Diabetic retinopathy, the leading cause of vision loss in the working-age population, is characterized by early vascular lesions such as the formation of acellular capillaries (AC) and pericyte loss (PL), which can contribute to retinal dysfunction. Studies suggest that retinal vascular basement membrane (BM) thickening, a histologic hallmark of diabetic retinopathy, may promote apoptosis and thus lead to vascular cell loss. Our recent study suggests that lysyl oxidase (LOX), a cross-linking enzyme critical for the development and maturation of the BM, is upregulated in retinal endothelial cells (RECs) grown in high-glucose (HG) medium and in diabetic rat retinas. Moreover, excess LOX has been shown to be involved in promoting endothelial cell monolayer permeability. However, the involvement of LOX in mediating HG-induced apoptosis and subsequent retinal vascular cell loss is still largely unknown.

LOX is synthesized as a 50-kD, N-glycosylated proenzyme (pro-LOX), which undergoes proteolytic cleavage resulting in a 32-kD, catalytically active, and mature enzyme (LOX). The integrity, stability, and functionality of the BM are largely dependent on proper cross-linking of collagen as these cross-links contribute to the physical and mechanical properties of collagen fibrils in forming a stable BM assembly. LOX plays a critical role in the posttranslational modification of collagen to form covalent cross-links that stabilize insoluble collagen, thus forming a functional extracellular matrix (ECM). However, excess cross-linking mediated by LOX can lead to BM thickening and compromised functionality due to the formation of disorganized assembly of the collagen fibrils, promoting abnormal ECM accumulation in fibrotic diseases. As the BM undergoes histologic and biochemical changes induced by HG insult, the development of AC and PL is exacerbated by the abnormally thickened BM, ultimately progressing to early-stage diabetic retinopathy.

While LOX is primarily known for its role in cross-linking, recent studies indicate that increased LOX levels can promote apoptosis. LOX upregulation may compromise the AKT signaling pathway in human osteosarcoma cells and ras-transformed NIH 3T3 cells, thereby promoting apoptosis. LOX was also identified as a tumor suppressor gene, further suggesting that LOX overexpression may trigger apoptosis. However, it is currently unknown whether LOX overexpression...
promotes retinal vascular cell loss associated with diabetic retinopathy.

The present study investigated whether HG-induced abnormal LOX overexpression may contribute to apoptosis, and whether reducing HG-induced LOX overexpression may facilitate AKT pathway activity and thereby prevent apoptosis in retinal endothelial cells. Additionally, to determine whether changes seen in LOX expression under HG condition were present in vivo, the retinas of LOX\textsuperscript{+/−} mice as well as those of streptozotocin (STZ)-induced diabetic mice were examined.

**METHODS**

**Cell Culture**

Capillary endothelial cells derived from rat retinas (RRECs) confirmed positive for von Willebrand factor (vWF) were used in this study, and were isolated as previously described.\textsuperscript{50} To determine the effect of abnormal LOX overexpression and apoptosis, RRECs were grown in normal (N, 5 mM glucose) or high-glucose (HG, 30 mM glucose) Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (Sigma-Aldrich Corp., St. Louis, MO, USA), antibiotics, and antimycotics for 7 days. To determine the effect of reducing LOX overexpression on cell viability, RRECs were grown in HG medium and transfected with LOX small interfering RNA (siRNA), or scrambled (Scram) siRNA as control. Cells from the experimental groups were then assayed for LOX expression, AKT activity, and caspase-3 activation.

