Lysyl Oxidase Activity in the Ocular Tissues and the Role of LOX in Proliferative Diabetic Retinopathy and Rhegmatogenous Retinal Detachment

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Purpose. Lysyl oxidase (LOX) cross-links the side chain of collagen and elastin and thereby contributes to extracellular matrix (ECM) integrity. ECM remodeling is seen in various ocular diseases. Until now, there have been no reports on the LOX enzyme's activity in ocular tissues. The purpose of this study was to estimate LOX activity and expression in human donor ocular tissues and to measure the specific activity of LOX in the vitreous of proliferative diabetic retinopathy (PDR) and rhegmatogenous retinal detachment (RRD).

Method. Human donor eyeballs obtained from an eye bank were used to study tissue distribution of LOX. Human vitreous specimens were obtained during vitreoretinal surgery from PDR ($n = 16$) and RRD ($n = 10$). LOX activity was estimated by *N*-acetyl-3,7-dihydroxyphenoxazine assay, immunohistochemistry, and real-time polymerase chain reaction (RT-PCR). Matrix metalloprotease (MMP)-2 and -9 were quantified in the vitreous by sandwich enzyme-linked immunosorbent assay (ELISA).

Results. The specific activity of LOX in ocular tissues was on the order of vitreous, iris ciliary body, lens, choroid RPE, and retina, which were comparable by mRNA expression and immunolocalization. The vitreous level of LOX activity decreased significantly in PDR and RRD, with an increase in total MMP-2 and -9 levels compared with normal donor vitreous.

Conclusions. LOX activity showed a statistically significant decrease in the vitreous of PDR and RRD relative to control specimens. This effect can contribute to the inadequate collagen cross-linking that causes the ECM changes that occur in these diseases. (*Invest Ophthalmol Vis Sci.* 2008;49: 4746–4752) DOI:10.1167/iovs.07-1550

The extracellular matrix (ECM) represents a heterogeneous group of macromolecules, including collagen, noncollagenous glycoproteins, elastic fibers, and proteoglycans. The ECM is under constant remodeling by simultaneous degradation and synthesis of matrix components with different turnover rates. Various disease processes, such as inflammatory reactions and neovascularization, occur in the ECM.[1] The structural integrity of ECM depends on the collagen and elastin cross-links. During the formation of intermolecular cross-links, collagen fibers become increasingly insoluble and refractory to the action of enzymes and show a progressive increase in tensile strength,[2] which is essential for normal connective tissue function and wound healing.

Lysyl oxidase (LOX; EC 1.4.3.13) is a copper-dependent amine oxidase that initiates the covalent cross-linking of collagen and elastin in ECM. It is secreted as a glycosylated proenzyme with a molecular weight (Mw) of 50,000, which is proteolytically processed by procollagen C proteinase (bone morphogenic protein-1) into a mature, biologically active Mw 32,000 form.[3,4] Isoforms of LOX, called LOX-like proteins (LOXL)—namely, LOXL, -2, -3, and -4—have been identified, which are fully functional but genetically distinct.[5] Each member of the LOX protein family is characterized by a highly conserved amino acid sequence at their C-terminal end that includes the copper-binding site, residues for carbonyl cofactor formation, and the cytokine receptor–like domain. The conserved C-terminal domains contribute to amine oxidase activity, and the unique N-terminal domains may determine individual functions. Both LOX and LOXL catalyze the oxidative deamination of lysine residues in collagen and elastin.[6] β-Aminopropionitrile (BAPN) is a potent irreversible inhibitor of LOX that binds covalently to the active site of LOX, with an inhibitory constant (K_i) of 3 to 5 μM.[7] BAPN specifically inhibits all LOX isoenzymes except LOXL2.[8] BAPN has been studied for its therapeutic implications as a LOX inhibitor in liver fibrosis[9] and lung fibrosis[10] in animal models.

LOX and LOXL are colocalized in the skin, aorta, heart, lung, liver, cartilage, kidney, stomach, small intestine, colon, retina, ovary, testis, and brain in mouse tissues.[11] There are very few reports showing amine oxidase activity in bovine retina and sclera,[12] including a report on the presence of human retina–specific amine oxidase in the retinal ganglion cell layer.[13] However, very little information is available on LOX in human ocular tissues, in both normal and pathologic conditions.

