Ldlr* genotype, and *Eln* genotype, while diastolic (Dias) pressures are affected only by diet. Heart rate (Hr) is not affected by any of the independent variables. Significance was determined by three-way ANOVA for diet, *Ldlr* genotype, *Eln* genotype and all two- and three-way interactions. Italics indicate $P < 0.05$. Data are presented as mean ± SD.

			Serum cholesterol (mg/dl)				blood pressure (mmHg)			
Diet	*Ldlr*	*Eln*	Total	Tri	LDL	HDL	Sys	Dias	Pulse	Hr (bpm)
ND	*Ldlr*+/+	*Eln*+/+	138 ± 32	62 ± 13	6 ± 3	69 ± 11	109 ± 12	74 ± 10	35 ± 4	497 ± 62
		Eln +/−	169 ± 39	55 ± 7	7 ± 3	83 ± 13	122 ± 19	79 ± 12	43 ± 11	514 ± 57
	Ldlr−/−	*Eln*+/+	441 ± 50	117 ± 35	123 ± 16	80 ± 14	114 ± 9	76 ± 7	37 ± 4	514 ± 68
		Eln +/−	432 ± 114	266 ± 181	170 ± 83	87 ± 13	122 ± 17	77 ± 11	44 ± 8	510 ± 82
WD	*Ldlr*+/+	*Eln*+/+	238 ± 77	66 ± 18	15 ± 5	120 ± 29	117 ± 12	78 ± 7	39 ± 6	484 ± 89
		Eln +/−	209 ± 77	58 ± 12	12 ± 3	114 ± 20	122 ± 12	81 ± 8	40 ± 7	506 ± 85
	Ldlr−/−	*Eln*+/+	1040 ± 143	740 ± 351	698 ± 229	99 ± 17	123 ± 16	82 ± 10	41 ± 8	482 ± 58
		Eln +/−	1001 ± 85	808 ± 215	596 ± 216	112 ± 10	141 ± 17	85 ± 9	56 ± 10	480 ± 58
P value		diet	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*0.002*	*0.011*	*0.006*	0.154
		Ldlr	*<0.001*	*<0.001*	*<0.001*	0.632	*0.014*	0.299	*0.001*	0.786
		Eln	0.596	0.215	0.628	0.122	*<0.001*	0.115	*<0.001*	0.565
	interactions		diet x *Ldlr* (*<0.001*)	diet x *Ldlr* (*<0.001*)	diet x *Ldlr* (*<0.001*)	–	–	–	diet x *Ldlr* (0.027), diet x *Ldlr* x *Eln* (0.018)	–
N/group			5–7	5–7	5–7	5–7	9–17	9–17	9–17	9–17