**Animals**

All animal studies were performed in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Twelve wild-type (WT) C57/BL6 albino male mice (Harlan Lab, Inc., Indianapolis, IN, USA) and 12 LOX\textsuperscript{+/−} mice bred into the C57/BL6 albino background kindly provided by Robert Mecham\textsuperscript{31} were used in the study. Genotypes were determined by polymerase chain reaction (PCR) at weaning using tail tip DNA, and then again at the time animals were killed. PCR reactions were performed with a PCR enzyme blend (PCR Master Mix; Promega, Madison, WI, USA) and included the following primers: primer 1, 5’-ACGGCCTTGTGAACGAAA-3’, primer 2, 5’-TGAATGACTG CAGGACGAG-3’, primer 3, 5’-ATCTGATGTCGGCGTCTTC-3’; primer 4, 5’-AGTCCAGGAGACTAAAGA-3’. Primers 1 and 2 amplify an approximately 1500-bp fragment from the LOX\textsuperscript{+/−} allele. Primers 3 and 4 amplify a 1022-bp fragment from the WT LOX allele. The LOX\textsuperscript{−/−} genotype was not used because it is perinatal lethal.\textsuperscript{31,32}

Six WT mice and six LOX\textsuperscript{+/−} mice were injected intraperitoneally with STZ (55 mg/kg body weight) to induce diabetes. The glucose concentrations in blood and urine were checked after 2 or 3 days following STZ injection to confirm diabetes status in the animals. The remaining six WT and six LOX\textsuperscript{+/−} animals served as nondiabetic controls. Blood glucose levels were measured in each animal two or three times weekly and at the time of death. The diabetic group represented mice with abnormal LOX overexpression after 2 or 3 days following STZ injection to confirm diabetes status in the animals. The remaining six WT and six LOX\textsuperscript{+/−} mice as well as those of streptozotocin (STZ)-induced diabetic mice were examined.

**Cell Transfection With LOX siRNA and β-Aminopropionitrile (BAPN) Treatment**

To determine the effect of reduced LOX expression on cell survival, RRECs were grown in HG medium and transfected with 33 nM LOX siRNA (Santa Cruz Biotechnology, Santa Cruz, CA, USA), or 20 μM Scram siRNA (Qiagen, Valencia, CA, USA) as a negative control in the presence of 8 μM Lipofectin (Invitrogen, Carlsbad, CA, USA). In this study, an optimal concentration of 33 nM LOX siRNA was used to achieve approximately 40% reduction in LOX expression in RRECs, as supported by our previous study.\textsuperscript{16} Additionally, to determine whether LOX activity influences cell survival, cells were exposed overnight to 500 μM BAPN (Sigma-Aldrich Corp.), a potent inhibitor of LOX activity,\textsuperscript{33} 24 hours prior to harvest.

**Western Blot Analysis**

To determine the expression of LOX, AKT, AKT phosphorylation, and caspase-3 activation, protein isolated from RRECs grown in N or HG medium were subjected to WB analysis. Similarly, protein samples isolated from diabetic or nondiabetic mouse retinas were subjected to WB analysis to examine LOX, AKT, AKT phosphorylation, cleaved caspase-3, and Bax expression. RRECs grown in N or HG medium, or retinal tissues of diabetic or nondiabetic mice, were washed with PBS and lysed in buffer containing 10 mM Tris, pH 7.5 (Sigma-Aldrich Corp.), 1 mM EDTA, and 0.1% Triton X-100 (Sigma-Aldrich Corp.) to yield total protein. Lysates were centrifuged at 13,000g for 20 minutes at 4°C. Protein samples from cell lysates and retinal tissues were then measured by bicinchoninic acid protein assay (Pierce Chemical, Rockford, IL, USA). An equal amount of protein (20 μg) was loaded in each lane and electrophoresed together with molecular weight standards (Bio-Rad, Hercules, CA, USA) in separate lanes on a 10% SDS-polyacrylamide gel. After electrophoresis, the proteins were transferred onto PVDF membranes (Millipore, Billerica, MA, USA) according to Towbin’s procedure\textsuperscript{34} using a semi-dry apparatus. The membrane was blocked with 5% nonfat dry milk for 2 hours and incubated overnight at 4°C with rabbit polyclonal LOX antibody (1:2000, Catalog No. NB110; Novus, Littleton, CO, USA), rabbit polyclonal Ser473 phosphorylated AKT (p-AKT) antibody (1:2000, Catalog No. 9271; Cell Signaling, Danvers, MA, USA), AKT antibody (1:1000, Catalog No. 9272; Cell Signaling), cleaved caspase-3 antibody (1:1000, Catalog No. 9661; Cell Signaling), or Bax antibody (1:500, Catalog No. 2772; Cell Signaling) solution in Tris-buffered saline containing 0.1% Tween-20 (TBBS) and 5% BSA. The following day, the membrane was washed with TTBS and incubated with a secondary antibody solution containing anti-rabbit IgG, AP-conjugated antibody (1:3000, Catalog No. 7054; Cell Signaling) for 1 hour in room temperature. After washing with TTBS, the membrane was subjected to Immuno-Star chemiluminescent substrate (Bio-Rad) and exposed to X-ray film (Fujifilm, Tokyo, Japan). The amount of protein loaded in the gel lanes was confirmed through Ponceau-S staining after transfer and by β-actin antibody (1:1000, Catalog No. 4967; Cell Signaling). To determine LOX, p-AKT, AKT, cleaved caspase-3, Bax, and β-actin protein expression, densitometric analysis of the chemiluminescent signal was performed at nonsaturating exposures and analyzed using ImageJ software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD, USA).