In vitreoretinal diseases such as proliferative diabetic retinopathy (PDR) and rhegmatogenous retinal detachment (RRD), there is extensive ECM remodeling.[14] PDR is a common complication of diabetes mellitus characterized by preretinal neovascularization and development of epiretinal fibrovascular traction and retinal detachment.[15] RRD is a complex wound-healing pathobiology of proliferative vitreoretinopathy (PVR).[16] Wound healing in PVR in general, involves inflammation, extracellular matrix deposition and tissue remodeling. The matrix metalloproteinases (MMPs), a ubiquitous family of enzymes, are known to play a role in the degradation of the ECM.[17] MMP 2 (72-kDa gelatinase) is constitutively found in normal human vitreous, where it is complexed with TIMP-2[18]

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and MMP 9 (92-kDa gelatinase) is constitutively expressed in the retinal ganglion cell layer.[19] These MMPs degrade denatured collagen (gelatin) and type IV collagen. Although many MMPs have been reported in retinal disease,[14] characteristic changes in the levels of MMP-2 and -9 activity attributing to ECM remodeling have been reported in both PDR[20–22] and RRD.[23,24] However, there are no reports on the LOX activity in the vitreous during ECM remodeling.

Hence, in the present study, we determined the ocular tissue distribution of LOX in human donor eye balls. We estimated LOX activity in the vitreous of PDR and RRD, in light of the changes seen in the levels of MMP-2 and -9.

Materials and Methods

Donor Eyes

All experiments involving human subjects adhered to the tenets of the Declaration of Helsinki. Human donor eye balls obtained from the CU Shah Eye Bank (Sankara Nethralaya, India), were used after light microscopic examination and cornea removal (age 65 ± 9, years; seven male; three female), for determining specific activity of LOX ($n = 10$), real-time gene expression, and immunohistochemistry ($n = 3$). Donors with a history of diabetes, hypertension, carcinoma, and sepsis were not included in the study. The donors had no history of ocular disease. All fine chemicals used were of laboratory grade unless specified.

Preparation of Tissue Extracts

The eyeball was dissected and the iris, ciliary body, vitreous, retina, and choroid/RPE were separated within 5 hours of death. The tissues were homogenized in 4 M urea in 0.02 M borate buffer (pH 8.0) at 4°C, whole vitreous jelly was homogenized at 4°C and all homogenate was centrifuged at 15,000*g* for 30 minutes at 4°C. The supernatants were analyzed immediately for LOX assay with and without BAPN.[25]

Activity assays for detection of LOX were performed as previously described.[26] Briefly, 100 μg of protein was added to the final reaction mixture (50 mM sodium borate [pH 8.2], 1.2 M urea, 50 μM *N*-acetyl-3,7-dihydroxyphenoxazine (Amplex Red; Molecular Probes-Invitrogen), 0.1 U/mL horseradish peroxidase, and 10 mM 1,5-diaminopentane substrate) in the presence or absence of 500 μM BAPN.

LOX acts on diaminopentane (pseudosubstrate) and produces H_2O_2. Horseradish peroxidase catalyzed oxidation of *N*-acetyl-3,7-dihydroxyphenoxazine by H_2O_2 produces fluorescent resorfurin, which is measured at an excitation of 563 nm and emission of 587 nm. The fluorescence was read for every 10 minutes for 2 hours at 37°C using a multimode plate reader (Triad; Dynex Technologies, Chantilly, VA). A standard graph was constructed using H_2O_2 (0.2–1.0 μM). The relative fluorescence unit obtained in test samples was calculated based on the standard and was expressed as specific activity of LOX (micromoles H_2O_2/minute/milligram protein). The interassay coefficient of variation (CV) was 15.4%.

RNA Extraction and Reverse Transcription for LOX

The eyeball was dissected and iris, ciliary body, (iris and ciliary body were processed separately unlike in activity assay) retina, choroid along with RPE were separated within 2 hours of death. Total RNA was extracted from different ocular tissues by the guanidine isothiocyanate and chloroform method (TRI Reagent; Sigma-Aldrich, St. Louis, MO). All RNA samples were treated with DNase (TURBO; Applied Biosystems/Ambion, Austin, TX) to remove DNA contamination. For all samples, 1 μg of total RNA was used to synthesize first-strand cDNA (SuperScript II reverse transcriptase; Invitrogen, Carlsbad, CA) and random primers.

