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Mechanical Regulation of Retinal Vascular Inflammation and Degeneration in Diabetes

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Vascular inflammation is known to cause degeneration of retinal capillaries in early diabetic retinopathy (DR), a major microvascular complication of diabetes. Past studies investigating these diabetes-induced retinal vascular abnormalities have focused primarily on the role of molecular or biochemical cues. Here we show that retinal vascular inflammation and degeneration in diabetes are also mechanically regulated by the increase in retinal vascular stiffness caused by overexpression of the collagen–cross-linking enzyme lysyl oxidase (LOX). Treatment of diabetic mice with LOX inhibitor β-aminopropionitrile (BAPN) prevented the increase in retinal capillary stiffness, vascular intracellular adhesion molecule-1 overexpression, and leukostasis. Consistent with these anti-inflammatory effects, BAPN treatment of diabetic mice blocked the upregulation of proapoptotic caspase-3 in retinal vessels, which concomitantly reduced retinal capillary degeneration, pericyte ghost formation, and the diabetes-induced loss of contrast sensitivity in these mice. Finally, our in vitro studies indicate that retinal capillary stiffening is sufficient to increase the adhesiveness and neutrophil elastase-induced death of retinal endothelial cells. By uncovering a link between LOX-dependent capillary stiffening and the development of retinal vascular and functional defects in diabetes, these findings offer a new insight into DR pathogenesis that has important translational potential.

ARTICLE HIGHLIGHTS

- The objective of this study was to determine whether retinal vascular inflammation and degeneration associated with early diabetic retinopathy are mechanically regulated by the increased stiffness of retinal capillaries.
- We provide the first direct evidence of retinal capillary stiffening in diabetes that is dependent on lysyl oxidase (LOX), promotes retinal vascular inflammation, and causes capillary degeneration by increasing retinal endothelial susceptibility to neutrophil elastase.
- We also show that pharmacological inhibition of LOX prevents the diabetes-induced loss of contrast sensitivity.
- These findings implicate LOX and capillary stiffening as new anti-inflammatory targets for the treatment of retinal vascular and functional defects associated with early diabetic retinopathy.

Retinal capillary degeneration is a clinical hallmark of early diabetic retinopathy (DR), a vision-threatening microvascular complication that affects nearly 40% of all individuals with diabetes. Retinal vascular inflammation is strongly implicated in the degeneration of retinal capillaries in DR (1,2). Past work has identified several molecular and biochemical factors as important mediators of retinal inflammation in diabetes (1,3). However, study of nonocular inflammatory conditions, such as atherosclerosis and sepsis-associated lung edema, has revealed that vascular inflammation and dysfunction are also independently regulated by vascular stiffening caused by

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upregulation of the collagen–cross-linking enzyme lysyl oxidase (LOX) (4–6). This effect is attributed to cellular mechanotransduction, wherein alterations in stiffness are transduced into intracellular biochemical signals that regulate vascular cell function at the transcriptional and/or translational levels (7,8). Given the significant role of vascular stiffening in these inflammatory conditions, coupled with the known association of diabetes with stiffening and inflammation of large vessels (aorta and arteries) (9,10), we asked whether LOX-dependent stiffening mechanically regulates retinal vascular inflammation and degeneration in diabetes.

Indeed, we and others have reported increased LOX expression in the retina and vitreous of rodents and humans with diabetes, respectively (11–13), whereas heterozygous LOX-knockout mice have been shown to be protected from diabetes-induced retinal capillary degeneration (14). Although these findings indicate a role for LOX in the development of retinal vascular lesions associated with DR, they do not explain how LOX achieves its degenerative effect. To this end, our recent studies using human retinal endothelial cell (HREC) cultures have shown that LOX upregulation by high glucose (HG) and advanced glycation end products (AGEs), major risk factors for DR, causes stiffening of subendothelial matrix that, in turn, promotes HREC activation characterized by upregulation of proinflammatory receptors (intracellular adhesion molecule-1 [ICAM-1] and receptor for AGE [RAGE]) and leukocyte-EC adhesion (12,15). These insights from past studies raise two important questions of mechanistic and translational significance, namely, whether LOX contributes to retinal capillary degeneration in diabetes via stiffening-dependent retinal EC activation and inflammation and whether pharmacological inhibition of LOX can block diabetes-induced retinal capillary degeneration as effectively as the previously reported genetic LOX inhibition (14).

Using the streptozotocin mouse model of DR and complementary HREC cultures, here we provide the first direct evidence of retinal capillary stiffening in diabetes that requires LOX, promotes retinal vascular inflammation, and causes capillary degeneration by increasing retinal endothelial susceptibility to inflammation-mediated apoptosis. Finally, we show that pharmacological inhibition of LOX also prevents the diabetes-induced loss of contrast sensitivity, a clinical hallmark of early DR.

RESEARCH DESIGN AND METHODS

Experimental Animals

All animal procedures were performed in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research and approved by the University of California, Los Angeles and University of California, Irvine Institutional Animal and Care Use Committees. Induction of diabetes in adult male C57BL/6J mice and administration of the LOX inhibitor β-aminopropionitrile (BAPN) in diabetic mice (fasting blood glucose ≥275 mg/dL) are described in the Supplementary Research Design and Methods. Body weights and fasting blood glucose levels of all groups of mice are summarized in Supplementary Table 1. Mice were euthanized at 10 weeks' (short-term) or 20–30 weeks' (longer-term) duration of diabetes, and eyes were harvested for further analysis.

Isolation of Mouse Retinal Vessels

Retinal vessels were isolated from fresh unfixed eyes (for gene expression analysis) or mildly fixed eyes (for stiffness measurement) of both short- and longer-term nondiabetic (ND), diabetic (D), or D + LOX inhibitor BAPN (D+BAPN) mice (n = 6–7/group) using our published protocol, which is described in the Supplementary Research Design and Methods.

Cell Culture and Treatments

HRECs and the human promyelocytic cell line HL-60 were purchased from commercial vendors and cultured according to vendor recommendations. HL-60 cells were differentiated with DMSO (dHL-60) to acquire the functional properties of neutrophils (16,17). Details of culture and differentiation conditions, as well as all in vitro treatments, are described in the Supplementary Research Design and Methods.

Subendothelial Matrix

Decellularized subendothelial matrices were obtained from HREC cultures grown in normal glucose (5.5 mmol/L) or HG (30 mmol/L) ± BAPN (0.1 mmol/L) medium according to our previously published protocol (18) that is described in the Supplementary Research Design and Methods.

