

Cardiovascular Protection by ApoE and ApoE-HDL Linked to Suppression of ECM Gene Expression and Arterial Stiffening

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SUMMARY

Arterial stiffening is a risk factor for cardiovascular disease, but how arteries stay supple is unknown. Here, we show that apolipoprotein E (apoE) and apoE-containing high-density lipoprotein (apoE-HDL) maintain arterial elasticity by suppressing the expression of extracellular matrix genes. ApoE interrupts a mechanically driven feed-forward loop that increases the expression of collagen-I, fibronectin, and lysyl oxidase in response to substratum stiffening. These effects are independent of the apoE lipid-binding domain and transduced by Cox2 and miR-145. Arterial stiffness is increased in apoE null mice. This stiffening can be reduced by administration of the lysyl oxidase inhibitor BAPN, and BAPN treatment attenuates atherosclerosis despite highly elevated cholesterol. Macrophage abundance in lesions is reduced by BAPN *in vivo*, and monocyte/macrophage adhesion is reduced by substratum softening *in vitro*. We conclude that apoE and apoE-containing HDL promote healthy arterial biomechanics and that this confers protection from cardiovascular disease independent of the established apoE-HDL effect on cholesterol.

INTRODUCTION

The mechanobiology of cells and tissues is a rapidly developing field of importance to development, physiology, and disease (Davies, 2009; Discher et al., 2005; Egeblad et al., 2010; Garcia-Cardena and Gimbrone, 2006; Schwartz and DeSimone, 2008). Increases in tissue stiffness and intracellular tension are common features of fibrosis-associated processes such as wound repair, cancer, and cardiovascular disease (Duprez and Cohn, 2007; Levental et al., 2009). A recurring theme in these processes is remodeling of the extracellular matrix

(ECM). Increased ECM synthesis is mediated by fibrotic factors such as TGF- β and PGF₂ (Border and Noble, 1994; Oga et al., 2009), but whether antifibrotic factors exist to antagonize aberrant ECM gene expression and maintain normal tissue elasticity is poorly understood.

Mechanical forces play a major role in the pathogenesis of atherosclerosis (Davies, 2009; Garcia-Cardena and Gimbrone, 2006; Gimbrone et al., 2000). As a result of disturbed blood flow patterns at sites of arterial curvature and branches, endothelial cell integrity is disrupted focally, ultimately allowing for entry of blood monocytes into the vessel. These monocytes develop into macrophages and foam cells, a process exacerbated by high cholesterol, and then secrete cytokines that act on vascular smooth muscle cells (VSMCs) to promote their dedifferentiation to a migratory and proliferative phenotype. Dedifferentiated VSMCs synthesize large amounts of ECM components (in particular, fibrillar collagens and elastin) and matrix-modifying enzymes that remodel the local ECM (Owens et al., 2004; Thyberg et al., 1997; Thyberg et al., 1990). Elastin makes arteries more compliant to large deformations, and fibrillar collagens make arteries stiffer (Diez, 2007; Lakatta, 2007). The mechanical properties of elastin and fibrillar collagens depend upon their crosslinking by the lysyl oxidases (Csiszar, 2001; Kagan and Li, 2003). VSMCs produce lysyl oxidase and are therefore poised to be major regulators of matrix remodeling and arterial stiffness.

Arterial stiffness increases with normal aging, and this process is exaggerated by the metabolic syndrome and diabetes (Lakatta, 2007; Stehouwer et al., 2008). Arterial stiffness is also a cholesterol-independent risk factor for a first cardiovascular event (Mitchell et al., 2010). Arterial stiffness is determined by vascular tone and the amount and composition of the ECM. While regulators of vascular tone have been very well studied (Bellien et al., 2008), little is known about effectors and mechanisms that might regulate arterial stiffness by limiting ECM production. Nor is it known if arterial stiffening is a cause or consequence of cardiovascular disease.

Here, we show that the expression of ECM genes in VSMCs and arterial stiffness is potently suppressed by apolipoprotein E (apoE) and apoE-containing high-density lipoprotein

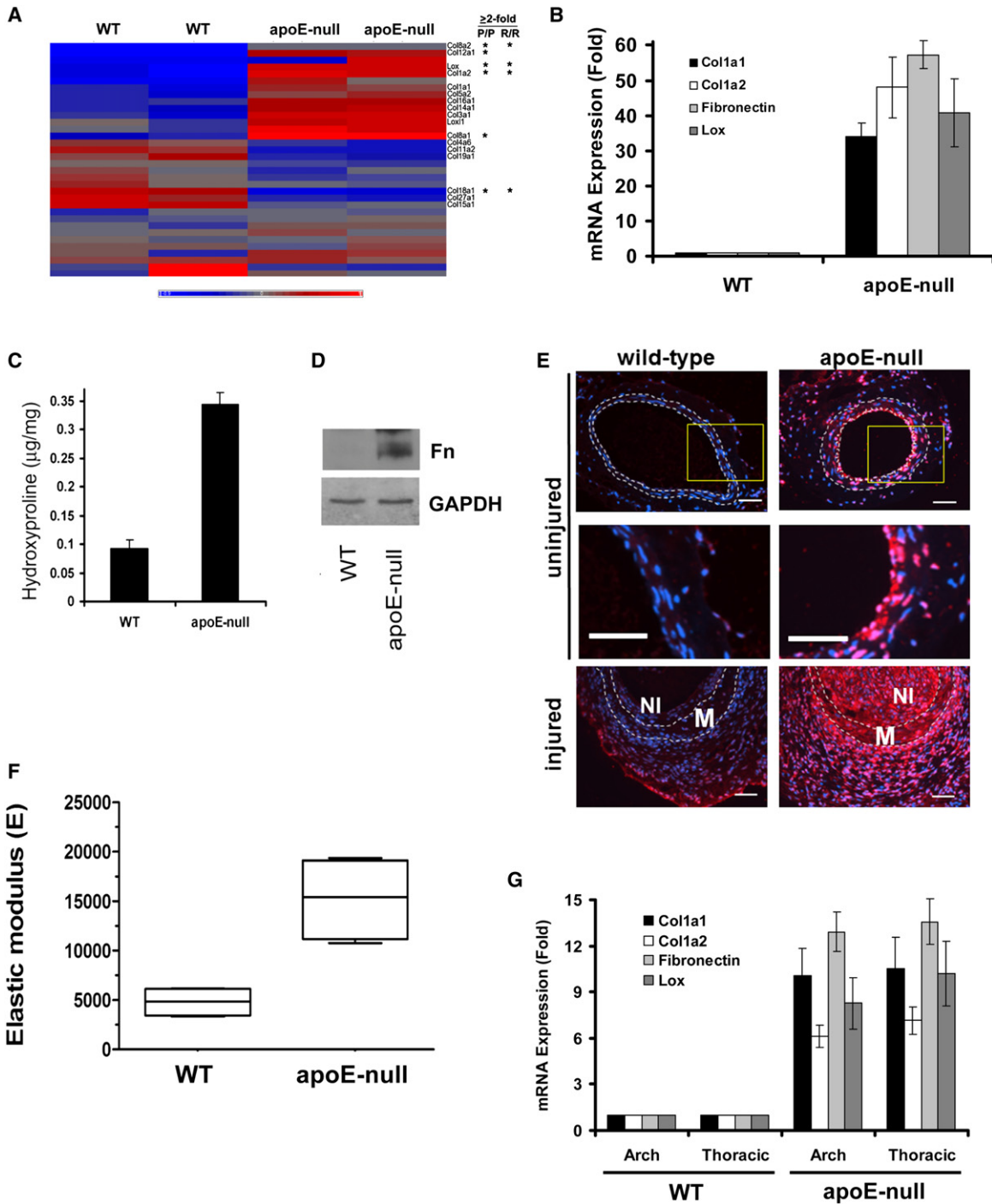


Figure 1. Altered ECM Gene Expression in ApoE Null Mice

(A) Heat map of collagen and Lox genes in WT and apoE null aortae. Duplicates represent Agilent dye-swaps. Asterisks indicate fold-change ≥ 2 in athero-prone (P/P) and resistant (R/R) regions. Scale: -0.9 to 1.0 .

