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Comparative analysis of endothelial cell and sub-endothelial cell elastic moduli in young and aged mice: Role of CD36.

Elizabeth Le Master^{1,2}, Ibra S. Fancher¹, James Lee², and Irena Levitan^{1,2}

¹Division of Pulmonary and Critical Care, Department of Medicine, University of Illinois at Chicago

²Bioengineering, University of Illinois at Chicago

Abstract

Objective: To perform comparative analysis of the role of scavenger receptor CD36 on endothelial vs. sub-endothelial elastic modulus (stiffness) in the aortas of young and aged mice.

Approaches and Results: Elastic moduli of endothelial and sub-endothelial layers of freshly isolated mouse aortas were quantified using atomic force microscopy. In young mice (4–6 months old), we found that while endothelial stiffness is markedly reduced in aortas of CD36^{-/-} mice, as compared to WT controls, no difference between CD36^{-/-} and WT aortas is observed in the stiffness of the sub-endothelial layer in denuded arteries. Additionally, inhibition of myosin phosphorylation also decreases the elastic modulus in the EC, but not the sub-EC layer in WT mice. Moreover, inhibiting CD36 mediated uptake of oxLDL in intact WT aortas abrogated oxLDL-induced endothelial stiffening. Further analysis of aged mice (22–25 months) revealed that aging resulted not only in significant stiffening of the denuded arteries, as was previously known, but also a comparable increase in the elastic modulus of the endothelial layer. Most significantly, this stiffening in the EC layer is dependent on CD36, whereas the denuded layer is not affected

Conclusions: Our results show that the role CD36 in stiffening of cellular components of intact aortas is endothelial-specific and that genetic deficiency of CD36 protects against endothelial stiffening in aged mice. Moreover, these data suggest that endothelial stiffness in intact mouse aortas depends more on the expression of CD36 than on the stiffness of the sub-endothelial layer.

Keywords

endothelial and sub-endothelial stiffness; CD36

Corresponding Author: Irena Levitan, Department of Medicine, University of Illinois at Chicago, 840 South Wood Street, Chicago, IL, 60612-7323, Phone: 312-996-0441, Fax: 312-996-4665, levitan@uic.edu.

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Disclosures
None declared.

Introduction

Endothelial stiffness has emerged as an important factor in guiding endothelial-leukocyte interactions (Martinelli et al., 2014; Schaefer & Hordijk, 2015; Schaefer et al., 2014), a key step in inflammation and endothelial dysfunction. Importantly, endothelial stiffness is critically distinct from a more thoroughly studied stiffness of the vascular wall, usually termed “vascular stiffness”. The stiffness of the arterial vascular wall reflects primarily the stiffness of the extracellular matrix and the multiple layers of vascular smooth muscle cells and, an increase in vascular stiffness of major arteries is recognized today to be associated with aging and the development of cardiovascular disease (Kohn, Lampi, & Reinhart-King, 2015). Mechanically, the stiffness of the endothelium, a single cell monolayer that constitutes the inner lining of the blood vessels is not expected to contribute to the general stiffness of the vascular wall. However, an increasing amount of evidence suggests that endothelial stiffness *per se* has significant physiological implications, including control of angiogenesis (Lee, Bai, Smith, Bayless, & Yeh, 2013; Oh et al., 2016) and recruitment of monocytes to the sites of inflammation (Schaefer & Hordijk, 2015). It is critical, therefore, to determine the mechanisms that govern endothelial stiffness. Several studies have shown that endothelial biomechanical properties depend on the stiffness (Byfield, Reen, Shentu, Levitan, & Gooch, 2009; Jalali, Tafazzoli-Shadpour, Haghighipour, Omidvar, & Safshekan, 2015) or molecular composition of the substrate they are grown on (Collins et al., 2014). For the *in vivo* situation, this would be equivalent to endothelial stiffness being governed by the stiffness of the vascular wall underneath. Studies from our group, however, discovered that endothelial stiffness can also be regulated by the uptake of oxidized lipids, independently of the stiffness of the substrate (Gundavaram et al., 2013). More specifically, we found that significant endothelial stiffening occurs as a result of the uptake of oxLDL mediated by a scavenger receptor CD36 (cluster of differentiation 36), and incorporation of specific oxysterols and oxidized phosphatidylcholines into the endothelial membrane (Le Master et al., 2017; Oh et al., 2016; Shentu et al., 2010). Mechanistically, we showed that CD36 mediates oxLDL-induced EC stiffening via the activation of RhoA/Rho kinase which facilitates the inhibition of myosin light-chain phosphatase and increases the phosphorylation of myosin light-chain (Le Master et al., 2017; Oh et al., 2016). Recently, our lab also found significant endothelial stiffening in the pro-atherogenic, disturbed flow region of the aortic arch in intact aortas *ex vivo*, an effect that was found to be CD36-dependent (Le Master et al., 2017). This is particularly interesting because it is known that genetic deletion of CD36 confers protection against the development of atherosclerosis in hypercholesterolemic murine models (Nicholson, Han, Febbraio, Silverstein, & Hajjar, 2001). Our current goal is to perform a comparative analysis of the elastic properties of the endothelial versus sub-endothelial layers in mouse aortas and evaluate the impact of CD36 expression in both layers. Furthermore, aging is well-known to be associated with increased vascular stiffness of whole arteries (Kohn et al., 2015), but the effect of aging on endothelial stiffness is not defined. We, therefore, also examine the effect of age on endothelial and subendothelial stiffness in wild type and CD36^{-/-} mice.