Real-Time RT-PCR Analyses

Gene expression assays for LOX (Hs00184706_m1; *Taq*Man) and two endogenous controls, GAPDH (Hs99999905_m1) and TBP (Hs99999910_m1), were obtained from Applied Biosystems (ABI; Foster City, CA). Quantification of gene expression was performed in a 20-μL volume in 96-well plates on a real-time PCR system (Prism 7300; ABI) in triplicate. Each reaction included 1× primer probe mix (*Taq*Man; ABI), 1× universal PCR master mix (*Taq*Man; ABI), and 100 ng cDNA. Cycling conditions were as follows: 2 minutes at 50°C, 10 minutes at 95°C, and 40 cycles of 15 seconds at 95°C plus 1 minute at 60°C. The retina was used as the calibrator for comparison with other ocular tissues. The normalization factor for each sample was calculated by taking the geometric mean of the two housekeeping genes (GAPDH and TBP) quantities using geNorm software (developed by Vandesompele J, et al., Center for Medical Genetics, Ghent University Hospital, Ghent Belgium and available at http://medgen.ugent.be/genorm/). Expression levels of the gene of interest (GOI) were calculated by dividing the GOI quantities for each sample by the appropriate normalization factor.[27]

Immunohistochemistry for LOX

Paraffin sections (5 μm) of donor eyeball were incubated with trypsin-EDTA for 30 minutes and washed with TBS. Further steps were performed with a polymer detection system (Novolink Min; Novacastra Laboratories Ltd., Newcastle-upon-Tyne, UK). The slides were blocked for peroxidase to avoid nonspecific binding. The slides were then incubated with a 1:200 diluted rabbit polyclonal antibody directed against human LOX followed by a polylink, as described by the suppliers. The sections were stained with 0.8% amino ethyl carbazole in acetate buffer (pH 5.0; Sigma-Aldrich, St. Louis, MO) or 3% diaminobenzidine. The sections were counterstained with hematoxylin. In negative control specimens, the entire procedure was followed with omission of primary antibody.[28]

Processing of Vitreous Specimens for LOX Assay

Undiluted vitreous samples from 16 patients (age, 54.9 ± 11.6 years: 15 men; 1 woman) with PDR and 10 patients (age 47.7 ± 16.3 year; 9 men and 1 woman) with RRD were collected by the vitreoretinal surgeon at the time of surgery. Clinical details of the patients are given in Tables 1 and 2. The samples were transported on ice and centrifuged at 3000 rpm for 10 minutes at 4°C. The centrifuged samples were frozen at −80°C until they were assayed with correspondingly stored control specimens. Control vitreous specimens were obtained from donor eyeballs $n = 23$ (age, 70 ± 11 years; 12 male and 11 female) from the CU Shah Eye Bank. The vitreous was aspirated with a syringe similar to that used in vitrectomy. Patients with a history of diabetes, hypertension, carcinoma, and sepsis were not included in the study. Care was taken to collect vitreous within 5 hours of death from donor specimens. Specific activity of total LOX was measured as described and normalized to levels of total vitreous protein in each sample.

ELISA Assay for Total MMP-2 and -9 in Vitreous

Total MMP-2 and -9 protein concentrations were determined in the vitreous specimen from PDR, RRD, and donor eyeballs by quantitative sandwich enzyme immunoassays according to the manufacturer's instructions (R&D Systems, Minneapolis, MN). The minimum detectable dose for MMP-2 ranged from 0.03 and 0.40 ng/mL and for MMP-9, <0.156 ng/mL. The ratio of MMP-9 to -2 was determined, to establish the disease background documented earlier at the level of MMPs.[23]

Statistics

With SPSS software (version 14.0; SPSS, Chicago, IL) the raw data were analyzed for statistical significance with the independent-samples *t*-test. $P < 0.05$ was considered significant.