(B) Cleaned aortae from 6-month-old male WT and apoE null mice analyzed by real-time qPCR. Results show mean \pm SE, $n = 4$. $p = 0.012$ by two-tailed t test.

(C and D) Cleaned aortae analyzed for hydroxyproline content (mean \pm SD, $n = 3$) or western blotted for FN, respectively.

(E) Lox immunostaining (red) of uninjured and injured femoral arteries. DAPI-stained nuclei are shown in blue. Dashed lines show the IEL and EEL. M, media; NI, neointima. Middle panels show enlargements of boxed regions. Scale bar represents $50 \mu\text{m}$.

(F) Thoracic aortae of four 6-month-old WT and four apoE null mice were cleaned of adventitia, opened longitudinally, and analyzed by AFM, indenting into the luminal surface at > 20 distinct nonlesioned locations. The mean elastic modulus was calculated for each mouse and graphed as a Tukey box and whisker plot; $p = 0.029$ by two-tailed Mann-Whitney test.

(apoE-HDL). ApoE-HDL has a well-established role in removing cholesterol from peripheral cells and delivering it to the liver in a process called reverse cholesterol transport, but several reports using cultured cells have indicated that the effects of apoE extend beyond regulation of plasma lipid levels (Ishigami et al., 1998, 2000; Kothapalli et al., 2004; Swertfeger and Hui, 2001; Symmons et al., 1994). Early in vivo studies even suggested that a lipid-independent effect of apoE could protect against atherosclerosis (Thorngate et al., 2003), though the basis for this observation has remained elusive. We now show that the inhibitory effect of apoE-HDL on ECM gene expression and arterial stiffening is cholesterol-independent and sufficient to attenuate atherosclerosis. Thus, in addition to its established effect on reverse cholesterol transport, HDL contributes to healthy arterial biomechanics, and this effect is causal for cardiovascular protection.

RESULTS

ECM Gene Expression Suppressed by ApoE and HDL

We interrogated GEO data set GSE13865 that transcript profiled atherosclerosis-prone and atherosclerosis-resistant regions of 4-month-old wild-type (WT) and apoE null mouse aortae (Figure 1A). Genes that were differentially expressed in the athero-prone regions were identified and subjected to enrichment analysis against the Gene Ontology (GO) database. This analysis ranked “Extracellular Region” (GO: 0005576) and “Extracellular Region Part” (GO: 0044421) as the most enriched within the “Cellular Component” (GO: 0005575) functional grouping (Figure S1A). Within the “Extracellular Region Part,” two functional groups were highly enriched: the “Extracellular Matrix” (GO: 0031012) and “Plasma Lipoprotein Particles” (Figure S1B), the latter of which was expected given the deletion of apoE. Several collagen genes, including the highly expressed type I collagen, were differentially expressed in apoE null aortae (Table S1A; Figure 1A, asterisks).

The effect of apoE knockout on several collagen mRNAs was confirmed by real-time quantitative PCR (qPCR) (Figure 1B; Table S1B). Real-time qPCR also revealed an apoE-dependent regulation of fibronectin (FN) mRNA that was not detected by transcript profiling (Figure 1B). Collagen protein (measured as hydroxyproline content) and FN protein levels were increased in the aortae of apoE null mice as compared to WT controls (Figures 1C and 1D, respectively). Similarly, increased collagen-I protein was readily detected in the media and neointima of immunostained aortic root sections from apoE null, but not WT, mice (Figure S1C). In contrast, elastin mRNA levels were not strongly affected by deletion of apoE (Figure S1D; Tables S1A and S1C).

Lysyl oxidase (Lox) crosslinks adjacent collagen triple helices and confers tensile strength to the collagen fibril (Csiszar, 2001; Kagan and Li, 2003). Our transcript profiling analysis revealed

an increase in the expression of Lox mRNA in apoE null arteries as compared to WT (Figure 1A, asterisk). Lox mRNA and protein induction in apoE null arteries was confirmed by real-time qPCR and immunostaining (Figures 1B and 1E, respectively; Table S1A). Note the increased Lox protein in apoE null versus WT arteries (Figure 1E, top and middle panels), and even greater increased expression in the media and neointima of apoE null mice after fine wire femoral artery injury (Figure 1E, bottom panels). In contrast, the gene expression of procollagen-lysine,2-oxoglutarate 5-dioxygenase 3 (PLOD3), which catalyzes the hydroxylation of lysine residues in collagens, was similar in WT and apoE null arteries (Figure S1E). Upregulation of collagen-I protein and enhanced crosslinking by elevated Lox expression has the potential to increase tissue stiffness; indeed, atomic force microscopy (AFM) in force mode showed an increase in the median elastic modulus of apoE null femoral arteries as compared to WT controls (Figure 1F). Further studies focused on collagen-I and Lox, which regulate tensile strength and tissue elasticity, and FN, which interacts with collagen functionally and has been associated with stiffness-dependent cell proliferation (Brüel et al., 1998; Kadler et al., 2008; Kagan and Li, 2003; Klein et al., 2009).

Deletion of apoE in mice leads to hyperlipidemia and spontaneous atherosclerosis as well as an exaggerated response to vascular injury (Ali et al., 2007; Matter et al., 2006; Piedrahita et al., 1992; Plump et al., 1992). Since VSMCs dedifferentiate at sites of injury and atherosclerotic lesion formation and begin to produce relatively large amounts of ECM, we considered the possibility that the matrix-regulatory effects detected in apoE null mice were secondary to lesion formation. However, several lines of evidence collectively indicated that the changes we observed in collagen-I, FN, and Lox gene expression were not merely consequences of disease. First, we could restore apoE expression in the liver of young apoE null mice by infection with AAV-apoE3 (Kitajima et al., 2006), and this resulted in near-WT levels of Col1a1, Col1a2, FN, and Lox gene expression quickly (within 2 weeks; compare Figures S1F and S1G to S1H and S1I). Second, the Col1a2, FN, and Lox genes were similarly upregulated in athero-resistant as well as athero-prone regions of the apoE null aortae as compared to WT (Figure 1A, asterisks, and Figure 1G; Table S1C). Third, purified apoE3, at a physiological concentration (Wientgen et al., 2004), reduced levels of Col1a1, Col1a2, FN, and Lox protein (Figures 2A and 2B) and mRNAs (Figures 2C–2F) in cultured VSMCs: these effects were specific to dedifferentiated VSMCs (Figure 2B) and dose and time dependent (Figures S2A–S2D). We conclude that apoE has a primary suppressive effect on expression of the VSMC collagen-I, FN, and Lox genes. A similar pattern of inhibition was seen in human VSMCs but not human aortic endothelial cells (Figures S2E and S2F).

The majority of apoE circulates as a component of triglyceride-rich lipoproteins and HDL, but apoE is not present in

(G) Cleaned aortae from 6-month-old male WT and apoE null mice were divided into arch (ascending) and thoracic (descending) regions. RNA was isolated from the aortae and analyzed by real-time qPCR for collagen-I, FN, and Lox gene expression. Results are normalized to 18S rRNA. Results show mean \pm SE, $n = 5$. $p = 0.036$ by two-tailed t test.

See also Figure S1 and Tables S1A–S1C.