Stiffness Measurements

Stiffness of isolated mouse retinal capillaries of ND, D, or D+BAPN mice (n = 6/group), cultured HRECs, and decellularized subendothelial matrices were measured using a NanoWizard 4 XP BioScience atomic force microscope (AFM; Bruker) in contact mode force spectroscopy mode, as we recently reported (15,19) and describe in the Supplementary Research Design and Methods.

Reverse Transcription–Quantitative PCR

mRNA expression levels in freshly isolated retinal vessels or whole retina from short- or longer-term ND, D, or D+BAPN mice (n = 5–7 mice/group) and HREC cultures were assessed according to our standard reverse transcription–quantitative PCR (RT-qPCR) protocol (19) using gene/species-specific TaqMan primers for LOX, ICAM-1, vascular cell adhesion molecule-1 (VCAM-1), E-selectin, caspase-3, tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), vascular endothelial growth factor A (VEGF-A), collagen IV, fibronectin, and the housekeeping gene GAPDH, which is described in the Supplementary Research Design and Methods.

Retinal Immunofluorescence

Levels of retinal vascular LOX protein from short-term ND and D mice were assessed by immunofluorescence labeling of 10-μm-thick retinal cryosections (obtained from unfixed flash-frozen eyes) with anti-LOX and anti-collagen IV primary antibodies, as described in the Supplementary Research Design and Methods.

Western Blot

LOX protein levels and caspase-3 activation in mouse retinas from longer-term ND, D, or D+BAPN mice (n = 6–8 mice/group) were assessed using our standard Western blotting protocol (19) involving target-specific primary antibodies for LOX or cleaved caspase-3 and loading control GAPDH, as described in the Supplementary Research Design and Methods.

Leukostasis

Mouse retinal leukostasis (leukocyte adhesion to mouse retinal vessels) from short-term ND, D, or D+BAPN mice (n = 6–8 mice/group) was assessed following fluorescein-concanavalin A lectin infusion, according to our previously reported protocol (20) described in the Supplementary Research Design and Methods.

Synthetic Matrix Fabrication

Fibronectin-coated polyacrylamide-based synthetic matrices of tunable stiffness, which mimic retinal capillary stiffness in ND (normal; 1 kPa) and D (stiff; 2.5 kPa) mice, were fabricated according to the published protocol (8,21) described in the Supplementary Research Design and Methods.

Leukocyte-EC adhesion

Adhesion of (neutrophil-like) dHL-60 cells to HRECs grown on normal or stiff synthetic matrices was assessed according to our previously published protocol (8,12) described in the Supplementary Research Design and Methods.

Flow Cytometry

Surface protein expression of ICAM-1, VCAM-1, and E-selectin in HRECs grown on normal or stiff synthetic matrices was investigated according to our previously published protocol (8,12) described in the Supplementary Research Design and Methods.

Retinal EC Apoptosis

Neutrophil elastase-induced apoptosis and death of HRECs grown on normal or stiff synthetic matrices were assessed by the addition of Biotium NucView 488 caspase-3 substrate (apoptosis) and propidium iodide (death) to cell cultures. Coculture of activated (TNF-treated) dHL-60 cells with HRECs grown on normal or stiff synthetic matrices were assessed for HREC apoptosis by addition of Biotium NucView 488 caspase-3 substrate. Caspase-3 mRNA expression in retinal vessels and caspase-3 activation in whole retinas (n = 6 mice/group) of longer-term ND, D, or D+BAPN mice were determined by RT-qPCR and Western blotting (for cleaved caspase-3), respectively. Details are described in the Supplementary Research Design and Methods.

Retinal Capillary Degeneration

Formation of acellular retinal capillaries and pericyte ghosts were assessed in retinal elastase digests from longer-term (30 weeks' duration) ND, D, and D+BAPN mice (n = 7–8 mice/group) that were stained with hematoxylin and periodic acid Schiff (PAS) reagent, according to our previously reported protocol (20), which is described in the Supplementary Research Design and Methods.

Contrast Sensitivity

Contrast sensitivity was measured from longer-term (30 weeks' duration) mice (n = 8 mice/group) by subjecting mice to a virtual optokinetic test at multiple spatial frequencies, as previously reported (22,23) and described in the Supplementary Research Design and Methods.

Statistics

GraphPad Prism 6.01 software was used to perform two-tailed unpaired Student t test (for two groups) or one-way ANOVA (for three groups), followed by the Tukey post hoc test. $P < 0.05$ was considered as statistically significant.

Data and Resource Availability

The data sets generated during the current study are available from the corresponding author on reasonable request. No applicable resources were generated or analyzed during the current study.

RESULTS

Retinal Capillaries Become Stiffer in Diabetes

To assess the role of LOX in the mechanical regulation of retinal vascular inflammation in diabetes, we first quantified the relative change in the stiffness of retinal capillaries isolated from nondiabetic and streptozotocin-induced diabetic mice. To ensure feasibility and reliability of this approach, we developed a mild fixation protocol for the eye that ensured sufficient durability of the isolated retinal vasculature to withstand handling during sample preparation steps and stiffness measurement by using an AFM. As shown in Supplementary Fig. 1, fixation with 5% formalin for 24 h at 4°C yielded sufficiently durable vessels for AFM measurement.

Our AFM measurements of the isolated mouse retinal vasculature revealed that capillary stiffness increases significantly (by ∼2.5-fold; $P < 0.001$) at 10 weeks' duration (short-term) of diabetes (Fig. 1*A*), with the increased stiffness persistent at longer duration of diabetes (20–30 weeks (Fig. 1*B*). Notably, this diabetes-induced increase in retinal capillary stiffness in vivo correlates with HG-induced stiffening of cultured HRECs (Fig. 1*C*) and their subendothelial matrix (Fig. 1*D*), thus indicating that ECs and the vascular basement membrane both likely contribute to the observed retinal capillary stiffening in diabetes. Predictably, the stiffer