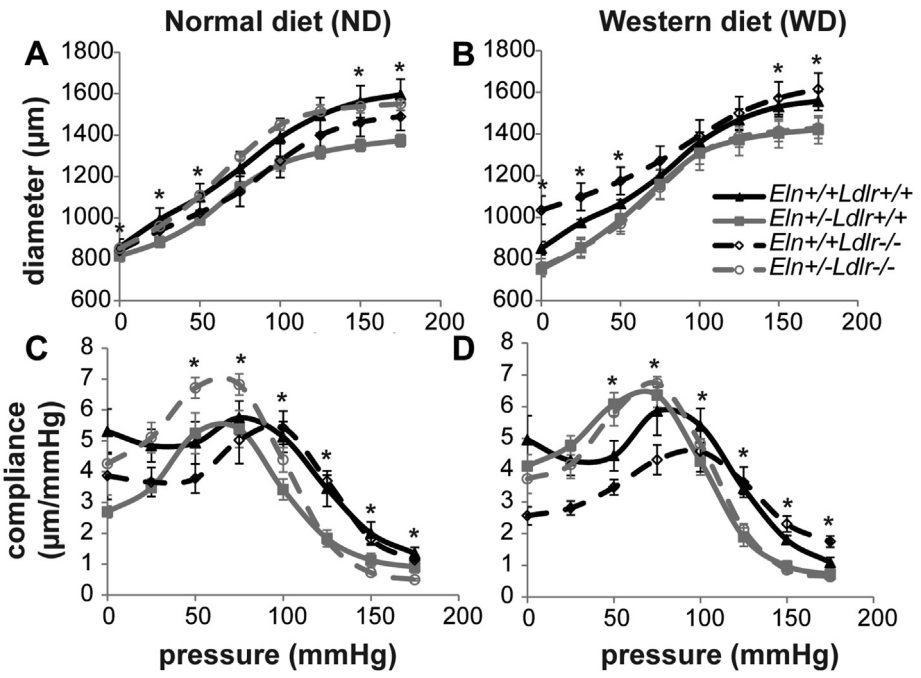

Fig. 1. Ascending aortic diameter is reduced in *Eln*+/− mice compared to *Eln*+/+ (A, B), as determined from in vitro mechanical tests. Ascending aortic compliance, calculated as the local slope of the pressure-diameter curve, is reduced in *Eln*+/− mice compared to *Eln*+/+ at pressures near and above systolic values (C, D). Diet and *Ldlr* genotype have no significant effects on the diameter or compliance at any pressure. N = 4–8/group. * = P < 0.05 for *Eln* genotype by three-way ANOVA.

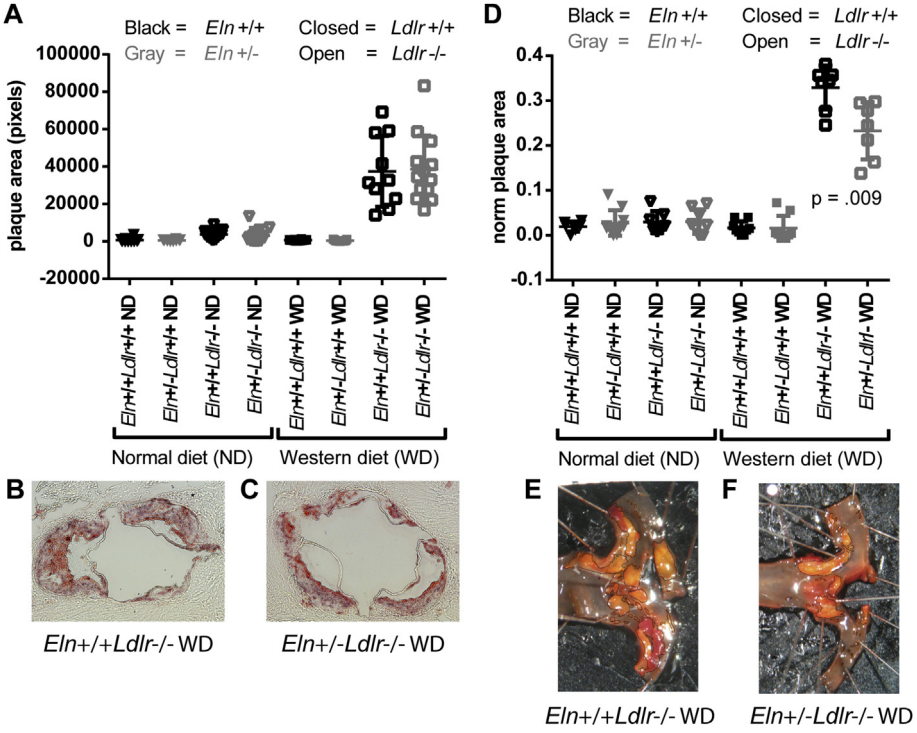

Fig. 2. Atherosclerotic plaque amounts are similar or reduced in *Eln*+/−*Ldlr*−/− mice on WD compared to *Eln*+/+*Ldlr*−/− mice on WD, despite high blood pressure and reduced aortic compliance in *Eln*+/−*Ldlr*−/− mice. Total plaque area at the level of the aortic valve is similar in *Eln*+/+*Ldlr*−/− and *Eln*+/−*Ldlr*−/− mice on WD (A), as quantified by Oil Red O staining of aortic root sections (B, C). N = 10–16/group. Normalized (norm) plaque area in the ascending aorta is reduced in *Eln*+/−*Ldlr*−/− mice on WD compared to *Eln*+/+*Ldlr*−/− mice on WD (D), as quantified by Oil Red O staining of en face preparations of the ascending aorta (E, F). N = 6–9/group. Significance between *Eln*+/+*Ldlr*−/− and *Eln*+/−*Ldlr*−/− mice on WD determined by student's t-test.

of the entire aorta is calculated, there are no significant differences in plaque amounts between *Eln*+/+*Ldlr*−/− and *Eln*+/−*Ldlr*−/− mice on WD (Supplemental Fig. I).