Results

Ocular Tissue Distribution of LOX

Figure 1 shows that the specific activity of LOX was greatest in the vitreous, 1.29 ± 0.33 micromoles/min/mg protein; fol-

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Table 1. Clinical Details of Patients with PDR

Subject	Age (y)/Sex	Stage of the Disease	Duration of Diabetes (y)	Patent Vessels	Vitreous Hemorrhage	Retinal Detachment
1	55/M	Advanced with macular hole	10	FVP over retina; active	−	No
2	63/M	Advanced	30	FVP over retina; active	+	Combined RD
3	48/M	Advanced with macular hole	13	FVP over retina; active	+	No
4	34/M	Active	10	No	+	Tractional band
5	67/M	Active	30	No	+	No
6	53/M	Active	24	FVP over retina; active	+	No
7	64/M	Active	6	FVP over retina; active	+	No
8	37/M	Advanced	7	FVP present	−	Tractional RD
9	54/M	Inactive	15	No	−	No
10	65/F	Active	10	FVP over retina; active	+	No
11	65/M	Active	5	No	−	Tractional RD
12	48/M	Advanced	20	No	+	RD
13	64/M	Advanced	3	FVP over retina; active	+	Combined RD
14	52/M	Active	15	FVP over disc	−	Tractional RD
15	39/M	Advanced	6	FVP over retina; active	+	No
16	68/M	Active	15	FVP present; active	+	No

Clinical profile of patients with PDR (n = 16). Fibrovascular proliferation (FVP) and vitreous hemorrhage were present in 11 cases and retinal detachment in 5 cases.

lowed by the iris/ciliary body (ICB), 1.15 ± 0.36 micromoles/min/mg protein; lens, 0.4 ± 0.07 micromoles /min/mg protein; choroid/RPE (CRPE), 0.35 ± 0.06 micromoles/min/mg protein; and retina, 0.2 ± 0.03 micromoles/min/mg protein.

Real-Time PCR Analyses

mRNA quantification of LOX expression using a gene expression assay (*Taq*Man; ABI) after normalizing with two endogenous controls (GAPDH and TBP) showed the least expression in retina compared with iris, choroid RPE and ciliary body. (Figs. 2A–C)

Immunohistochemistry

Immunolocalization of LOX (Fig. 3) showed cytoplasmic and stromal positivity in iris, ciliary body, lens epithelial cells, choroid with RPE, optic nerve head and retinal photoreceptor cells. The negative control showed no reaction.

Therefore in control eyeballs, the specific activity, mRNA expression, and localization of LOX in different ocular tissues were relatively similar.

Specific Activity of LOX in Vitreous Specimens Obtained from PDR and RRD

The level of total protein in the vitreous was increased threefold in PDR (3.30 ± 0.46 mg/mL) and ninefold in RRD (9.10 ± 2.76 mg/mL) when compared with the control (1.28 ± 0.14 mg/mL) which is considered a measure of the retinal barrier permeability, as observed earlier by Descamps et al.[29] The specific activity of LOX, with and without the specific inhibitor BAPN, was analyzed and is expressed as micromoles per minute per milligram protein. In control specimens, the mean specific activity of total LOX was found to be 0.675 ± 0.17 micromoles/min/mg protein with the range from 0.022 to 2.93 micromoles/min/mg protein. Relative to the control the specific activity of total LOX showed a fourfold decrease in RRD (P = 0.010, mean of 0.164 ± 0.06; range, 0.01–0.484 micromoles/min/mg protein). Similarly, in PDR the specific activity of total LOX showed a 3.5-fold decrease (P = 0.014) compared with the control (mean, 0.197 ± 0.04 micromoles/min/mg protein; range, 0.017–0.702 micromoles/min/mg protein) In summary, the specific activity of total LOX showed a strong and statistically significant (3- to 4-fold) decrease in PDR and RRD, relative to the control. In both the control and diseased vitreous, the BAPN inhibited LOX activity was found to be nearly 50% of total LOX activity in the control, 43% in PDR, and 50% in RRD (Table 3).

Ratio of MMP-9 to -2 in Vitreous Specimens Obtained from PDR and RRD

The mean MMP-9 to -2 ratio was highest in the vitreous samples of patients with PDR (0.027 ± 0.018; P = 0.000) followed by RRD (0.013 ± 0.011; P = 0.004) when compared with the

Table 2. Summary of Patient Profile with RRD

Subject	Age (y)/Sex	PVR Grade	Quadrants Involved	Macula Detached	Vitreous Hemorrhage	Complications after Surgery	Resurgery
1	56/M	Not done	4	Yes	+	Nil	No
2	38/M	Not done	4	Yes	−	Nil	No
3	50/M	Not done	3	Yes	−	Preretinal hemorrhage	Had phaco IOL + SOR
4	22/M	Not done	4	Yes	−	Nil	No
5	20/M	C	4	Yes	−	Nil	No
6	52/M	A	4	Yes	−	Nil	No
7	56/F	Not done	1	No	−	Nil	No
8	57/M	A	4	Yes	−	Nil	No
9	72/M	Not done	3	Yes	+	Macular hole	No
10	54/M	Not done	3	Yes	−	Redetachment	Yes

Clinical profile of patients with RRD (n = 10). Vitreous hemorrhage was present in two cases. IOL, intraocular lens; SOR, silicone oil removal.