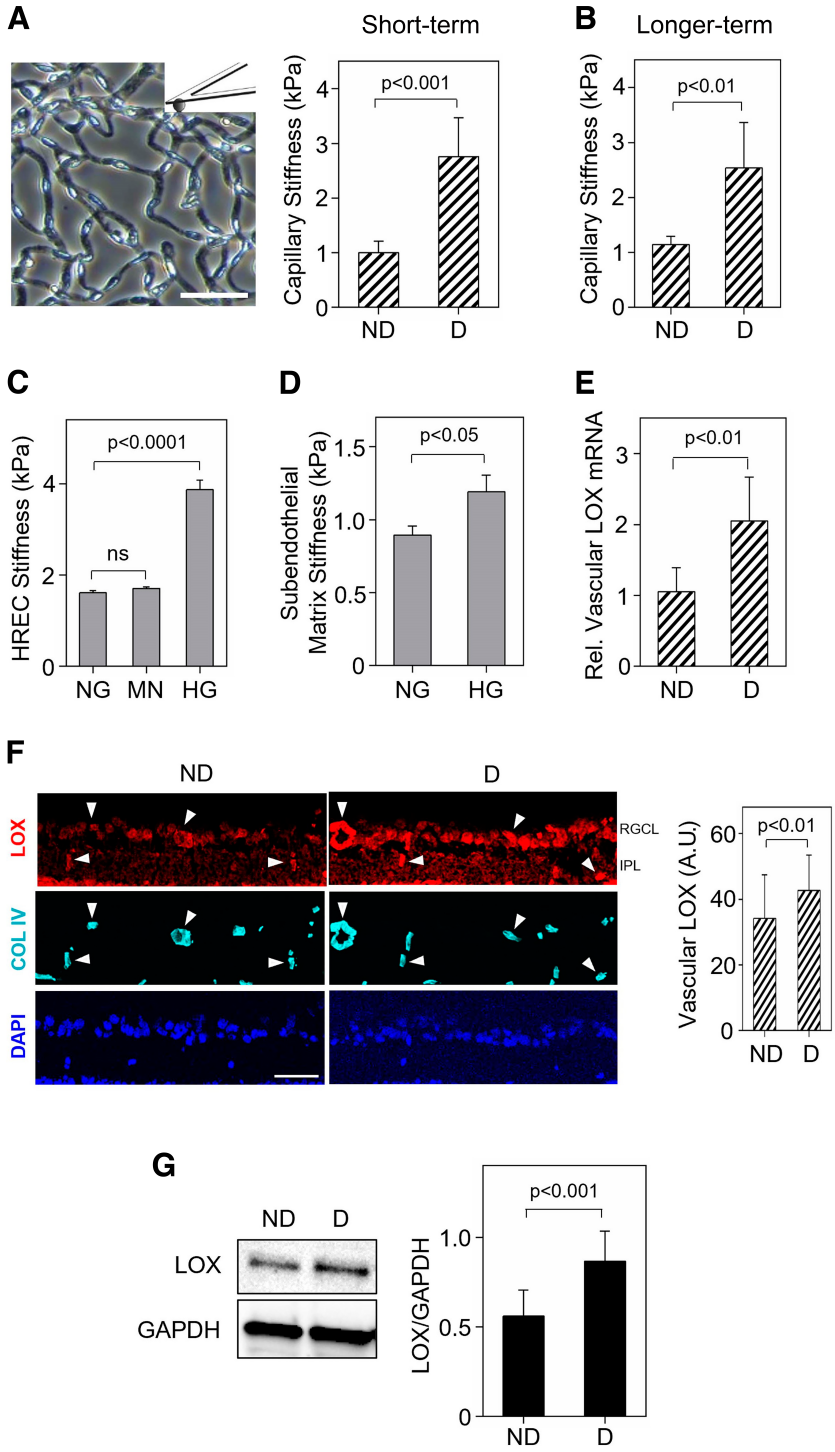

Figure 1—Retinal capillaries become stiffer in diabetes. *A* and *B*: Stiffness of retinal capillaries isolated from ND and D mice (representative image) was measured using a biological grade AFM (n = 6/group). Quantitative analysis of force-indentation curves from ≥12 locations on the capillary network revealed an ∼2.5-fold increase ($P < 0.01$) in stiffness following both short-term (10 weeks' duration) (*A*) and longer-term (20–30 weeks' duration) (*B*) diabetes. Scale bar: 50 μm. *C* and *D*: Quantitative analysis of AFM force-indentation curves from multiple ($n \geq 20$) cultured HRECs or multiple locations ($n \geq 20$) on the unfixed decellularized subendothelial matrix revealed that 10-day HG treatment (30 mmol/L) leads to a significant increase in both HREC (*C*) and matrix stiffness (*D*). *E*: RT-qPCR analysis of freshly isolated mouse retinal vessels (n = 6–7/group) revealed a twofold increase ($P < 0.001$) in retinal vascular LOX mRNA in short-term (10 weeks' duration) D mice. *F*: Representative fluorescent images show mouse retinal sections from ND and short-term (10 weeks' duration) D mice immunolabeled with anti-LOX, anti-collagen (Col) IV (vascular basement membrane), and DAPI (nucleus). LOX intensity analysis from multiple ($n \geq 160$) vessels/group in the retinal ganglion cell layer, the inner plexiform layer, and the inner plexiform layer/inner nuclear layer boundary indicated that diabetes leads to a significant increase in LOX protein expression in retinal vessels (arrowhead). A.U., arbitrary units. Scale bar, 50 μm. *G*: Western blot analysis of whole retinas from mice (n = 6–8 mice/group) revealed a 1.5-fold increase ($P < 0.001$) in retinal LOX protein levels in longer-term D mice. Bars indicate mean ± SD (in vivo data) or mean ± SEM (in vitro data).

retinal vessels in diabetic mice expressed significantly higher levels of LOX, as judged by the significant increase in vascular LOX mRNA (Fig. 1*E*), stronger vascular LOX immunolabeling in retinal cryosections (Fig. 1*F*), and higher levels of retinal LOX protein (Fig. 1*G*).

LOX Promotes Retinal Capillary Stiffening and Leukostasis in Diabetes

To determine the extent to which LOX contributes to diabetes-induced retinal capillary stiffening, diabetic mice were systemically treated with BAPN, a specific and irreversible LOX inhibitor, for the entire 10 weeks' duration (short-term) of diabetes. Our AFM measurements revealed that BAPN treatment, which inhibits the diabetes-induced increase in retinal vascular LOX by 75% ($P < 0.01$) (Supplementary Fig. 2), blocks retinal capillary stiffening in diabetic mice (Fig. 2*A*). As shown in Supplementary Fig. 3, this ability of LOX to increase retinal capillary stiffness is associated with a significant increase in the expression of major vascular basement membrane proteins collagen IV and fibronectin.