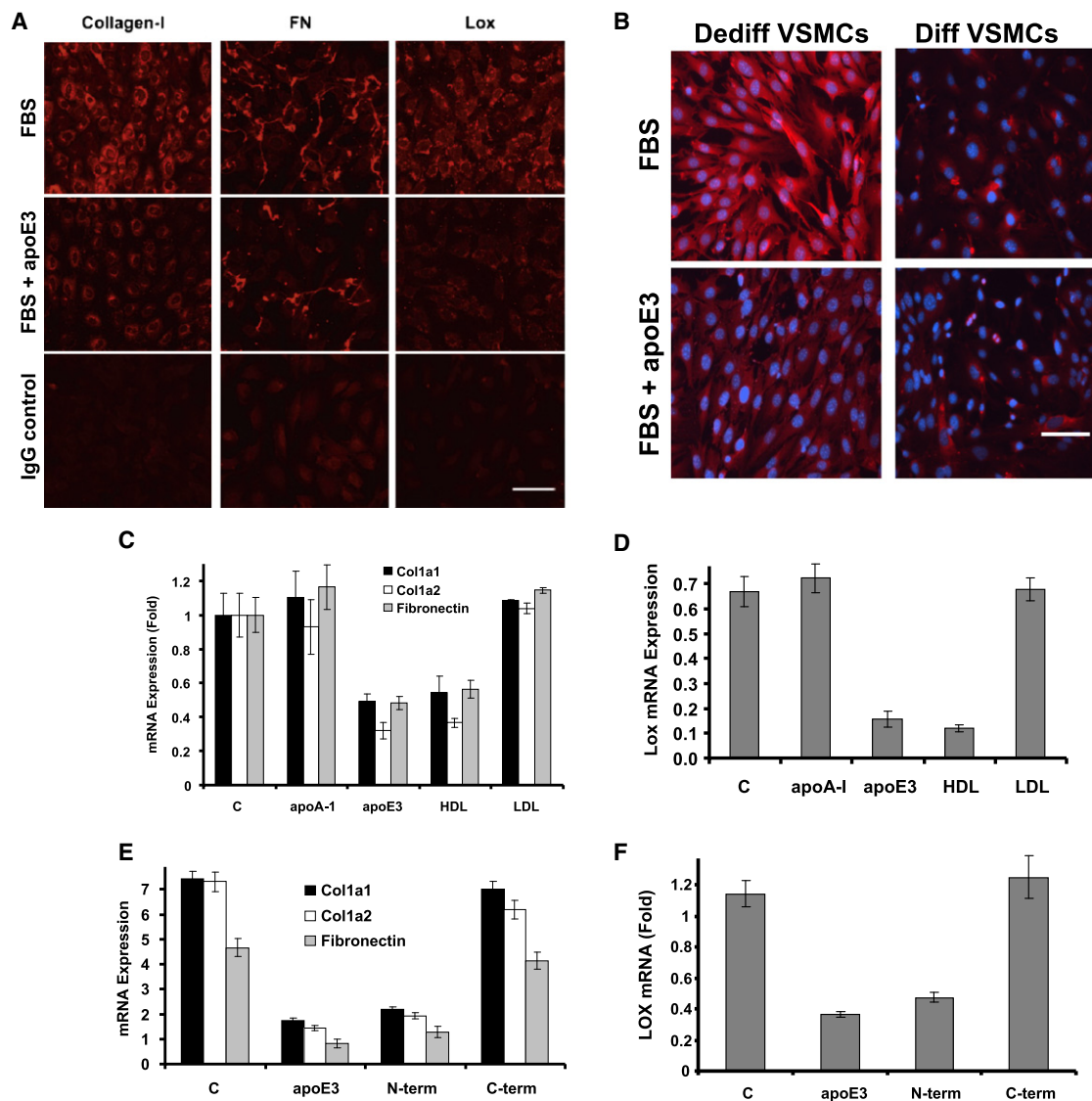


Figure 2. ApoE and HDL Inhibit ECM Expression in Dedifferentiated VSMCs

(A) Serum-starved VSMCs isolated from WT mice were incubated with 10% FBS in the absence (control; C) or presence of 2 μ M apoE3 for 24 hr. The cells were fixed and stained for collagen-I, FN, or Lox and visualized by immunofluorescence microscopy. Scale bar represents 50 μ m. The IgG control used FBS-stimulated cells.

(B) VSMCs were isolated from C57BL/6 aortae by explant cultures (dedifferentiated phenotype) or by collagenase digestion (differentiated phenotype). Asynchronous cells of each phenotype were incubated with 10% FBS in the absence (control, C) or presence of 2 μ M apoE3 for 24 hr and then stained for collagen-I (red). Blue: DAPI-stained nuclei. Scale bar represents 50 μ m. The figure shows a representative result.

(C and D) Serum-starved VSMCs isolated from WT mice were incubated with 10% FBS in the absence (control, C) or presence of 2 μ M apoA-I or apoE3 or 50 μ g/ml HDL or LDL for 24 hr. RNA levels were determined by real-time qPCR.

(E and F) The experiment in (C) and (D) was repeated except we compared the effects of 2 μ M apoE3 to its N- and C-terminal fragments. Real-time qPCR results show mean \pm SD of duplicate PCR reactions and are representative of at least three independent experiments.

See also Figure S2.

low-density lipoprotein (LDL). We therefore compared HDL and LDL for their abilities to regulate matrix protein gene expression in primary mouse VSMCs. HDL efficiently suppressed collagen-1, FN, and Lox gene expression, while LDL failed to inhibit these genes (Figures 2C and 2D). ApoA-I, the major apolipoprotein in HDL, was unable to decrease expression of

these matrix genes (Figures 2C and 2D), nor was HDL after depletion of apoE (Figure S2G). All three isoforms of human apoE inhibited ECM gene expression (Figure S2H). Thus, suppression of collagen-I, FN, and Lox gene expression is a selective property of apoE and apoE-containing HDL rather than a general property of apolipoproteins and lipoproteins.

ApoE3 consists of a 22 kDa N-terminal domain that binds to the LDL receptor and a 10 kDa C-terminal domain required for lipid binding and regulation of reverse cholesterol transport (Weisgraber, 1994). Expression of Col1a1, Col1a2, FN, and Lox mRNAs were all repressed by the N-terminal domain but not by the C-terminal lipid-binding domain of apoE3 (Figures 2E and 2F).

Mechanosensitive Collagen-I and Fibronectin Gene Expression Regulated by Cox2 and Circumvented by ApoE

Collagen-I expression correlates directly with ECM stiffness in fibroblasts (Liu et al., 2010). We therefore asked if the effects of apoE on ECM gene expression might be affected by substratum stiffness itself. VSMCs were cultured on biocompatible FN-coated polyacrylamide hydrogels prepared with elastic moduli that span the stiffness range of healthy and diseased arteries (~2,000 and 25,000 Pa; Klein et al., 2009; hereafter called low and high stiffness, respectively). The expression of Col1a1, Col1a2, FN, and Lox mRNAs (Figures 3A and 3B) as well as Lox enzymatic activity (Figure S3A) were all positively regulated by substratum stiffness, and this stiffness-dependent upregulation was blocked by apoE3 (Figures 3A, 3B, and S3A). We conclude that apoE is an inhibitor of mechanosensitive ECM gene expression.

Collagen-I gene expression is inversely proportional to the expression of Cox2 in fibroblasts (Liu et al., 2010) and VSMCs (Figures 3A versus 3E; Figures S2E versus S2F). Previously, we reported that apoE3 and its N-terminal domain stimulate the expression of Cox2 mRNA and protein in VSMCs (Ali et al., 2008; Kothapalli et al., 2004). Consistent with these results, the Cox2 inhibitor, nimesulide, prevented suppression of the collagen-I and FN genes by apoE3 in VSMCs whereas the Cox1 inhibitor, SC560, was without effect (Figure 3C). Cox2 production in VSMCs leads to the production of PGI₂, and VSMCs treated with apoE3 have increased amounts of PGI₂ in their conditioned medium (Ali et al., 2008; Kothapalli et al., 2004). The PGI₂ mimetic, cicaprost, phenocopied the effect of apoE3 on collagen-I and FN mRNAs (Figures 3D, S3C, and S3D), and these effects of apoE3 and PGI₂ are linked mechanistically because deletion of the PGI₂ receptor, called IP, prevented suppression of Col1a1, Col1a2, and FN mRNAs by apoE (Figures 3D, S3C, and S3D). Thus, regulation of VSMC collagen-I and FN gene expression by apoE is mediated by the Cox2-PGI₂-IP signaling pathway.