Materials and Methods

Mouse models:

Wild Type (WT, C57BL/6) and CD36^{-/-} (B6.129S1-Cd36^{tm1Mfe}) male mice were purchased from Jackson laboratories and bred in-house. VE-cadherin Cre mice, Tg(Cd5-cre/ERT2)1 Rha were a kind gift from Dr. Kishore Wary and used for the purpose of imaging the intact endothelial and denuded sub-endothelial layers. Euthanasia was performed with CO₂ followed by cervical dislocation. All animal protocols adhered to the guidelines established by UIC's Institutional Animal Care and Use Committee.

Elastic modulus measurements were performed using an Asylum MFP-3D-Bio atomic force microscope (Santa Barbara, CA), as previously described (Le Master et al., 2017). Briefly, the elastic modulus (stiffness) is quantified by the indentation of the cell's surface by a cantilever tip to produce force-distance curves.

Denuding the endothelial layer was performed as previously described (Peloquin, Huynh, Williams, & Reinhart-King, 2011). In brief, a cotton-tipped applicator was wetted with Hank's buffering solution and gently rubbed on the endothelial layer ~10 times then washed to remove debris. Treatments for select experiments of 4–6 month old WT male mice *ex vivo* aortic arch tissues included one hour treatment with blebbistatin (Santa Cruz Biotechnologies, 50, 500 and 2500 μ M) (Kovacs, Toth, Hetenyi, Malnasi-Csizmadia, & Sellers, 2004; Oh et al., 2016) or SSO (sulfo-N-succinimidyl oleate, Cayman Chemical, 25 μ g/mL, 1 hour) (Kuda et al., 2013), the latter followed by 1 hour oxLDL exposure (50 μ g/mL, Alfa Aesar). For experiments to visualize and ensure EC removal, the endothelial layers were fixed, washed and staining for PECAM1, an endothelial cell marker.

Imaging was performed using a Ziess confocal microscope, to visualize one micron z-stack slices, or a Ziess Axiovert fluorescent microscope.

Results

Stiffness of the intact endothelial monolayer in the aortic arch, but not the denuded subendothelial layer, is dependent on CD36

To perform a comparative analysis of the endothelial and sub-endothelial layers, we measured the elastic moduli of the intact and denuded aortas, with both measurements performed on the same vessel, same day. Specifically, the measurements were first performed on an intact vessel, then denuded and repeated in the same area after the denudation. As shown in Figure 1A, the endothelial layer (FITC) is preserved in the intact EC layer (left) whereas the denudation process reveals the sub-endothelial layer (rhodamine) below. Furthermore, the intact layer is visualized by *en face* PECAM1 (endothelial junctional marker) staining (Figure 1B, left), whereas no staining is observed in the denuded layer (Figure 1B, right).

In Figure 2, we show the elastic modulus of the intact and denuded monolayer surfaces of the athero-prone region of the aortic arch in 'young' (4–6 month old) wild type and CD36^{-/-} male mice using atomic force microscopy. First, these data show that in wild type mice, the

subendothelial layer is significantly stiffer (6.8 ± 0.5 kPa) compared to the intact EC layer, as represented as a rightward shift in the elastic modulus values (Figure 2A, top row, B). More importantly, we show that CD36 expression has a differential effect on the elastic moduli of intact versus denuded aortas. Consistent with our previous study (Le Master et al., 2017), endothelial stiffness is markedly reduced in the aortic arch of CD36^{-/-} mice as compared with WT controls (Figure 2A, left column, B), but further analysis revealed that the loss of CD36 does not affect elastic modulus values of the sub-endothelial layer (5.3 ± 1 kPa, Figure 2A, right column, B), as is shown in the EC layer. This indicates that the role CD36 has on vascular stiffening is an endothelial phenomenon.