Since LOX cross-links collagen, we reasoned that BAPN prevents retinal capillary stiffening by blocking LOX-dependent crosslinking/stiffening of collagen IV-rich subendothelial matrix (basement membrane). Indeed, AFM measurement of decellularized HREC-derived subendothelial matrix demonstrated that BAPN treatment almost completely prevents HG-induced matrix stiffening (Supplementary Fig. 4). Importantly, inhibition of subendothelial matrix stiffening by BAPN simultaneously prevented the HG-induced increase in HREC stiffness (Fig. 2*B*), thus confirming that HRECs sense and mechanically reciprocate the stiffness of their underlying matrix. Notably, treating HRECs with varying doses of recombinant LOX did not significantly increase cell stiffness (Fig. 2*C*). Thus, we conclude that LOX increases retinal capillary stiffness in diabetes by cross-linking/stiffening the basement membrane (subendothelial matrix) that, via reciprocal interaction, simultaneously causes stiffening of the overlying retinal ECs.

To determine the inflammatory effects of LOX-dependent retinal capillary stiffening in diabetes, we measured the mRNA levels of vascular ICAM-1, whose upregulation promotes leukocyte-EC adhesion (leukostasis), a major determinant of retinal capillary degeneration in early DR (2,24,25). As shown in Fig. 2*D*, the twofold increase ($P < 0.01$) in retinal vascular ICAM-1 mRNA in short-term diabetic mice was completely blocked by the LOX inhibitor BAPN, which predictably led to a significant reduction by ~60% ($P < 0.05$) (Fig. 2*E*) in leukostasis.

Retinal Capillary Stiffening Alone Can Enhance Leukocyte-EC Adhesion

LOX exists in both soluble and matrix-localized forms, with the latter form implicated in matrix cross-linking and tissue stiffening (26). To determine whether matrix LOX-mediated capillary stiffening can mechanically regulate leukostasis independent of any potential inflammatory effects of soluble LOX, we assessed the adhesion of (neutrophil-like) dHL-60 cells to HRECs plated on "synthetic" matrices of tunable stiffness that mimic retinal capillary stiffness of nondiabetic (1 kPa) or diabetic (2.5 kPa) mice (refer to Fig. 1*A* and *B*). As shown in Fig. 3*A*, HRECs plated on the stiff (2.5 kPa) matrix for 24 h exhibited a twofold increase ($P < 0.001$) in dHL-60 cell adhesion compared with those grown on normal (1 kPa) matrix. Predictably, this increased HREC adhesivity on stiff matrix was associated with a significant increase in the mRNA (Fig. 3*B*) and protein (Supplementary Fig. 5*A*) levels of ICAM-1, a major EC adhesion molecule that contributes significantly to retinal capillary degeneration in DR (2,27). This ICAM-1 upregulation in HRECs grown on stiff matrix for 18 h was associated with a significant increase in mRNA levels of DR-related proinflammatory cytokines, namely, TNF-α, IL-1β, and VEGF-A (Fig. 3*C*). Remarkably, however, mRNA (Fig. 3*B*) and protein (Supplementary Fig. 5*A*) levels of other HREC adhesion molecules, namely VCAM-1 and E-selectin, remained unaltered on stiff matrix. Interestingly, this unique mechanical regulation of ICAM-1 is associated with a preferential constitutive expression of ICAM-1, but not VCAM-1 and E-selectin, in HRECs (Supplementary Fig. 5*B*). Together, these findings indicate that LOX-dependent retinal capillary stiffening alone can promote vascular inflammation by selectively increasing ICAM-1–dependent leukostasis.

Retinal Capillary Stiffening Exacerbates EC Apoptosis and Capillary Degeneration Associated With DR

We showed earlier that diabetes-induced retinal capillary stiffening promotes leukostasis in vivo and neutrophil-EC adhesion in vitro. The adherent leukocytes, particularly neutrophils via their secreted elastase, play a causal role in retinal EC apoptosis and capillary degeneration associated with DR (28). Thus, here we asked whether increased retinal capillary/EC stiffness increases EC susceptibility to neutrophil elastase-induced apoptosis. Indeed, 12-h neutrophil elastase treatment of HRECs grown for 48 h on the stiff matrix, which mimics retinal capillary stiffness in diabetic mice, led to a 1.5-fold increase ($P < 0.01$) in the activation of the proapoptotic marker caspase-3 (Fig. 4*A*), which was predictably associated with a concomitant 40% increase ($P < 0.05$) in propidium iodide labeling of dead HRECs (Fig. 4*B*). Notably, this increased apoptosis of HRECs on stiff matrix was also seen in cocultures with activated (neutrophil-like) dHL-60 cells that acquire the cytotoxicity of diabetic neutrophils (Supplementary Fig. 6) (29). Importantly, this stiffening-dependent increase in retinal EC apoptosis in vitro was mirrored in vivo where the prevention of retinal capillary stiffening with BAPN (Fig. 4*C*) blocked the 1.7-fold increase ($P < 0.05$) in vascular caspase-3 mRNA expression (Fig. 4*D*) and 2.2-fold increase ($P < 0.01$) in retinal caspase-3 activity (caspase-3 cleavage) (Fig. 4*E*) in longer-term diabetic mice.

Inflammation-induced EC apoptosis leads to retinal capillary degeneration, a major clinically recognized