Cross-sections of the aortic root for *Eln*+/+*Ldlr*−/− and *Eln*+/

−Ldlr−/− mice on WD were analyzed for plaque composition. Representative F4/80 images for macrophage amount and localization are shown in Fig. 3A and B. The plaques have a layer of macrophages on top of a necrotic core, with no differences in the amount of F4/80 positive cells (Fig. 3E). Fluorescence staining for elastin, collagen, SMCs, and cell nuclei for the same plaques are shown in Fig. 3C and D. A layer of SMCs is located on top of the necrotic core, where there are few cell nuclei. There are no differences in the amount of cells staining positively for αSMA (Fig. 3F). Collagen stains brightly in the valves and faintly under the SMCs. There is a significant increase in area of the plaque staining positively for collagen in *Eln+/−Ldlr−/−* mice on WD compared to *Eln+/+Ldlr−/−* mice on WD ($p = 0.02$) (Fig. 3G).

3.5. Circulating cytokine levels are not affected by Eln genotype

Immunoassays were used to measure serum levels of inflammatory cytokines and TGF-β1 in *Eln+/+Ldlr−/−* and *Eln+/−Ldlr−/−* mice on ND and WD. IL6, IL10, CXCL1, and TNF are increased 140–600% with WD, but are not affected by *Eln* genotype (Table 2). TGF-β1 levels are not affected by diet or *Eln* genotype (Table 2).

3.6. Elastic fibers are not fragmented in Eln+/− arteries

Cross-sections of the left common carotid artery were processed for histology to visualize the wall structure away from the plaque area of *Eln+/+Ldlr−/−* and *Eln+/−Ldlr−/−* mice on WD (Fig. 4). H&E staining shows similar cell density and orientation. VVG staining shows that *Eln+/−Ldlr−/−* arteries have thinner, more numerous elastic lamellae than *Eln+/+Ldlr−/−* arteries, with no evidence of increased elastic fiber fragmentation. PSR staining shows that collagen organization appears similar in *Eln+/+Ldlr−/−* and *Eln+/−Ldlr−/−* arteries.

4. Discussion

Eln+/+ and *Eln+/−* mice were bred with *Ldlr+/+* and *Ldlr−/−* mice and fed ND or WD for 16 weeks after weaning to determine if hypertension and decreased aortic compliance in *Eln+/−Ldlr−/−* mice increases atherosclerotic plaque accumulation compared to *Eln+/+Ldlr−/−* mice. WD increases total cholesterol, triglyceride, LDL and HDL levels. *Ldlr−/−* genotype increases total cholesterol, triglycerides, and LDL levels. Total cholesterol and triglyceride changes are consistent with previous studies of *Ldlr* mice on WD [29–31]. LDL and HDL levels are also consistent with previous results for *Ldlr* mice on WD [32]. The serum cholesterol measurements confirm that the combination of diet and *Ldlr* genotype has the expected results on *Eln* mice.

WD, *Ldlr−/−* genotype, and *Eln+/−* genotype increase systolic blood pressure and pulse pressure, with *Eln+/−* genotype having the most significant effect. WD has been shown to increase blood pressure in wild-type mice [33,34]. To our knowledge, *Ldlr−/−* genotype has not previously been associated with increases in systolic or pulse pressure. *Eln+/−* mice are known to have increased systolic and pulse pressure [19,35]. Our blood pressure results confirm previous studies and show that *Eln+/−Ldlr−/−* mice on WD have the highest systolic and pulse pressure of all groups. The increase in pulse pressure for *Eln+/−* mice is similar to the increase in pulse pressure for aging humans [36]. In clinical studies, there are significant associations between high blood pressure and atherosclerotic plaque accumulation [3–6], but animal studies of relationships between hypertension and atherosclerosis have been contradictory [37].

One complication in animal studies is that modulation of the renin-angiotensin system (RAS) is often used to alter blood pressure. Ang II is the active end product of the RAS and has a range of effects on cells including encouraging proliferation and apoptosis and activating inflammatory and oxidative stress pathways [38]. Infusing Ang II in *Apoe−/−* mice accelerates atherosclerosis [39],