If Cox2 is causally linked to the effect of apoE3 on mechanically driven collagen-I and FN gene expression, then its response to apoE3 should be mechanosensitive. Indeed, we found that Cox2 levels (Figure 3E) and activity (Figure S3B) decline when VSMCs are cultured at a high stiffness characteristic of vascular lesions. ApoE3 prevents this downregulation and maintains elevated Cox2 gene expression (Figure 3E) and enzymatic activity (Figure S3B) despite substrate stiffening. Moreover, the apoE3 effect on mechanosensitive Cox2 expression can explain the suppressive effects of apoE3 on collagen-I and FN gene expression because (1) Cox2 inhibition with nimesulide was sufficient to increase levels of collagen-I and FN mRNAs in cells cultured on a low stiffness substratum (Figure 3F),

and (2) ectopic expression of Cox2 was sufficient to reduce levels of collagen-I and FN mRNAs in VSMCs cultured on a rigid substratum (Figure 3G). Thus, the downregulation of Cox2 on a stiff substratum leads to increased collagen-I and FN gene expression in VSMCs, and apoE limits synthesis of these ECM proteins by preventing the stiffness-dependent downregulation of Cox2 (Figure 3H). Similar results were seen with collagen-coated hydrogels (Figures S3E and S3F), indicating that the effects were stiffness-specific rather than matrix-protein specific.

We considered the possibility that the suppressive effects of apoE3 and/or a soft substratum on ECM mRNAs might be due to increased VSMC differentiation because ECM synthesis is minimal in differentiated (also called “contractile”) VSMCs (Owens et al., 2004; Thyberg et al., 1990, 1997). However, three different markers of differentiated VSMCs were slightly decreased, rather than increased, in response to a low-stiffness substratum (Figure S3G) and apoE3 had no effect on SM-marker expression regardless of substratum stiffness (Figure S3G). Thus, apoE3 directly controls mechanosensitive ECM production in dedifferentiated VSMCs rather than indirectly inhibiting ECM synthesis downstream of a primary effect on VSMC differentiation.

miR-145 Mediates the Suppressive Effect of ApoE on Lox Gene Expression

In contrast to collagen-I and FN, ectopic expression of Cox2 on a rigid substratum (Figure 3G) or Cox2 inhibition on a soft substratum (Figure S4A) failed to alter Lox gene expression. Deletion of IP (Figure S4B) or inhibition of Cox2 also failed to overcome the inhibitory effect of apoE3 on Lox gene expression (Figure S4C) or enzymatic activity (Figure S4D). Thus, the effect of apoE3 on mechanosensitive Lox gene expression is Cox2 independent. We used a genome-wide approach to identify potential upstream regulators of Lox gene expression in vivo. We injured the femoral arteries of smooth muscle actin (SMA)-GFP transgenic mice, a line in which GFP levels are controlled by the α -SMA promoter (Yokota et al., 2006). Injury-induced VSMC proliferation can be visualized in this mouse line by the loss of GFP fluorescence because the SMA promoter is not expressed in proliferating (dedifferentiated) VSMCs (Klein et al., 2009). We microdissected these GFP-negative femoral artery regions and determined global mRNA expression patterns relative to uninjured contralateral controls. Differentially expressed mRNAs and miRNAs were superimposed on the Ingenuity database of molecules experimentally demonstrated or highly predicted to regulate Lox. This analysis identified miR-145 as a putative direct upstream Lox mRNA regulator (Figure 4A, box).

miR-145 levels were strongly reduced in apoE null arteries as compared to WT (Figure 4B), consistent with upregulation of Lox mRNA seen under the same conditions (refer to Figure 1B). Moreover, apoE3 increased miR-145 in cultured VSMCs (Figure 4C), consistent with the downregulation of Lox mRNA (refer to Figure 2D). This inverse relationship between miR-145 and Lox mRNA is causal, because ectopic miR-145 expression reduced Lox mRNA levels whereas an anti-miR-145 blocked the upregulation of Lox mRNA in response to apoE3 (Figure 4D).

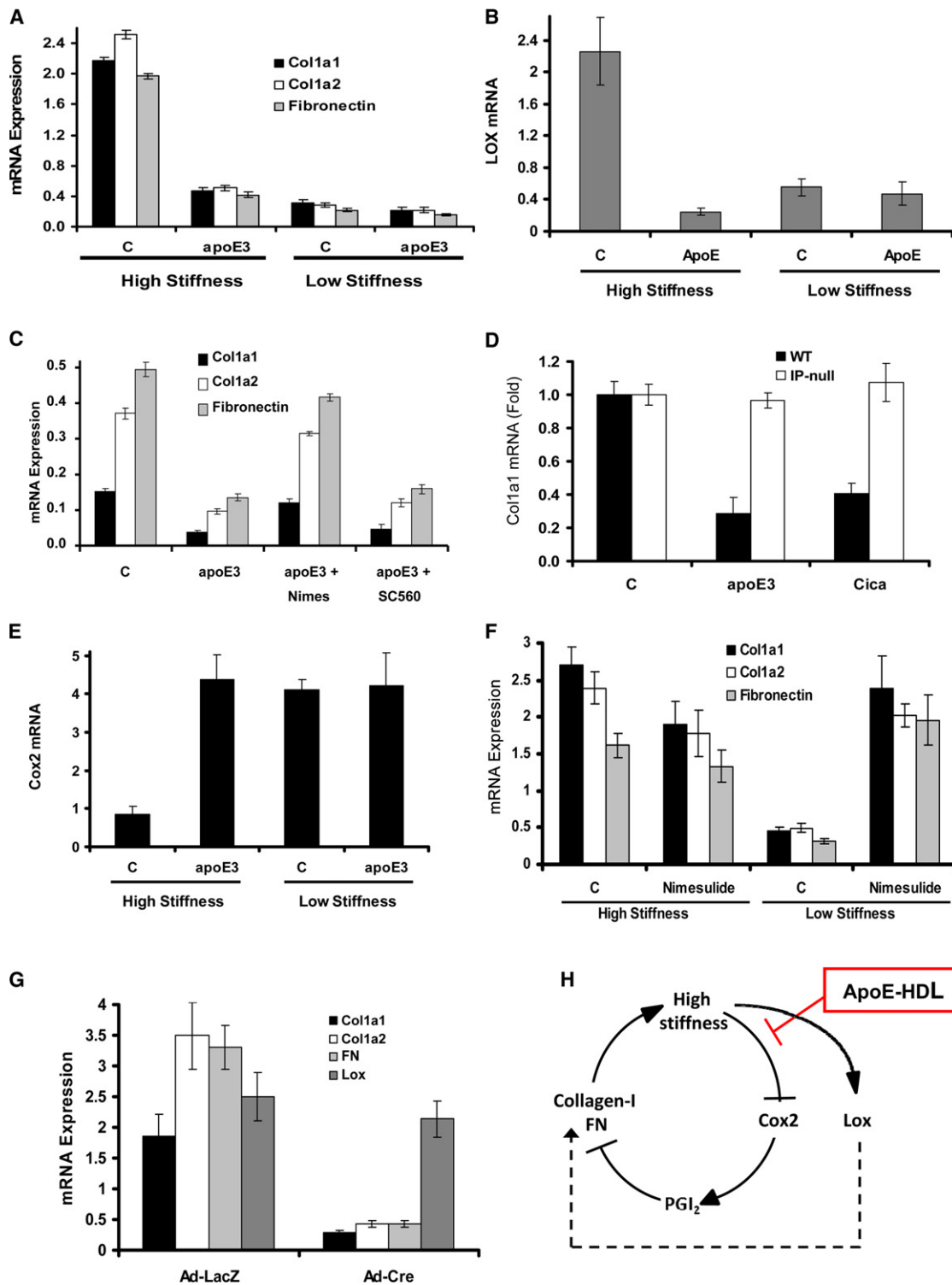


Figure 3. Mechanosensitive Gene Expression Regulated by ApoE

For all panels, Col1a1, Col1a2, FN, Lox, or Cox2 mRNAs were quantified by real-time qPCR and plotted relative to 18S rRNA. For panels (A)–(G), results show mean \pm SD of duplicate PCR reactions and are representative of at least three independent experiments.