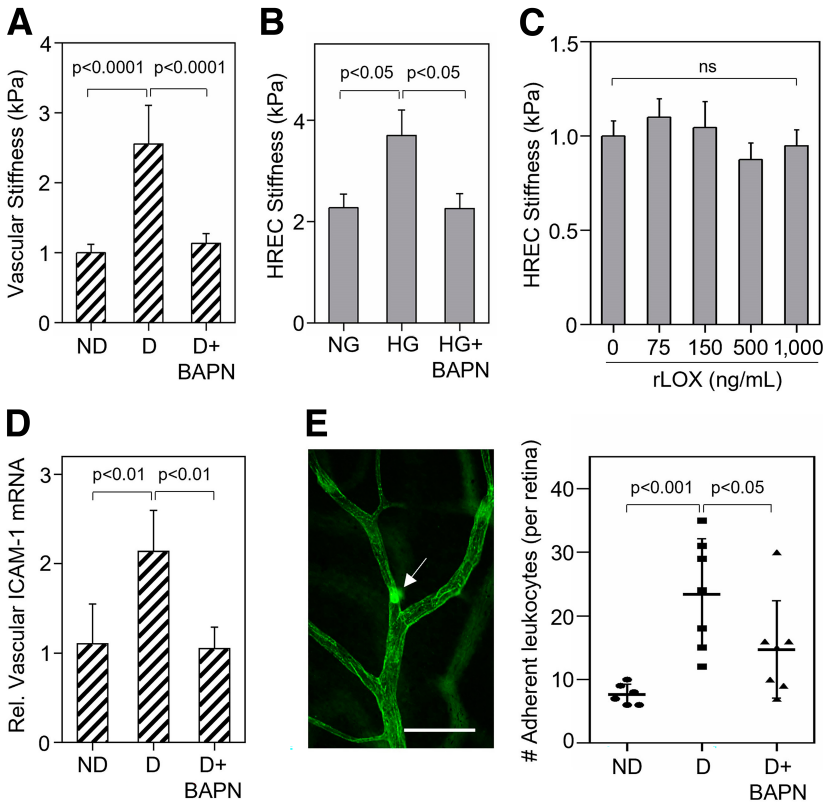

Figure 2—LOX promotes retinal capillary stiffening and leukostasis in diabetes. *A*: Quantitative analysis of AFM force-indentation curves from six or more locations on the capillary network (n = 6/group) revealed that the ~2.5-fold increase ($P < 0.0001$) in stiffness seen in short-term (10 weeks' duration) D mice is almost completely prevented by LOX inhibition using BAPN (3 mg/kg). *B*: AFM stiffness measurement from multiple cells ($n \geq 30$) indicates that LOX inhibitor BAPN blocks the significant HREC stiffening caused by 10-day HG treatment (30 mmol/L). NG, normal glucose. *C*: HRECs treated with varying doses of recombinant LOX for 18 h reveal no significant (ns) change in cell stiffness, as determined by the analysis of AFM force-indentation curves from multiple cells ($n \geq 30$/dose). *D*: RT-qPCR analysis of freshly isolated mouse retinal vessels (n = 6–7/group) shows that treatment of short-term D mice with LOX inhibitor BAPN (3 mg/kg) prevents the diabetes-induced twofold increase ($P < 0.01$) in retinal vascular ICAM-1 mRNA expression level. *E*: Representative retinal whole-mount fluorescent image shows an adherent fluorescein-concanavalin A–labeled leukocyte (arrow). Quantification of adherent leukocytes (n = 6–8 mice/group) revealed that the threefold increase in leukostasis seen in short-term D mice is significantly inhibited by BAPN treatment. Scale bar: 50 μm. Bars indicate mean ± SD (in vivo data) or mean ± SEM (in vitro data).

vascular lesion of early DR that is marked by the formation of acellular capillaries and pericyte ghosts (1,20). Consistent with this, the BAPN-mediated inhibition of retinal EC caspase-3 in longer-term (30 weeks' duration) diabetic mice was associated with a concomitant and significant reduction in the formation of acellular retinal vessels (Fig. 5*A*) and pericyte ghosts (Fig. 5*B*).

LOX Inhibition Mitigates the Diabetes-Induced Loss of Contrast Sensitivity

Loss of contrast sensitivity is a hallmark of early DR that, in mice, is expected to occur concurrently with our observed LOX-mediated retinal inflammation and capillary degeneration (22). Thus, we asked whether LOX also contributes to this loss of visual function in diabetes. Our findings revealed that longer-term diabetes predictably causes a substantial loss of contrast sensitivity in almost all spatial frequencies, which was significantly mitigated by treatment with the LOX inhibitor BAPN (Fig. 6).

DISCUSSION

Despite the well-recognized role of vascular inflammation in the development of retinal capillary lesions of DR (1,2), the underlying molecular mechanisms remain insufficiently understood. Findings from this study have introduced a new paradigm for retinal vascular inflammation and degeneration in DR that identifies LOX-dependent retinal capillary stiffening as a crucial, independent, and previously unrecognized regulator of endothelial ICAM-1 upregulation, leukostasis, and endothelial apoptosis. These findings, which are consistent with the causal role of vascular stiffening in cardiovascular and lung inflammation associated with atherosclerosis and sepsis (4–6), bring mechanical signaling at par with biochemical signaling in significance for inflammation-mediated DR pathogenesis.

Diabetes has long been associated with stiffening and inflammation of larger vessels, such as aorta and arteries (9,10,30,31), which correlates significantly with the incidence