Stiffening of ECs but not sub-ECs is dependent on the phosphorylation of myosin II

Previous studies from our group have shown that oxLDL-induced endothelial stiffening *in vitro* is mediated by CD36 via increased levels of phosphorylated light chain myosin II (Oh et al., 2016). To determine whether the same mechanism contributes to endothelial stiffness of the aortic endothelium *in vivo*, excised intact or denuded aortas were exposed to increasing concentrations of blebbistatin, a pharmacological inhibitor of myosin II phosphorylation (Kovacs et al., 2004) for 1 hour. The AFM measurements were performed before and after the application of blebbistatin in the same aortas. As shown in Figure 3A, endothelial stiffness is significantly reduced by the application of blebbistatin in a dose-dependent manner, whereas the stiffness of the denuded sub-endothelial layer is unaffected (Figure 3B). Consistent with the data shown in Figure 2, sub-endothelial stiffness is greater than the stiffness of the endothelial layer in both experimental cohorts of mice. Furthermore, these new data present additional evidence that the stiffness of the endothelial monolayer can be regulated independently of the stiffness of the sub-EC layer via the myosin II pathway.

Inhibition of CD36 prevents oxLDL-induced endothelial stiffening in the aortic arch

CD36 has been shown to be involved in cellular lipid accumulation (Nicholson et al., 2001) and oxLDL uptake-induced endothelial stiffening *in vitro* (Le Master et al., 2017; Oh et al., 2016; Shentu et al., 2010). Since we have shown above that CD36-dependent changes take place in the EC, but not the sub-EC layer (Figure 2), we further tested the role of CD36 in oxLDL-induced endothelial stiffening in intact aortas. The irreversible inhibition of CD36-mediated uptake of oxLDL was achieved by the application of SSO (sulfosuccinimidyl oleate, 25 μ g/mL for 1 hour). Our results (Figure 4A, top row, B) show an increase in elastic modulus values (stiffness) of the intact endothelial layer in the arch exposed to oxLDL (50 μ g/mL, 1 hour) as compared to control. Inhibiting CD36-mediated uptake, through the use of SSO, prior to oxLDL treatment abrogated this response (Figure 4A, bottom row, B).

Stiffening of endothelial monolayer with age depends on CD36

To determine the potential contribution that age may have on the stiffness of the EC layer, we extended our studies to mice that were 22–25 months old, a generally accepted range for advanced aged mice (Kohn et al., 2015). Consistent with earlier studies (Huynh et al., 2011), elastic modulus of the sub-endothelial layer significantly increased in aged mice (17 ± 2 kPa in 22–25 months old mice versus 6.8 ± 0.5 kPa in 4–6 months old mice). Our current data show that the elastic modulus of the endothelial layer of intact aortas was also significantly

increased with age: 15 ± 2 kPa in the aged mice, as compared to 4 ± 1 kPa range in the younger mice, suggesting that the stiffening occurs both in the extracellular matrix and in endothelial cells.

Our further data indicate that the loss of CD36 results in a significant softening of the endothelial layer in aged mice, as compared to WT aged controls (6 ± 2 versus 15 ± 2 kPa in CD36^{-/-} and WT ECs respectively). In contrast, the elastic modulus of the sub-endothelial layer of the aortic vasculature in aged mice remains unchanged regardless of the presence or absence of CD36 (Figure 5A, right column, B). It is interesting to note that the loss of CD36 does not completely abrogate the aged-related increase in stiffness of the endothelial monolayer (2 ± 0.5 versus 6 ± 2 kPa) for young versus aged EC monolayer in CD36^{-/-} mice.

Discussion

The main findings of this study are: (i) the EC stiffness in intact mouse aortas depends on the CD36 scavenger receptor via myosin phosphorylation independently of the stiffness of the subendothelial layer, (ii) blocking oxLDL uptake by CD36 abrogates this stiffening effect; (iii) there is age-related stiffening of the endothelial monolayer, which critically depends on CD36 expression, whereas the sub-EC layer does not.