(A and B) Serum-starved primary mouse VSMCs were incubated with 10% FBS in the absence (control, C) or presence of 2 μ M apoE3 on high stiffness or low stiffness FN-coated hydrogels for 24 hr.

(C) The experiment in (A) and (B) was repeated except that apoE-treated VSMCs on plastic were given 1 μ M nimesulide (Nimes; Cox2 inhibitor) or 1 mM SC560 (Cox1 inhibitor).

Neither collagen-I nor FN mRNA levels were affected by ectopic expression or inhibition of miR-145 (Figure 4E and SF4E). Thus, miR-145 induction selectively transduces the apoE3 signal to repress Lox mRNA. The combined effects of apoE3 on Cox2 and miR-145 account for its regulation of the collagen-I, FN, and Lox genes (Figure 4F).

Inhibition of Arterial Stiffening Reduces Atherosclerosis

If suppression of ECM expression and arterial stiffening contribute to the cardiovascular protective effect of apoE, then a blockade of arterial stiffening should reduce atherosclerosis in apoE null mice. ApoE null mice on a high-fat diet were treated with the selective lysyl oxidase inhibitor, BAPN (Brüel et al., 1998; Kagan and Li, 2003; Tang et al., 1983). The lysyl oxidase family crosslinks collagen fibers and confers its tensile properties (Brasselet et al., 2005; Brüel et al., 1998; Wells, 2008). Lysyl oxidases also crosslink elastin, but several studies have shown that the net effect of BAPN treatment has been a reduction in tissue stiffness (Brüel et al., 1998). Blood pressure is not affected by BAPN (Berry et al., 1981; lwatsuki et al., 1977; Kanematsu et al., 2010).

We treated apoE null mice on a high-fat diet with vehicle or BAPN. BAPN led to a notable reduction in arterial stiffness as compared to vehicle-treated controls (Figure 5A). BAPN also inhibited the development of atherosclerosis as determined by Oil Red O-staining of isolated aortae (Figures 5B and 5C). Moreover, the degree to which lesion formation was reduced (~50%) agreed reasonably well with the degree to which BAPN reduced arterial stiffness (Figure 5A). These inhibitory effects occurred despite the extraordinary high plasma cholesterol levels seen in apoE null mice on a high-fat diet (Figure 5D). Body weight was unaffected by BAPN (Figure 5E). Thus, the inhibitory effect of apoE on arterial stiffness confers protection against atherosclerosis, and pharmacologic regulation of arterial biomechanics can attenuate disease even when plasma cholesterol remains extremely high.

BAPN also reduced lesion area in aortic roots (Figures 6A and 6B), and the magnitude of the effect was similar to what we observed in the thoracic aorta. We then analyzed the composition of these aortic root lesions. Second harmonic generation two-photon microscopy revealed a clear reduction in highly structured collagen within the neointimas of lesions from the BAPN-treated mice (Figure 6C, top panel; Figure 6D, left) whereas total collagen-I levels were minimally affected (Figure 6C, bottom panels; Figure 6D, right). We observed chimeric staining patterns for α -SMA (a marker of differentiated VSMCs) in lesions regardless of BAPN treatment (Figures 7A and 7B). However, CD68-staining revealed that macrophage abundance in lesions was markedly reduced in the BAPN-treated mice

(Figures 7A and 7B). We then prepared ECM-coated hydrogels at the stiffness of healthy and diseased arteries to determine if the adhesion of THP-1 monocytes (Figure 7C) or primary mouse thioglycollate-elicited peritoneal macrophages (Figure 7D) was affected by substratum stiffness. Although the extent of cell adhesion depended on the subendothelial ECM protein used to prepare the hydrogel, in every case cell adhesion to ECM was strongly reduced when we softened the ECM from the high stiffness of lesions to the low stiffness of healthy arteries (Figures 7C and 7D). Thus, the effect of BAPN on macrophage abundance in vivo can be phenocopied by direct manipulation of ECM stiffness in vitro. Collectively, these results reveal a specific aspect of atherogenesis affected by arterial stiffness and connect stromal ECM production and arterial stiffening to the inflammatory component of lesion development.

DISCUSSION

Our results identify apoE and apoE-containing HDL as negative regulators of ECM gene expression and arterial stiffening. This effect does not require the apoE lipid binding domain and confers cardiovascular protection independent of plasma cholesterol levels. We detected the suppressive effect of apoE on collagen-I, FN, and Lox expression selectively in dedifferentiated VSMCs. This result suggests that apoE-HDL does not affect homeostatic arterial stiffness in contractile VSMCs, but rather acts when dedifferentiated VSMCs begin to synthesize ECM and stiffen their microenvironment during the onset of CVD.

Mechanosensitive Regulation of Cox2 and Lox Expression Opposed by ApoE-HDL

Our initial transcript profiling results, as well similar results by others (Hui and Basford, 2005), suggested that apoE would be an overall inhibitor of ECM gene expression. However, a more detailed analysis of apoE action on deformable substrata indicated that apoE-HDL actually has a much more subtle role, and that it selectively interferes with the increase in collagen-I, FN, and Lox gene expression that occurs in response to substratum stiffening (Figure 4F). These results suggest the existence of a mechanically sensitive feed-forward loop that can accelerate ECM synthesis, matrix remodeling, and arterial stiffening (Figure 4F). By short-circuiting this loop, apoE-HDL would restrain stiffening during the progression of atherosclerosis.

Cox2 regulates the production of PGI₂ in VSMCs, and PGI₂ inhibits collagen-I and FN gene expression. Moreover, Cox2 gene expression is mechanosensitive, with efficient expression seen only when VSMCs are on soft surfaces characteristic of normal arteries. We posit that the inverse relationship between ECM stiffness and Cox2 expression represses collagen-I/FN

(D) The experiment in (C) was repeated using WT and IP null VSMCs treated with 2 μ M apoE3 or 200 nM cicaprost (Cica; stable PGI₂ mimetic).

(E) The experiment in (A) was analyzed for Cox2 mRNA.

(F) Serum-starved primary mouse VSMCs were incubated with 10% FBS in the absence (control, C) or presence of 1 μ M nimesulide on high stiffness or low stiffness FN-coated hydrogels for 24 hr.

(G) Primary VSMCs were isolated from mice expressing Cre-dependent Cox2. The cells were seeded overnight on FN-coated coverslips, serum-starved, infected with adenoviruses encoding LacZ or Cre, and directly stimulated with 10% FBS for 24 hr.

(H) Model of the stiffness- and apoE-regulatory effects on Cox2, collagen-I, FN, and Lox expression.

See also Figure S3.