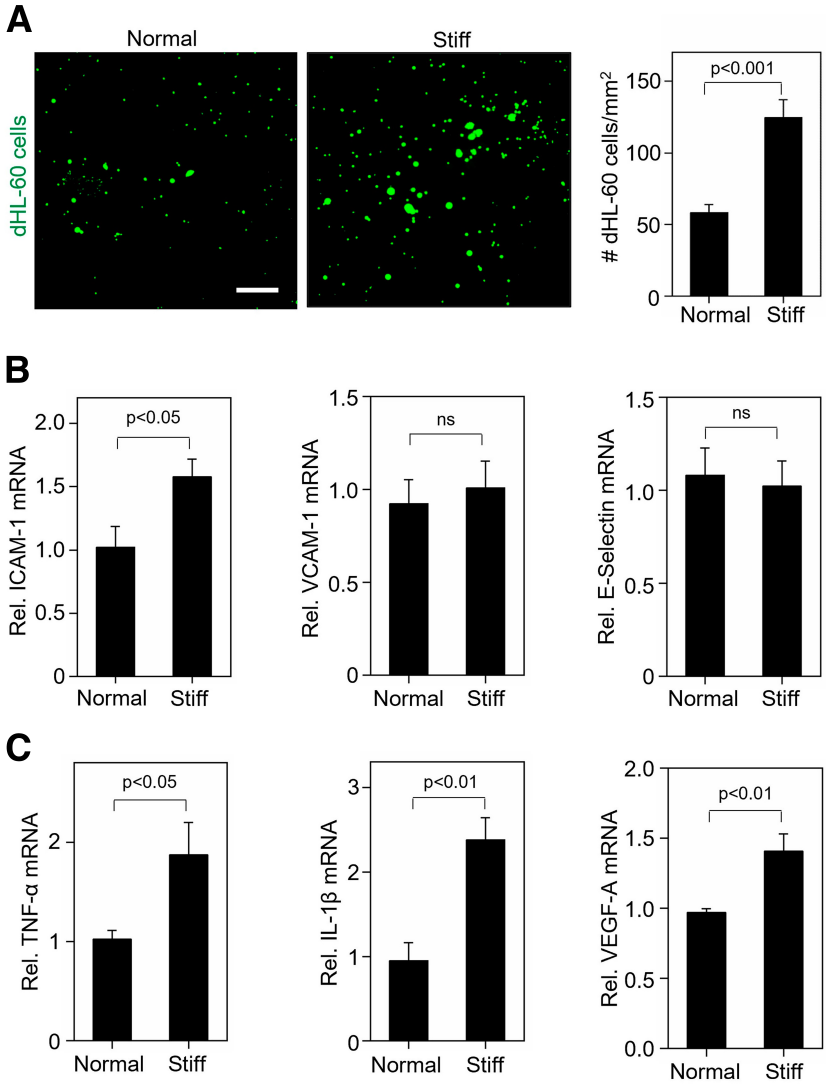

Figure 3—Retinal capillary stiffening alone can enhance leukocyte-EC adhesion. *A*: Representative fluorescent images of adherent (neutrophil-like) dHL-60 cells and subsequent cell count ($n \geq 6$ images/condition from three replicates) revealed a twofold greater ($P < 0.001$) dHL-60 cell adhesion to HRECs grown on stiff (2.5 kPa) synthetic matrices (for 24 h) than those grown on normal matrices (1 kPa). Scale bar: 200 μm. *B*: RT-qPCR analysis of HRECs grown on normal (1 kPa) or stiff (2.5 kPa) synthetic matrices for 18 h revealed that matrix stiffening preferentially increases the mRNA levels of ICAM-1 (by 1.6-fold; $P < 0.05$) but not VCAM-1 or E-selectin. *C*: RT-qPCR analysis of HRECs grown on normal (1 kPa) or stiff (2.5 kPa) synthetic matrices ($n = 3$/condition) for 18 h revealed that matrix stiffening increases the mRNA levels of TNF-α (by 1.8-fold; $P < 0.05$), IL-1β (by 2.3-fold; $P < 0.01$), and VEGF-A (by 1.4-fold; $P < 0.01$). Bars indicate mean ± SEM.

of microvascular dysfunction, including retinopathy (9,31). Thus, we asked whether diabetes also causes stiffening of the retinal microvasculature that, in turn, mechanically promotes inflammation-mediated DR pathogenesis. We began to address this question by measuring the diabetes-induced changes in retinal capillary stiffness. As we and others have shown previously, AFM offers the most reliable, accurate, and sensitive method to directly measure the stiffness of soft biological samples, including cells, extracellular matrix, intact tissues, and tissue engineering biomaterials (12,19,32–34). Our AFM measurement of isolated intact mouse retinal vasculature has provided the first direct evidence of significant retinal capillary stiffening in diabetes, which aligns with the reported diabetes-induced stiffening of larger vessels in the cardiovascular system (9,10). Isolation of intact retinal vessels for stiffness measurement required mild fixation of the mouse eye because unfixed eyes yielded fragmented retinal vessels unfit for AFM studies. Since this mild fixation may contribute to the absolute stiffness values obtained for retinal capillaries, we suggest that our AFM measurements be interpreted more in terms of relative changes in stiffness rather than its absolute values.

Another recent study used AFM to measure the stiffness of intact vessels in lightly fixed mouse retinal flat mounts (35). However, those measurements reflect the combined stiffness of the inner limiting membrane and