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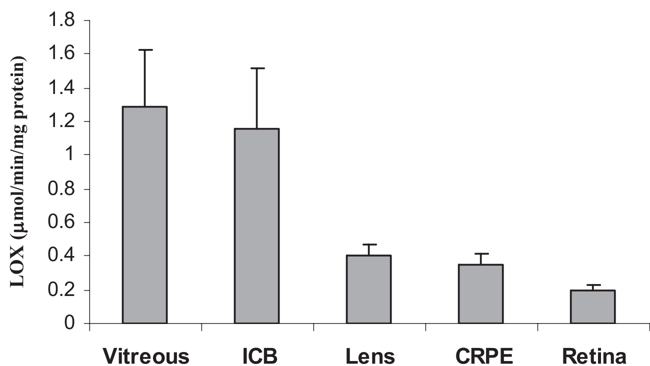

FIGURE 1. Specific activity of total LOX in ocular tissues ($n = 10$). Data are expressed as the mean ± SEM.

control (0.003 ± 0.005). In addition, the mean MMP-9 to -2 ratio from patients with PDR was significantly higher than in patients with RRD ($P = 0.035$).

DISCUSSION

The present study was prompted by the lack of information on the enzyme LOX in ocular tissues. Collagen, the substrate for LOX is the most abundant connective tissue protein in vertebrates. The metabolism of collagen and its regulation are of vital interest in several clinically important diseases that are characterized by excessive matrix synthesis, degradation, or remodeling. However, there is little knowledge on the levels and distribution of LOX, an enzyme that mediates collagen cross-linking in ocular tissues. Imamura et al.[13] have reported human retina specific amine oxidase in human retina; and, recently, Hewitt et al.[30] have reported expression of LOXL1 mRNA and protein in human donor ocular tissue. In the present study, a detailed distribution profile of LOX activity in ocular tissues from normal donor eyeballs was obtained. This study revealed the presence of LOX in the various ocular tissues in terms of specific activity, localization, and mRNA expression. Vitreous showed the highest specific activity among the ocular tissues studied. This distribution profile of LOX was found to be similar to the collagen content in the ocular tissue reported by Siddiqi et al.[31] The variation in LOX distribution and activity could be reflective of the inherent differences in the collagen structure and composition in these tissues. Therefore, the present study helps in understanding the ocular tissues further with respect to the extent of collagen cross-linking activity. Retina reportedly had the lowest content of soluble and insoluble collagen, which we found to correlate with the lowest LOX expression.

Despite progress in the treatment of PDR and PVR, these vitreoretinal diseases continue to be major causes of visual impairment. Changes in the activity of MMP-2 and -9 have already been documented in both PVR and PDR. Although a low activity of MMP-2 is constitutively present in the vitreous, the MMP-9 activity is reported to be increased in both PDR and PVR.[20–24] The ratio of total MMP-9 to -2 as determined in this study (Fig. 4) shows that it is similar with that reported by Abu El-Asrar et al.,[23] which was highest in PDR followed by RRD.

Although the depredatory enzyme MMP has been detected in the vitreous, fibrovascular, and neovascular membranes, LOX may also play a crucial role in the ECM changes, this possibility has not been looked into. This study shows that there is a significant decrease in the level of LOX-specific activity that is possibly associated with the ECM remodeling. This is the first report showing a significant decrease in LOX-specific activity in the vitreous of PDR and RRD.