Multiple studies showed that endothelial biomechanical properties depend on the stiffness of the extracellular matrix and it has been generally assumed that extracellular matrix plays a dominant role in determining endothelial stiffness (Huynh et al., 2011; Jalali et al., 2015; Kohn et al., 2015). We have shown, however, that endothelial stiffness is also regulated by the uptake of oxidized lipids independently of the stiffness of the substrate (Le Master et al., 2017; Oh et al., 2016; Shentu et al., 2010). However, the contribution of these two processes in intact vasculature was not known. Therefore, we measured the elastic moduli in freshly-isolated aortas examining both the endothelial monolayer and the sub-endothelial extracellular matrix. Our current study provides two lines of evidence that the stiffness of the endothelial monolayer *in vivo* can be regulated independently of the stiffness of the sub-endothelial layer, which constitutes the substrate for the endothelium. We show that both a global knockout of CD36 and the inhibition of myosin phosphorylation result in significant softening of the endothelial monolayer of mouse aortas without any change in stiffness of the extracellular matrix. Furthermore, exposure of intact aortas to oxLDL *ex vivo* results in endothelial stiffening in a CD36-dependent way. Since we already know that CD36 is required for endothelial uptake of oxLDL, which leads to endothelial stiffening via RhoA/ROCK/MLCII cascade (Huveneers, Daemen, & Hordijk, 2015), taken together these findings suggest that it is the uptake of the oxidized lipids that have the dominant effect on aortic endothelial stiffness *in vivo*.

Furthermore, our recent studies determined that endothelial stiffening is enhanced in regions of pro-atherogenic disturbed flow both *in vitro* and *in vivo* in the aortic arch, as compared to the descending aorta (Le Master et al., 2017), an effect attributed to increased CD36-mediated uptake of oxidized lipids. We proposed, therefore, that endothelial stiffening may contribute to the development of atherosclerosis in these regions. Since it is well known that genetic deficiency of CD36 confers an athero-resistant phenotype (Nicholson et al., 2001),

our current data showing that the lack of CD36 decreases endothelial but not sub-endothelial stiffness supports the notion that abrogating EC stiffening in the athero-susceptible site of the aortic arch may contribute to the athero-resistant phenotype of CD36^{-/-}.

Finally, in terms of aging, it is well documented that the stiffness of the aortic vascular wall increases with age (Huynh et al., 2011) and is a predictor of cardiovascular disease (Palombo & Kozakova, 2015). However, the effect of aging on endothelial stiffness was unknown. We show here that significant endothelial stiffening develops in aged aortas and that this effect depends primarily on CD36 independently of the stiffness of the sub-endothelial layer. These findings provide a model to discriminate between the roles of endothelial and sub-endothelial stiffness in age-related vascular dysfunction.

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Figure 1: Images of intact and denuded endothelial layer in the pro-atherogenic region of the aortic arch in mice.

A. Confocal z-stack images of intact (left) and denuded (right) endothelial cell (EC) layer (green) and the sub-EC layer (red) in the aortic arch in mice. Scale bar = 20 microns. **B.** Fluorescent staining of endothelial marker, PECAM1, in intact (left) and denuded (right) aortic mouse samples. Scale bar = 20 microns.

Figure 2: Stiffness of the endothelial layer, but not the sub-endothelial layer, is CD36 dependent.

A. Histograms of the elastic modulus (stiffness) of the intact endothelial (left) and denuded sub-endothelial (right) layer in the athero-susceptible region of the aortic arch of ‘young,’ 4–6 month old, WT (top) and CD36^{-/-} (bottom) mice. **B.** Average elastic modulus for the intact EC and denuded sub-EC layers in the aortic arch of younger WT and CD36^{-/-} mice [6–10 sites (50–90 measurements) per sample, n=5 mice per condition]. * $p < 0.05$. NS = not significant.

Figure 3: Inhibition of myosin pathway reduces EC but not denuded sub-EC stiffening in the aortic arch.

A. Average elastic moduli of the sites of the endothelial monolayer in the aortic arch of WT mice ($n=3-4$ mice per condition) exposed to increasing concentrations of the myosin II phosphorylation inhibitor, blebbistatin. **B.** Average elastic moduli for the measured sites from the denuded sub-EC layer in the aortic arch of 4-6 month old WT mice treated with blebbistatin (0, 50, 500 μM) [6-10 sites (50-90 measurements) per sample, $n=3-5$ mice per condition]. * $p < 0.05$.

Figure 4: Inhibition of CD36 abrogates oxLDL-induced stiffness in the aortic arch.

A. Histograms of the elastic modulus of the intact endothelial layer in the athero-susceptible region of the aortic arch of 4–6 month old WT mice with and without oxLDL exposure in the presence or absence of CD36 inhibitor, SSO (sulfo succinimidyl oleate). **B.** Average elastic modulus for the conditions described in A. [6–10 sites (50–90 measurements) per sample, n=3 mice per condition]. * p < 0.05.