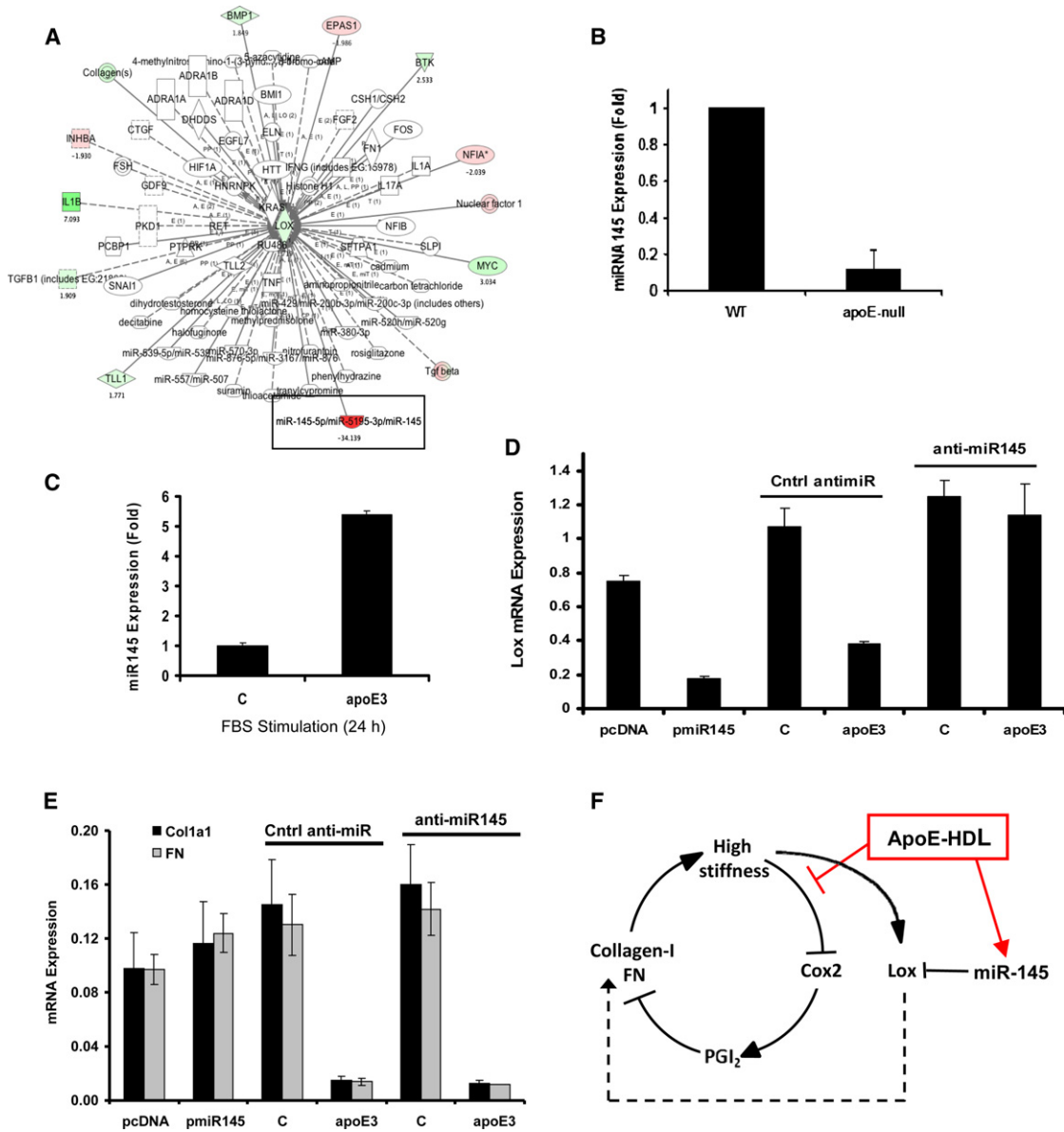


Figure 4. miR145-Dependent Lox Gene Expression by ApoE

(A) Upstream regulators of Lox showing differential gene expression after vascular injury as determined by Ingenuity Pathway Analysis. Green and red represent induction and repression, respectively.

(B) Aortae from 6-month-old WT or apoE null mice were harvested and analyzed by real-time qPCR for miR-145. Results show mean \pm SE, $n = 4$, $p = 0.0002$ by two-tailed t test.

(C) Serum-starved primary mouse VSMCs were incubated with 10% FBS in the absence (control, C) or presence of 2 μ M apoE for 24 hr. RNA was isolated and analyzed by real-time qPCR for miR-145.

(D and E) VSMCs were transfected with pmiR-145 or anti-miR-145, serum starved for 48 hr and stimulated with 10% FBS in the absence (control, C) or presence of 2 μ M apoE3 for 24 hr. RNA was isolated and analyzed by real-time qPCR for Lox, Col1a1, and FN.

(F) Regulation of collagen-I, FN, and Lox gene expression by apoE through Cox2 and miR-145.

In (C–E), results show mean \pm SD of duplicate PCR reactions and are representative of at least three independent experiments.

See also Figure S4.

synthesis and contributes to healthy compliance in healthy vessels (Figure 4F). Upon an atherogenic insult, however, the efficacy of this mechanism is reduced as dedifferentiated VSMCs accumulate and ECM production increases. ApoE-HDL helps to maintain negative feedback on collagen-I and FN

synthesis by circumventing mechanical control of Cox2 gene expression (Figure 4F). In apoE null mice, and perhaps in humans with insufficient apoE-HDL, the stiffening-dependent loss of Cox2 would proceed unabated and exacerbate ECM synthesis, arterial stiffening, and lesion development. Since Cox2 limits

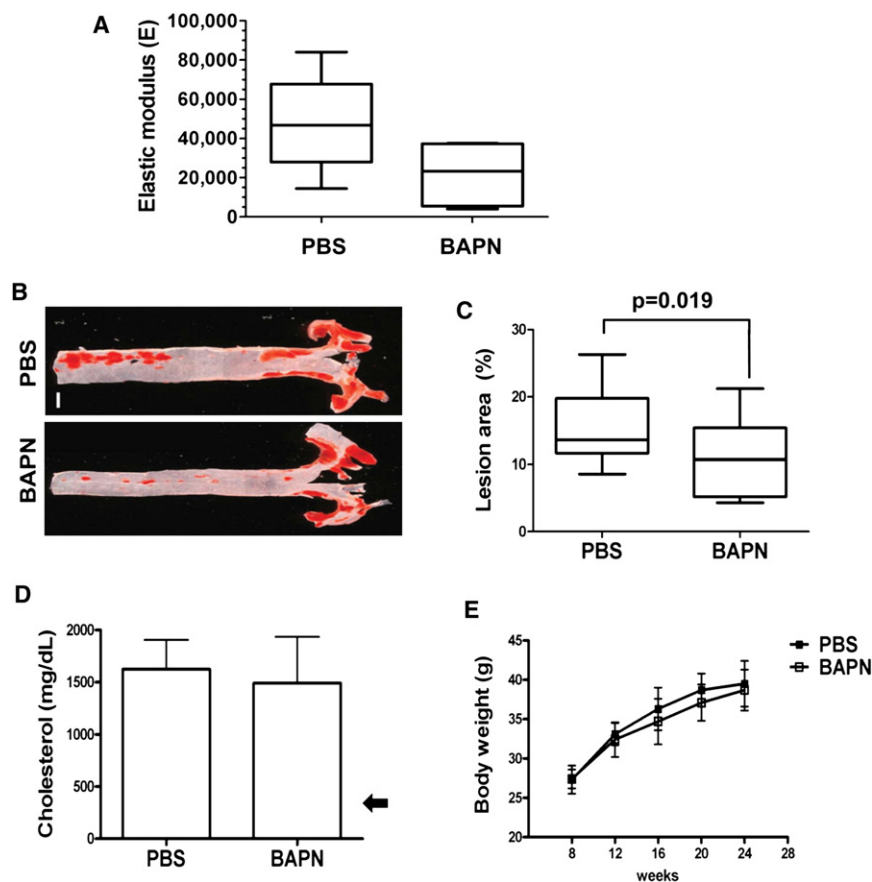


Figure 5. Reduced Atherosclerosis in ApoE Null Aortae Softened In Vivo with BAPN

(A) AFM of aortae from ~2-month-old old apoE null mice fed a high-fat diet and treated with vehicle (PBS; $n = 5$) or BAPN ($n = 4$) for 16 weeks. Data are presented as a Tukey box and whisker plot with a 1-tailed Mann-Whitney test for softening by BAPN; $p = 0.032$.