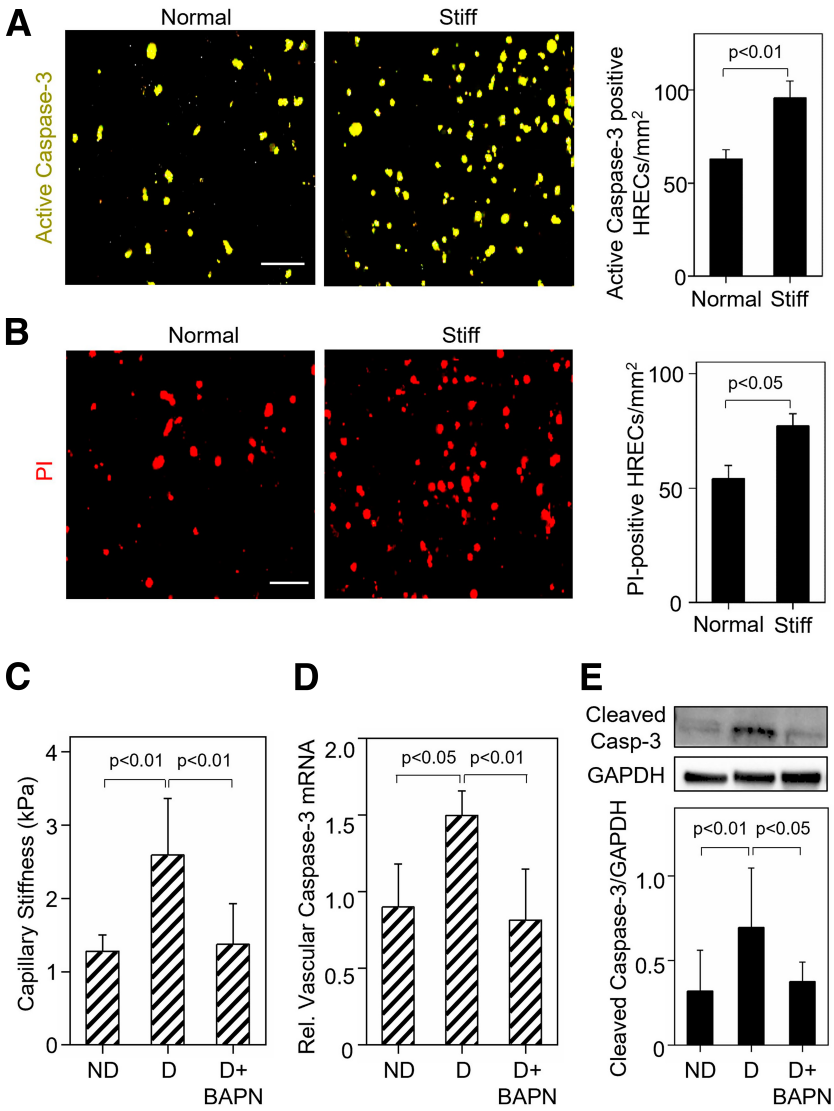

Figure 4—Retinal capillary stiffening exacerbates EC apoptosis associated with DR. Representative fluorescent images and quantitative analysis of HRECS treated with neutrophil elastase (50 nmol/L) for 12 h grown on normal (1 kPa) or stiff (2.5 kPa) synthetic matrices (n = 2–3/condition) for 48 h revealed that matrix stiffening significantly increases both HREC apoptosis (caspase-3 activity) (*A*) and necrosis (propidium iodide [PI] labeling) (*B*). *C*: Quantitative analysis of AFM force-indentation curves from ≥12 locations on the vasculature (n = 6/group) revealed that the approximately twofold increase ($P < 0.01$) in retinal vascular stiffness seen in longer-term D mice is almost completely blocked by LOX inhibitor BAPN. RT-qPCR analysis of unfixed mouse retinal vessels and Western blot analysis of whole mouse retinas (n = 6–8 mice/group) show that treatment of longer-term D mice with LOX inhibitor BAPN (3 mg/kg) prevents the diabetes-induced increase in both vascular caspase-3 mRNA expression (*D*) and retinal caspase-3 activity (*E*), respectively. Bars indicate mean ± SEM for in vitro data or mean ± SD for in vivo data.

the superficial capillaries that span across the surface of the nerve fiber layer while, crucially, leaving out the intermediate and deep capillary plexus that are particularly affected in patients with early DR (36). Thus, we believe that our AFM-based approach offers a direct, more comprehensive, and thereby, clinically relevant assessment of stiffness alterations in intact retinal vessels. Importantly, we show that retinal capillary stiffness increases within 10 weeks of diabetes, which coincides with the onset of early inflammatory changes in the retina. Thus, retinal capillary stiffening might serve as an early diagnostic marker for DR, an idea that will need to be validated in DR patients.

Consistent with the causal role of LOX in HG- and AGE-induced subendothelial matrix stiffening in vitro (12,15), we found significant LOX upregulation in the stiffer retinal vessels of diabetic mice. Our current findings and past reports reveal that this upregulation of retinal vascular LOX is associated with increased expression of basement membrane collagen IV, a LOX substrate (26), and fibronectin, which regulates LOX activity (37), in the retinas of mice and humans with diabetes (12,38,39). Importantly, systemic administration of the LOX inhibitor BAPN prevented the diabetes-induced increase in retinal capillary stiffness as well as retinal mRNA levels of collagen IV and fibronectin.

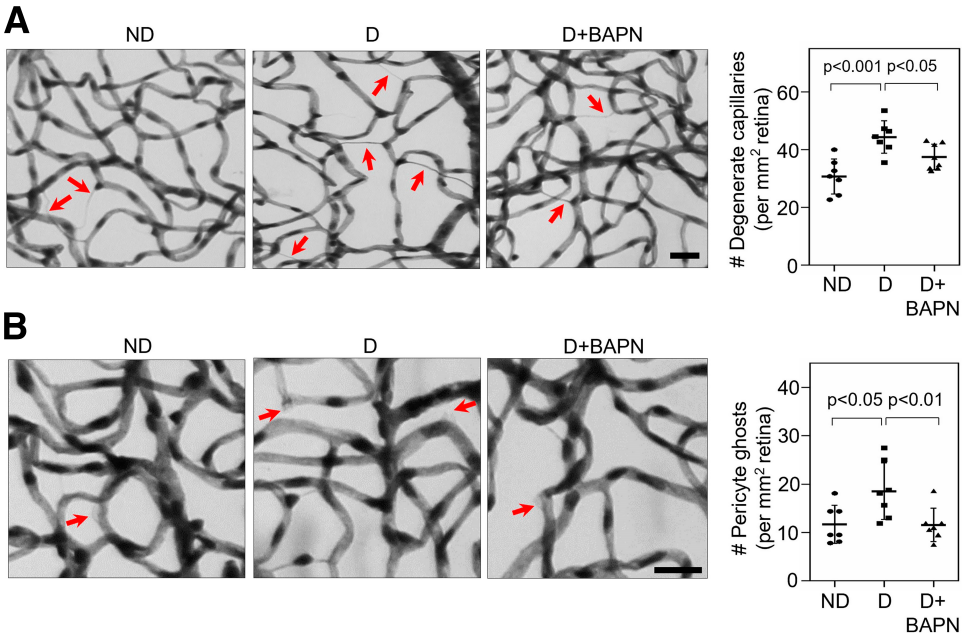

Figure 5—LOX-dependent stiffening promotes retinal capillary degeneration in diabetes. *A* and *B*: Representative images of elastase-digested retinal vessels and subsequent quantitative analysis revealed that the increased formation of degenerate (acellular) capillaries (indicated by arrows in *A*) and pericyte ghosts (indicated by arrows in *B*) in longer-term (30 weeks' duration) D mice is significantly inhibited by blocking LOX-dependent capillary stiffening using BAPN (3 mg/kg). Scale bar: 25 μm (n = 8 mice/group).

Our subsequent in vitro studies revealed that LOX inhibition using BAPN prevents the HG-induced stiffening of both subendothelial matrix and HRECs. Since we also showed that recombinant LOX does not directly stiffen HRECs, we conclude that LOX contributes to retinal EC stiffening by virtue of the cell's ability to sense and respond to LOX-dependent matrix stiffening by undergoing a similar change in its own stiffness. This phenomenon, known as mechanical reciprocity, is exhibited by all adhesion-dependent cells, including ECs (18,32,40). Further, EC stiffening associated with higher cell contractility can, in turn, feed back to increase vascular stiffness (41). Taken together, these findings assert that reciprocal interaction between retinal ECs and the basement membrane (subendothelial matrix) actively promotes LOX-dependent stiffening of retinal capillaries in diabetes. Yet, surprisingly, a recent study found that the basement membrane of decellularized retinal vessels from human donor eyes is softer in diabetes (38). One plausible explanation for this contradictory finding is that the lack of retinal ECs leads to basement membrane flattening into a two-dimensional sheet where AFM indentation of the less–cross-linked and less-dense membranes from eyes from donors without diabetes may produce stiffness artifacts from the underlying hard glass surface, thereby increasing the measured stiffness values.