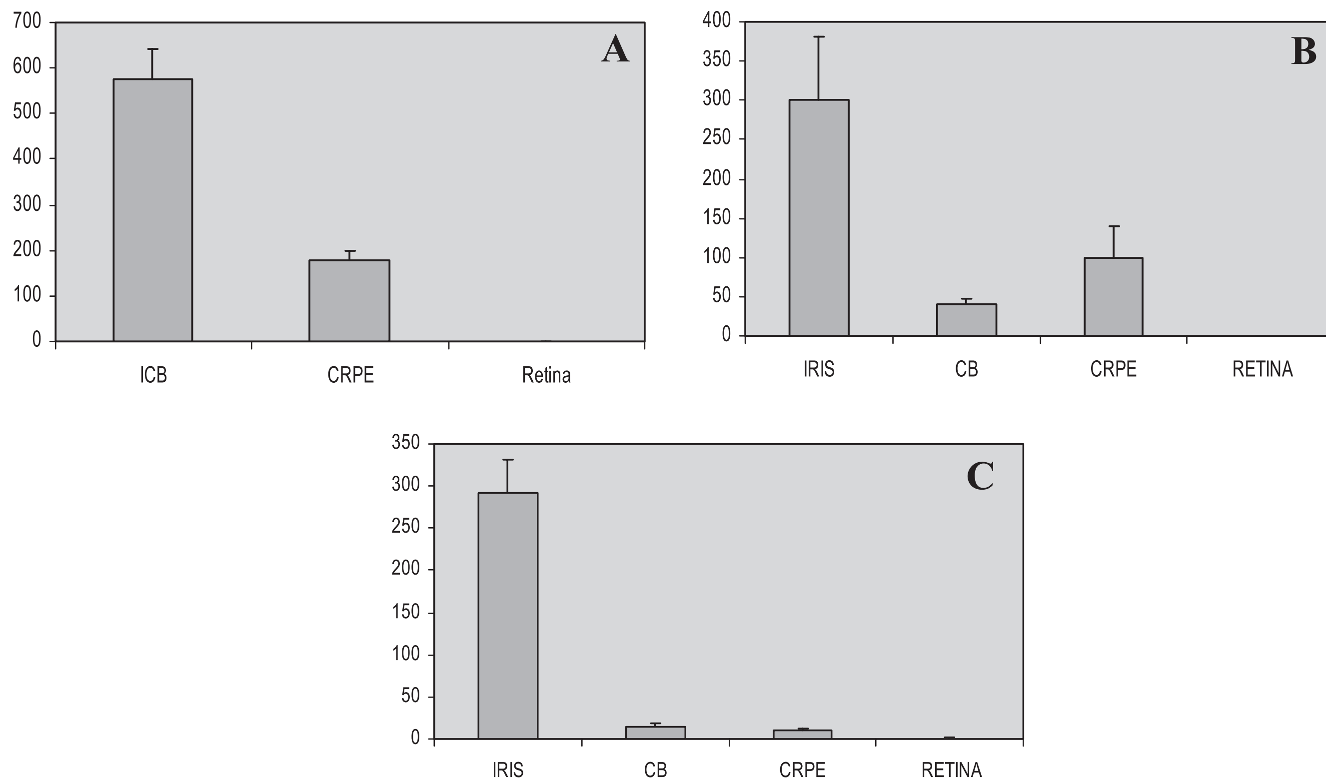

FIGURE 2. LOX mRNA expression in human ocular tissues calibrated to retina. Error bars represent SE of the relative expression levels, normalized against GAPDH and TBP. (**A–C**) LOX expression in individual eye balls.