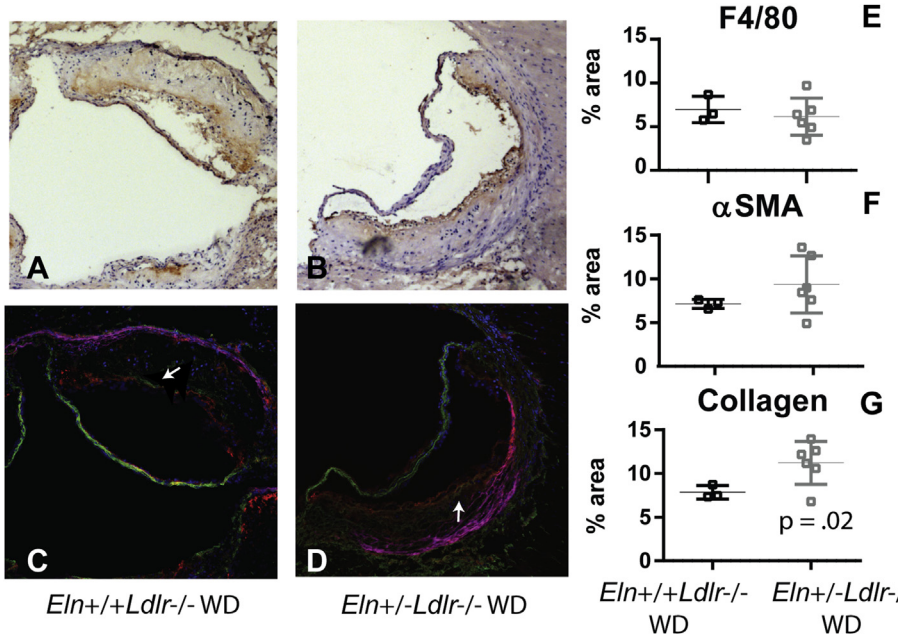

Fig. 3. Macrophage and SMC amounts are similar, but collagen amount is increased for aortic root plaques in *Eln+/−Ldlr−/−* mice on WD compared to *Eln+/+Ldlr−/−* mice on WD. F4/80 staining shows a layer of macrophages on top of a necrotic core (A, B). Fluorescent staining of the same plaque (C, D) shows αSMA positive cells (red) on top of the necrotic core containing few cell nuclei (blue). Collagen (green) stains brightly in the valve tissue and shows patchy staining (arrows) below the layer of αSMA positive cells. Elastic fibers (magenta) are visible in the aortic wall. Quantification of the percent area of the plaque staining positively for F4/80 (E), αSMA (F), and collagen (G) shows an increase in collagen amounts for *Eln+/−Ldlr−/−* mice. N = 3–6/group.

Table 2
Levels of inflammatory cytokines and TGF-β1 were measured using electrochemiluminescence immunoassays on serum from *Eln*+/+*Ldlr*−/− and *Eln*+/−*Ldlr*−/− mice fed ND or WD for 16 weeks after weaning. IL6, IL10, CXCL1, and TNF levels are significantly affected by diet, but not *Eln* genotype. TGF-β1 levels are not affected by diet or *Eln* genotype. Significance was determined by two-way ANOVA for diet, *Eln* genotype and the interaction between diet and *Eln* genotype. Italics indicate P < 0.05. Data are presented as mean ± SD.

			Inflammatory cytokines (pg/ml)				TGF-β1 (ng/ml)
Diet	*Ldlr*	*Eln*	IL6	IL10	CXCL1	TNF	TGF-β1
ND	*Ldlr*−/−	*Eln*+/+	12 ± 5	10 ± 2	29 ± 10	8 ± 2	39 ± 10
		Eln +/−	13 ± 3	10 ± 3	26 ± 8	7 ± 1	52 ± 12
WD	*Ldlr*−/−	*Eln*+/+	89 ± 28	29 ± 20	185 ± 160	23 ± 7	28 ± 30
		Eln +/−	86 ± 73	20 ± 6	202 ± 144	29 ± 15	37 ± 26
P value		Diet	*0.021*	*0.005*	*0.019*	*0.007*	0.332
		Eln	0.964	0.231	0.913	0.697	0.412
	Diet x *Eln*		0.956	0.274	0.873	0.579	0.879
N/group			2–6	2–6	2–6	2–6	2–6