(B and C) Oil red O staining and lesion quantification in aortae of apoE null mice treated with PBS ($n = 17$) or BAPN ($n = 15$). Data are presented as Tukey box and whisker plots; $p = 0.019$ by two-tailed Mann-Whitney test. Scale bar represents 1 mm.

(D) Plasma cholesterol levels (mean \pm SD) in PBS ($n = 10$) and BAPN ($n = 9$) treated mice measured at the time of sacrifice. The arrow shows the cholesterol level in wild-type mice on a western diet (Nakashima et al., 2004).

(E) Mice treated with PBS ($n = 17$) or BAPN ($n = 17$) from both groups were weighed every 4 weeks until sacrifice. $p > 0.05$ by two-way ANOVA. Results show mean \pm SD.

collagen-I expression, an increase in arterial stiffness may be a contributing factor to the cardiovascular hazards associated with chronic use of selective Cox2 inhibitors (FitzGerald, 2003).

ApoE also suppresses Lox expression in VSMCs. The effect of apoE on Lox is independent of Cox2 but strongly dependent on the upregulation of miR-145. It is not yet known if apoE-HDL signaling to miR-145 and Cox2 involves distinct apoE receptors or divergent signaling downstream of a single receptor. The best-studied apoE receptors are LDL receptor (LDLR), LRP, and heparin sulfate proteoglycan (Boucher et al., 2003; Nimph and Schneider, 2000; Strickland et al., 2002). Our previous report indicated that Cox2 induction by apoE is likely independent of these receptors (Ali et al., 2008). Downregulation of miR-145 and its bicistronic partner, miR-143, has been implicated in the phenotypic switch of VSMCs from contractile (differentiated) to synthetic (dedifferentiated) states (Boettger et al., 2009; Cordes et al., 2009). Consistent with these reports, we saw a large decrease in miR-145 at sites of vascular injury, a setting in which VSMC dedifferentiation is occurring. However, apoE3 strongly increases miR-145 but does not affect smooth muscle differentiation as judged by our marker analysis. Thus, miR-145 downregulation may contribute to the dedifferentiation of VSMCs, but its upregulation is insufficient for VSMC differentiation. The collective work of others (Boettger et al., 2009; Cordes et al., 2009; Xin et al., 2009) also suggests a complex and incompletely understood role for miR-145 in VSMC differentiation.

movement of excess cholesterol from peripheral tissues back to the liver for excretion from the body (Mahley et al., 2006; Rothblat and Phillips, 2010). However, two major studies have recently questioned the importance of high HDL levels in cardiovascular protection. One clinical study, AIM-HIGH, increased HDL in response to slow-delivery niacin yet failed to show reduced cardiovascular risk (Nicholls, 2012; Nofer, 2012). Moreover, a large-scale mendelian randomization analysis found that increases in plasma HDL are not sufficient to reduce the risk of myocardial infarction (Voight et al., 2012). Our data may help to reconcile these conflicting reports because apoE-HDL is relatively rare, comprising only ~6% of the total HDL protein (Weisgraber and Mahley, 1980). Cardiovascular protective effects associated with elevated apoE-HDL may escape detection in clinical and population genetics analyses that rely on changes in total plasma HDL levels.

Arterial Biomechanics and CVD

Atherosclerosis develops focally at sites of disturbed flow (Davies, 2009; Garcia-Cardena and Gimbrone, 2006; Gimbrone et al., 2000). In this context, we note that Cox2 production by endothelial cells is increased at sites of disturbed flow (Dai et al., 2004), and that PGI₂ is an important Cox2 product of endothelial cells (Fitzgerald et al., 1987; Narumiya et al., 1999) as it is in VSMCs. Thus, disturbed flow should enhance PGI₂ production focally, which could then act in a paracrine

Implications for HDL Biology

Many studies have concluded that HDL protects against cardiovascular disease, and pharmacological treatments and/or lifestyle changes that elevate HDL have become engrained into Western medical practice. Canonically, HDL is thought to confer cardiovascular protection by stimulating reverse cholesterol transport, the

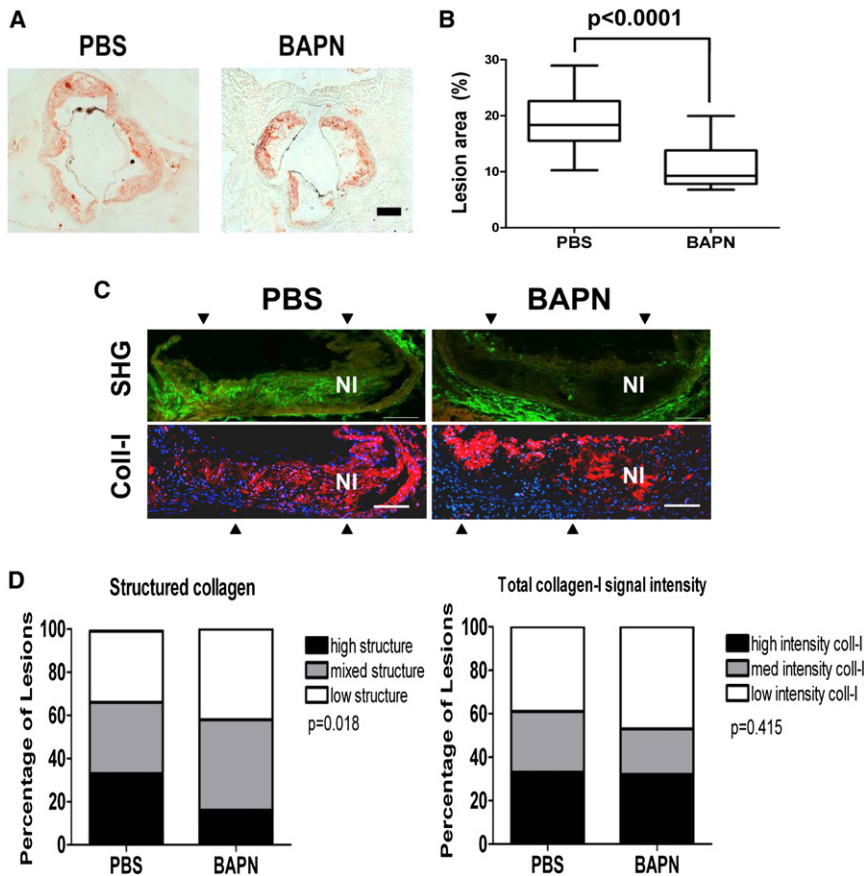


Figure 6. Reduced Collagen Structure and Macrophage Abundance in Atherosclerotic Lesions of BAPN-Treated Mice

(A and B) Oil red O staining and lesion quantification in aortic roots of apoE null mice treated with PBS (n = 15) or BAPN (n = 17). Data presented as Tukey box and whisker plots; $p < 0.0001$ by two-tailed Mann-Whitney test. Scale bar represents 200 μ m.

(C) Second harmonic generation detection of neo-intimal collagen. Top panels show composites of three serial second harmonic generation (SHG) images of an aortic root lesion from a PBS- or BAPN-treated mouse; collagen SHG signal and the elastin autofluorescence signal are pseudo-colored green and red, respectively. Bottom panels show composites of the same lesions stained for total collagen-I (red) and nuclei (DAPI; blue). Composite positions are indicated by arrowheads. Scale bar represents 100 μ m. NI, neo-intima.