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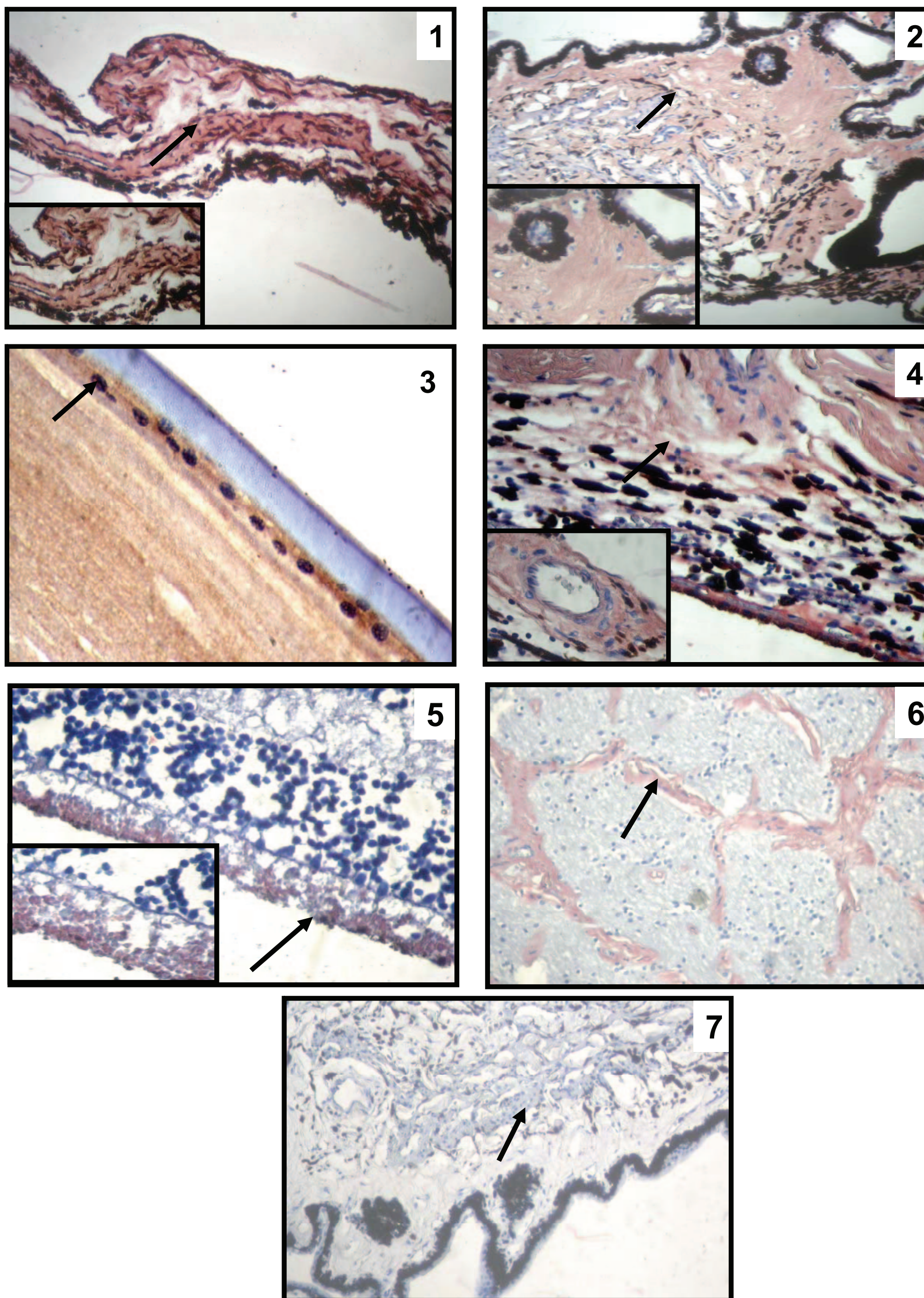

Figure 3. Immunolocalization of LOX protein in paraffin-embedded sections of normal human donor ocular tissue detected by streptavidin biotin labeling. *Arrow*: positive labeling (*reddish brown*) of the iris (**1**), ciliary body (**2**), lens (**3**), CRPE (**4**), retina (**5**), optic nerve head (**6**), and negative control (**7**). Hematoxylin was used as the counterstain. *Inset*: magnification of area indicated by *arrow*. Magnification, ×100.

In PDR, immature collagen cross-linking is reported due to the nonenzymatic glycosylation of collagen as sugars can compete for the same lysine and hydroxylysine residues that serve as substrates for LOX, and there could be substrate unavailability.[32] It has been reported that glucose inhibits collagen fibril formation in vitro.[33] The degree of decreased collagen fibril

Table 3. Specific Activity of LOX in Vitreous Specimens of PDR and RRD

Conditions	Protein (mg/mL)	Specific Activity of LOX (μmol/min/mg Protein)	BAPN Inhibited LOX (μmol/min/mg Protein)
Control (n = 23)	1.28 ± 0.14	0.675 ± 0.173	0.336 ± 0.067
PDR (n = 16)	3.30 ± 0.46*	0.197 ± 0.049†	0.114 ± 0.028†
	P = 0.002	P = 0.014	P = 0.005
RRD (n = 10)	9.10 ± 2.76*	0.164 ± 0.060*	0.083 ± 0.030*
	P = 0.020	P = 0.010	P = 0.002

Data are expressed as the mean ± SEM. *P,* control vs. *RRD and †PDR.