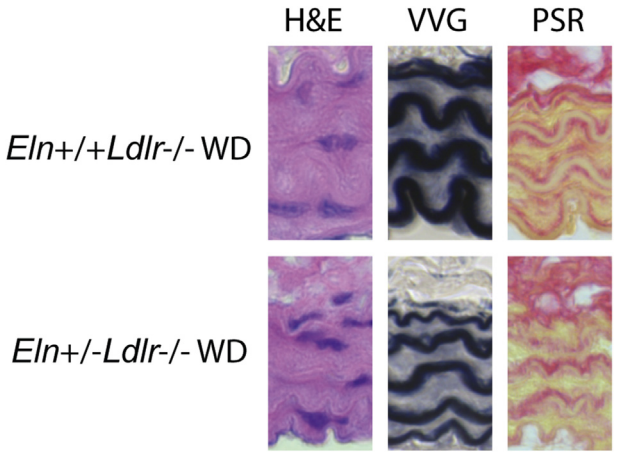

Fig. 4. Representative histology images of carotid artery sections stained with H&E. for cell nuclei (purple), Verhoeff Van Gieson (VVG) for elastic fibers (black) and picrosirius red (PSR) for collagen (red). *Eln*+/−*Ldlr*−/− mice on WD have thinner, more numerous elastic lamellae across the arterial wall than *Eln*+/+*Ldlr*−/− mice on WD, but the lamellae are not fragmented. N = 6–7/group. There is also no evidence of elastic fiber fragmentation at the level of the aortic valve under the plaque (Fig. 3).

while inhibiting Ang II in *Apoe*−/− mice through pharmaceutical [40] or genetic [41] approaches reduces plaque accumulation, independent of blood pressure lowering effects. When norepinephrine or Ang II are infused in *Apoe*−/− mice to obtain equivalent increases in blood pressure, there are much more modest increases in plaque amounts with norepinephrine than Ang II compared to control. However, norepinephrine still causes increased blood pressure and increased plaque deposition compared to control [42]. Previous studies showed that renin activity is high in *Eln*+/− mice, suggesting increased levels of Ang II, but aldosterone levels are normal, suggesting that downstream effects of Ang II may be mediated differentially in *Eln*+/− mice [19]. Increased blood pressure and increased renin activity in *Eln*+/− mice would be expected to increase atherosclerotic plaque accumulation in a hyperlipidemic model, but our results show that *Eln*+/−*Ldlr*−/− mice on WD have similar or reduced plaque amounts compared to controls.

Two recent studies investigate links between hypertension and atherosclerosis in animals without RAS activation. Vitamin D deficiency coupled with WD in *Ldlr*−/− and *Apoe*−/− mice increases blood pressure and increases atherosclerotic plaque accumulation [43]. However, vitamin D deficiency also activates macrophage ER stress and accelerates atherosclerosis by inducing phenotypic changes in macrophages. Plaque accumulation in vitamin D deficiency is decreased by a chemical chaperone that decreases ER stress, without any effects on blood pressure, indicating that macrophage ER stress is the driving factor for plaque accumulation, not blood pressure. Compared to ND, a low sodium diet given to *Apoe*−/− mice results in normal blood pressure, increased inflammatory markers, and increased atherosclerotic plaque accumulation [44]. Contrarily, high sodium diet given to *Apoe*−/− mice results in high blood pressure, decreased inflammatory markers and decreased atherosclerotic plaque accumulation, indicating that inflammatory markers are associated with atherosclerosis, but high blood pressure is not. The data from the vitamin D and sodium studies highlight the role of inflammation over hypertension as a factor in atherosclerosis susceptibility.

The results of the vitamin D and sodium studies are consistent with our findings that *Eln*+/−*Ldlr*−/− mice with hypertension, but no indication of abnormal inflammation in the aortic wall, do not have increased plaque accumulation compared to *Eln*+/+*Ldlr*−/− mice on WD. Serum levels of inflammatory cytokines, IL6, IL10, CXCL1, and TNF, which all play important roles in atherogenesis [45], are increased in *Ldlr*−/− mice on WD compared to ND, but are not different in *Eln*+/− mice compared to *Eln*+/+. Macrophage amounts and localization are not different in aortic root plaques in *Eln*+/+*Ldlr*−/− and *Eln*+/−*Ldlr*−/− mice on WD, indicating that the infiltration of inflammatory cells in the plaque is not affected by elastin haploinsufficiency and the resulting hypertension and reduced aortic compliance. Although SMC amounts are similar, collagen amounts are higher in aortic root plaques for *Eln*+/−*Ldlr*−/− mice on WD compared to *Eln*+/+*Ldlr*−/− mice on WD, indicating increased plaque stability [2].

Aortic compliance is significantly lower in *Eln*+/− mice than *Eln*+/+ at physiologic pressures, consistent with previous studies [19,23]. Diet and *Ldlr* genotype have no significant effects on aortic compliance. In B6D2F1 mice, WD decreases aortic compliance as measured by pulse wave velocity [46] and in vitro mechanical tests [47]. Previous studies have found reduced aortic compliance in *Ldlr*−/− mice [48]. Differences in the effects of diet and *Ldlr* genotype on aortic compliance may be due to differences in mouse strain, diet protocol, and measurement methods. Despite significantly decreased aortic compliance, *Eln*+/−*Ldlr*−/− mice on WD do not show increased atherosclerotic plaque accumulation or decreased plaque stability.