(D) Double-blind quantitation of lesion images from mice treated with PBS (n = 18) and BAPN (n = 19). Statistical significance was determined by chi-square test.

manner to limit collagen-I and FN synthesis by proximal dedifferentiated VSMCs. While this effect should be atheroprotective, the fact that lesions prefer to form at sites of disturbed flow indicates that reduced collagen and FN expression are not sufficient to overcome other atherostimulatory effects of disturbed flow. Nevertheless, our data do support the notion that loss of this effect (e.g., in apoE null mice and perhaps humans with low apoE-HDL) likely contributes to arterial stiffening and lesion formation by reducing endothelial PGI₂ production. Arterial stiffness in vivo is regulated by vascular tone as well as ECM composition, and PGI₂ is a potent vasodilator (Fitzgerald et al., 1987; Narumiya et al., 1999). Tone and ECM composition may be linked through the Cox2-PGI₂ pathway.

Monocyte/macrophage infiltration into the vessel wall contributes profoundly to atherosclerosis because subendothelial macrophages develop into the lipid-laden foam cells that populate lesions. Circulating monocytes adhere to intimal endothelial cells and begin the process of macrophage differentiation and transmigration into the subendothelial space. Our in vivo studies show that arterial stiffness is critical to this process. Arterial softening with BAPN strongly reduces macrophage abundance in lesions, and our hydrogel experiments show that ECM softening inhibits the adhesion of monocytes and macrophages to FN and collagen-IV, components of the subendothelial ECM. Endothelial permeability is also sensitive to ECM stiffness (Huynh et al., 2011). These results raise the possibility

that pharmacological control of arterial stiffness may complement the effect of cholesterol-lowering drugs in treating CVD by regulating the inflammatory component of atherosclerosis. Although the extrapolation of results from mice to humans requires caution, our data fit

well with epidemiologic studies identifying arterial stiffness as a cholesterol-independent risk factor for a first cardiovascular event in humans (Duprez and Cohn, 2007; Mitchell et al., 2010).

EXPERIMENTAL PROCEDURES

Expression Profiling

Gene Expression Omnibus (GEO) data set GSE13865 was downloaded, processed, and analyzed using Partek Genomics Suite. The same software was used to determine differential gene expression in injured versus uninjured arteries of SMA-GFP mice. Genes differentially expressed at sites of injury were then analyzed using Ingenuity Pathway Analysis software (<http://www.ingenuity.com>). See Extended Experimental Procedures for details.

Cell Culture

Primary explant murine VSMCs were isolated from 10- to 12-week-old male C57BL/6 (WT), IP null, and conditional Cox2-expressing mice as described (Cuff et al., 2001). Near confluent monolayers were serum-starved for 48 hr before stimulation with 10% fetal bovine serum (FBS) with or without apolipoprotein, lipoprotein, or cicaprost. Fibronectin-coated hydrogels were prepared with elastic moduli that approximate the stiffness of healthy or diseased arteries (Klein et al., 2009). Differentiated murine aortic VSMCs and thioglycolate-elicited peritoneal macrophages were isolated similarly to described procedures (Golovina and Blaustein, 2006; Hodge-Dufour et al., 1997).

BAPN Treatment of ApoE Null Mice

The effect of BAPN on atherosclerosis was determined using 8-week-old males fed a high-fat diet and given PBS or BAPN for 16 weeks. Aortae were isolated from the heart to the diaphragm, and a small portion of the thoracic

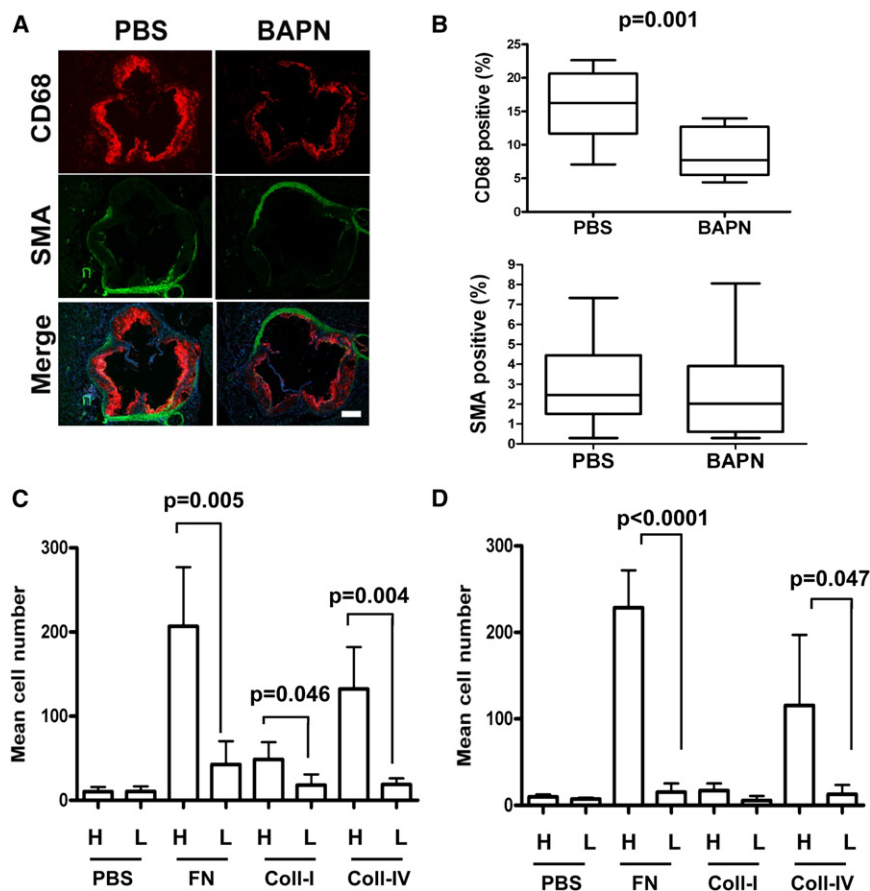


Figure 7. Matrix Stiffness Regulates Monocyte/Macrophage Adhesion to Substratum

(A) Aortic root lesions of apoE null mice treated with PBS (n = 15) or BAPN (n = 16) were stained with markers for macrophages (anti-CD68, red) and VSMCs (anti-SMA, green). Scale bar represents 200 μ m.

(B) Quantification of aortic root sections stained positive for CD68 (p = 0.001) and SMA (p = 0.36). Data are presented as Tukey box and whisker plots. The p values are from two-tailed Mann-Whitney tests.

(C and D) THP-1 monocytes (C) or primary murine thioglycollate-elicited peritoneal macrophages (D) were fluorescently labeled by incubation with calcein-AM and added to high-stiffness (H) or low-stiffness (L) ECM-coated hydrogels for 4 hr at 37°C. The total numbers of Calcein-AM labeled cells were counted in five randomly selected fields. Results show mean \pm SD; n = 4. The p values were determined by two-tailed t test.

aortae near the diaphragm was used to determine the elastic modulus. The remaining portions of aortae and the aortic roots were used to quantify atherosclerotic lesion formation by Oil-Red O staining. Sectioned aortic roots were also stained for CD68 and SMA. Blood was collected at the time of sacrifice for determination of total plasma cholesterol levels. See [Extended Experimental Procedures](#) for further details.

ACCESSION NUMBERS

The GEO accession number for the injury transcript profiling experiment reported in this paper is GSE40637.

SUPPLEMENTAL INFORMATION

Supplemental Information includes [Extended Experimental Procedures](#), four figures, and three tables and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2012.09.018>.

LICENSING INFORMATION

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