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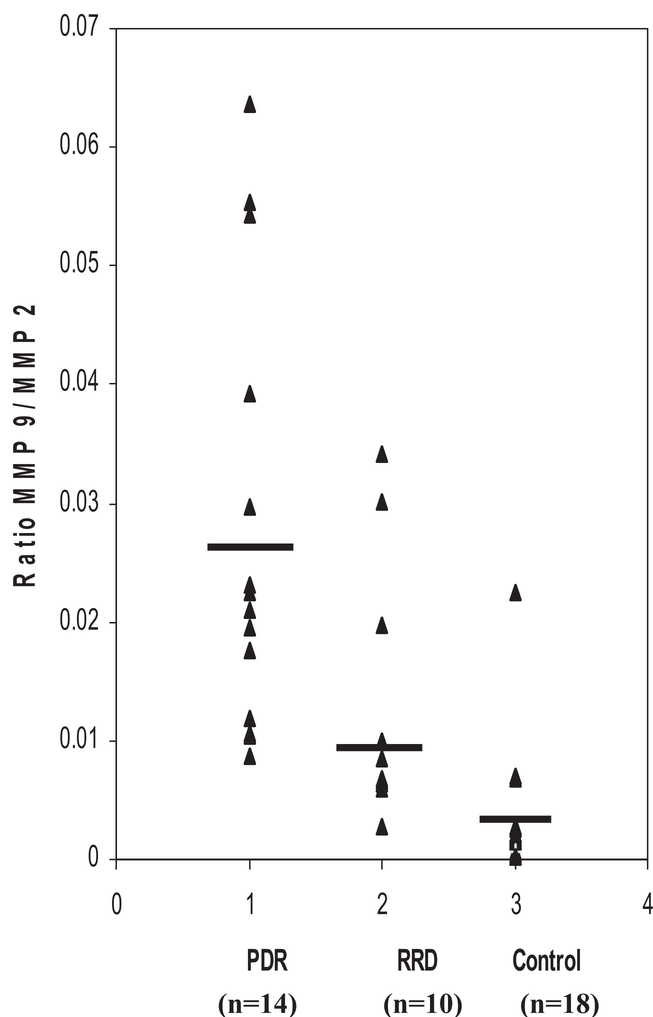

Figure 4. Ratio of MMP-9 to -2 in vitreous samples of patients with PDR or RRD and of control donors. *Horizontal lines*: mean ratio.

formation has been shown to correlate with the loss of ability of collagen to serve as a substrate for LOX.[33] Despite collagen turnover, the collagen fibrils may not be adequately cross-linked, owing to the decreased LOX activity as observed in the study. However, additional work is needed to determine the relative roles of nonenzymatic glycosylation versus decreased LOX activity, with respect to immature collagen cross-linking in PDR.

Decreased LOX activity was also observed in the vitreous of patients with RRD, compared with control subjects. The decrease in LOX activity in RRD is intriguing, as these patients were nondiabetic. LOX expression has been shown to be regulated by transforming growth factor β,[34] tumor necrosis factor (TNF)-α,[35] platelet-derived growth factor retinoic acid, and fibroblast growth factor.[6] Endothelial dysfunction induced by TNF-α has been shown to be associated with a decrease in LOX expression as studied in human umbilical vein endothelial cells and porcine aortic endothelial cells.[36]

Notably Pischon et al.[37] have also found that TNF-α inhibits the expression and activity of LOX in osteoblasts cultures but does not inhibit collagen synthesis, thereby contributing to perturbed collagen cross-linking and accumulation.[37] TNF-α is reported to be increased in the vitreous of PDR and RRD. It is produced locally as an inflammatory response.[38,39] Although PVR and PDR have different causes and clinical characteristics, retinal membranes from both conditions share the features of fibroplasia, excessive matrix protein deposition, and cellular infiltration. The decrease in LOX could be due to growth factors that are common in both PDR and RRD. However, more studies are needed to aid in understanding the regulation of LOX by growth factors in these diseases.

The only other report showing the relevance of LOX in ocular disease comes from a recent study conducted in a LOXL1-knockout mouse model in which there is defective elastin fiber that has been associated with increased susceptibility to laser induced choroidal neovascularization.[40] In the present study, we report that there was decreased LOX activity in the vitreous of eyes with PDR and RRD as part of the altered ECM activity. We hypothesize that the decrease in LOX activity contributes to inadequate or improper collagen cross-linking and increased proteolysis due to elevated levels of MMPs causing a net vitreous degradation leading to liquefaction. For a deeper understanding of the role of LOX, additional structure–function studies have to be performed at the level of collagen in the vitreoretinal diseases such as PDR and RRD.

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