C1039G+/−*Apoe*−/− mice have decreased aortic compliance and increased plaque accumulation and decreased plaque stability, with no changes in blood pressure compared to control mice [13]. *C1039G*+/− mice have fragmented elastic fibers [16,17] and dysregulated TGF-β signaling [15]. Injecting elastin-derived peptides increases plaque accumulation in *Apoe*−/− mice on ND and *Ldlr*−/− mice on WD[18]. The effect is blocked in the absence of immune PI3Kγ, indicating that fragmentation of elastic fibers, release of elastin-derived peptides, and the subsequent immune response may contribute to increased plaque accumulation in *C1039G*+/

–Apoe–/– mice. TGF-β has complex effects on atherosclerosis and immune reactivity in mouse and man [49,50], but it is reasonable to believe that altered TGF-β regulation may also affect atherosclerosis accumulation in *C1039G+/–Apoe–/–* mice, independent of reductions in aortic compliance. *C1039G+/–* mice have higher levels of circulating TGF-β1 that increase with age and correlate with aortic root dilation [51]. We found no significant differences in circulating TGF-β1 levels between *Eln+/+Ldlr–/–* and *Eln+/–Ldlr–/–* mice on ND or WD. TGF-β signaling could be further investigated through quantification of gene and protein levels of TGF-β pathway members within the aortic wall at different time points, but our current results show no evidence of altered TGF-β signaling in *Eln+/–* mice.

In human studies, a 3–21% reduction in arterial compliance is observed in individuals with atherosclerotic plaques, compared to individuals with no detectable plaques [7,8]. The reduction in compliance is consistent with that observed in *Eln+/–* mice at systolic blood pressure. However, human studies generally include older individuals (mean age > 65) [7,8]. Elastin degrades and fragments with age, leading to decreased aortic compliance [52,53]. Because elastin degradation and decreased aortic compliance are intimately related, it is difficult to separate them in human studies with aging individuals or in mouse models with fragmented elastic fibers. *Eln+/–* mice are unique in that they have reduced aortic compliance, but no indication of elastic fiber fragmentation. Our data show that *Eln+/–Ldlr–/–* mice on WD do not have increased atherosclerotic plaque accumulation or instability compared to *Eln+/+Ldlr–/–* mice on WD. Our results suggest that additional insults, besides high blood pressure and low aortic compliance, such as activation of inflammatory pathways through elastic fiber fragmentation or TGF-β dysregulation, are necessary to alter atherosclerosis progression.

4.1. Limitations

Atherosclerosis in mice is different than in humans. However, mice allow us to investigate specific contributions to atherosclerosis in a controlled manner that cannot be done in human studies. We used *Ldlr–/–* mice as our atherogenic model and may have observed different results in another model. We measured plaque accumulation at one specific time point, so we may have missed differences in the time course of plaque progression that depend on blood pressure or aortic compliance. We examined plaque composition and cytokine levels in a subset of *Eln+/+Ldlr–/–* and *Eln+/–Ldlr–/–* mice on WD. More comprehensive analyses of plaque composition and progression and cytokine signaling are needed to determine if *Eln+/–* genotype affects characteristics of atherosclerotic disease other than plaque amounts. We measured anesthetized blood pressure, which will be different than ambulatory blood pressure. Although absolute pressure values vary between anesthetized and awake animals, the differences between groups are likely to remain. We suggest that additional insults, such as elastic fiber fragmentation, are necessary to alter atherosclerosis progression. Alternate mouse models, such as fibulin-5 knockout mice (*Fbln5–/–*), that have reduced aortic compliance, as well as fragmented elastic fibers [54,55] could be used to further investigate this idea.

Acknowledgements

Trey Coleman at the Washington University School of Medicine is gratefully acknowledged for providing assistance with the plaque quantification methods. The Washington University Digestive Diseases Research Core Center (DDRCC) performed the F4/80 staining. The Immunomonitoring Laboratory in the Center for Human Immunology and Immunotherapy Programs at Washington University performed the immunoassays. The Saint Louis University Research Microscopy and Histology Core did the histological processing. This work was supported in part by NIH R01HL105314 (JEW), NIH R01HL115560 (JEW), American Diabetes Association grant 7-13-JF-16 (CSC), Washington University Nutrition and Obesity Research Center grant P30DK056341 (CSC), and NIH P30DK052574 (DDRCC).

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.atherosclerosis.2016.03.022

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