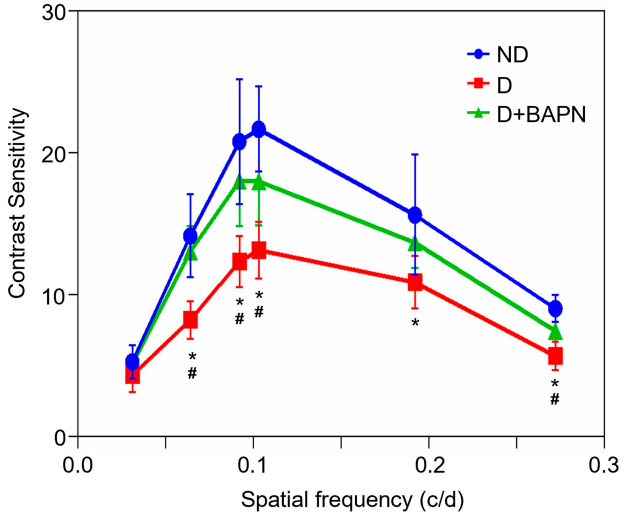

Figure 6—LOX inhibition mitigates the diabetes-induced loss of contrast sensitivity. Contrast sensitivity was assessed by subjecting mice to an optokinetic test. Quantitative analysis of their tracking response to moving grating lines of varying contrast at multiple spatial frequencies (0.031 to 0.272 cycles/degrees [c/d]) revealed that the substantial loss of contrast sensitivity seen in longer-term D mice can be significantly mitigated by LOX inhibitor BAPN (3 mg/kg). Line graphs indicate mean ± SD (n = 8 mice/group). *$P < 0.001$ for D vs. ND; #$P < 0.01$ for D vs. D+BAPN.

Our previous in vitro studies showed that LOX-dependent subendothelial matrix stiffening promotes retinal EC activation (12,15). Consistent with this, we found that prevention of LOX-dependent retinal capillary stiffening (using BAPN) in diabetic mice simultaneously prevented ICAM-1 upregulation and leukostasis. ICAM-1 is widely known to play a causal role in animal models of early DR, where blocking ICAM-1 prevents diabetes-induced leukostasis, EC death, and vascular leakage (2,27).

In contrast, VCAM-1 and E-selectin have not been strongly implicated in early DR pathogenesis. As we show here, this may be due to the unique ability of ICAM-1 (but not VCAM-1 and E-selectin) to be constitutively expressed at high levels in retinal ECs, becoming even more abundant in response to proinflammatory cytokines such as TNF-α.

Further, HRECs grown on synthetic matrices of varying stiffness revealed that LOX-dependent capillary stiffening is likely sufficient to promote ICAM-1–dependent retinal vascular inflammation. Interestingly, these studies on synthetic matrices also showed that matrix stiffening preferentially increases the levels of ICAM-1 but not VCAM-1 or E-selectin. However, these endothelial adhesion molecules are all upregulated by proinflammatory cytokines, such as TNF-α and IL-1β, that activate nuclear factor (NF)-κB (42). Thus, ICAM-1 expression appears to be uniquely regulated by both mechanical and cytokine (biochemical) signaling, likely via distinct (mechanotransduction) signaling pathway(s) that need to be properly elucidated. To this end, we recently showed that the matrix stiffening-dependent increase in ICAM-1 expression is prevented by NF-κB inhibition (15), thus indicating that the mechanical regulation of ICAM-1 expression is mediated by mechanosensitive NF-κB. However, matrix stiffness sensing involves various mechanotransduction pathways, including signaling via the integrins, cytoskeleton, and Hippo-signaling pathway (YAP/TAZ) (7). Future studies will aim to identify the role of these pathways in the mechanical regulation of retinal endothelial ICAM-1 expression. Further, it remains to be seen whether this distinct mechanochemical regulation of ICAM-1 occurs specifically in retinal ECs, which were recently shown to respond uniquely to diabetic stressors by exhibiting the greatest increase in endothelial ICAM-1 among the major vascular beds (43). Together with the upregulation of HREC-derived proinflammatory cytokines (TNF-α, IL-1β, and VEGF-A) seen on stiffer matrix, these findings identify LOX-dependent capillary stiffening as a previously unknown but major determinant of retinal vascular inflammation in DR.

Although the current findings reveal that LOX mechanically promotes retinal vascular inflammation, its own expression is enhanced by diabetes-associated retinal proinflammatory factors such as AGE and its receptor RAGE (15). This interdependence is believed to establish a feedforward mechanism that sustains the LOX-dependent mechanical regulation of retinal vascular inflammation and dysfunction during DR progression. Indeed, we show that mouse retinal capillaries remain stiffer (in a LOX-dependent manner) following prolonged diabetes, which promotes endothelial apoptosis and retinal capillary degeneration. Based on our HREC culture studies, this likely results from increased susceptibility of the stiffer retinal ECs to activated neutrophils that secrete neutrophil elastase, a major cytotoxic factor that is upregulated in diabetes. Vascular/EC stiffening has similarly been shown to increase EC susceptibility to other proinflammatory factors, such as lipopolysaccharide and complement factors, that cause vascular dysfunction associated with pulmonary edema and early age-related macular degeneration (4,19).

Finally, we show that the LOX inhibitor BAPN simultaneously reduces pericyte ghost formation and loss of contrast sensitivity in diabetic mice, thereby implying that LOX also contributes to diabetes-induced pericyte and neuronal dysfunction. Although these findings further underscore a crucial role for LOX in diabetes-induced retinal abnormalities, future studies will be needed to determine whether LOX exerts these (nonendothelial) effects directly via a cell-specific manner or indirectly via its mechanical regulation of retinal vascular inflammation, or both. These studies will be significant because whereas diabetes-induced pericyte loss is strongly implicated in the formation of acellular retinal capillaries (44), reduced contrast sensitivity reflects inner retinal (visual) dysfunction, a clinical hallmark of early DR (22). Also noteworthy is that the vascular protective effects resulting from systemic pharmacological LOX inhibition were comparable to those seen in heterozygous LOX-knockout mice (14), which provide important proof-of-concept for the development of novel LOX-targeting drugs for future clinical use.

In summary, our current findings have introduced a new paradigm for DR pathogenesis that identifies LOX-dependent vascular stiffening as a crucial and independent regulator of retinal vascular inflammation and degeneration in diabetes. A deeper understanding of the mechanical regulation of DR has the potential to identify entirely new classes of anti-inflammatory targets (e.g., LOX) for more effective DR therapies in the future. Successful validation studies in human donor eyes may also provide rationale to develop novel imaging techniques for clinical assessment of retinal capillary stiffness in the future.

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