**Differential Staining Assay to Identify Apoptotic Cells**

To identify apoptotic cells, a differential dye staining method\textsuperscript{35} was performed, which relies on the uptake of fluorescent dyes,
acridine orange (AO) and ethidium bromide (EB). The condition of the cell membrane integrity and the properties of the DNA binding dyes facilitate the distinction of viable versus early- or late-stage apoptotic cells. RRECs grown on coverslips as specified in the experimental conditions were exposed to a dye mixture containing 25 µg/mL ethidium bromide (Catalog No. E-8751; Sigma-Aldrich Corp.) and 25 µg/mL acridine orange (Catalog No. A-64014; Sigma-Aldrich Corp.) for 10 minutes, washed with PBS, fixed, and mounted in SlowFade Antifade Kit (Catalog No. S2828; Invitrogen, Eugene, OR, USA). The cells were then visualized using a 4',6-diamidino-2-phenylindole (DAPI) filter, and imaged using a digital camera attached to a fluorescence microscope (Nikon Diaphot, Tokyo, Japan). Ten random fields of approximately 1000 cells/field per sample were counted. Data are pooled from four independent experiments. The number of apoptotic cells per field was expressed as a percentage of the total number of cells in the field, also known as the apoptotic index. Apoptotic cells appear orange or bright green while viable cells appear uniformly dark green.

**Statistical Analysis**

All data are expressed as mean ± standard deviation (SD). Values of the control groups were normalized to 100%, and values from all other groups were expressed as percentages of control. Statistical analysis was performed using the normalized values. Comparisons between groups were performed using 1-way ANOVA followed by Bonferroni’s post hoc test. A level of P < 0.05 was considered statistically significant.

**RESULTS**

**Effect of HG on LOX Protein Expression in RRECs**

RRECs grown in HG medium had significantly increased LOX expression compared to cells grown in N medium (163 ± 23% of N versus 100 ± 17% of N; P < 0.05; n = 4; Figs. 1A, 1B). In addition, cells grown in HG medium and transfected with LOX siRNA showed significantly reduced LOX expression compared to cells grown in HG alone (124 ± 8% of N versus 163 ± 23% of N; P < 0.05; n = 4; Figs. 1A, 1B). As expected, cells grown in HG medium transfected with Scram siRNA did not show a significant difference in LOX expression compared to cells grown in HG medium alone (165 ± 12% of N versus 163 ± 23% of N; P > 0.05; n = 4; Figs. 1A, 1B).

**Effect of HG and LOX Downregulation on AKT Activity and Caspase-3 Activation in RRECs**

Western blot analysis indicated that the ratio of p-AKT to total AKT was significantly decreased in cells grown in HG medium compared to those grown in N medium (65.8 ± 12.3% of N versus 100 ± 7.01% of N, P < 0.05; n = 4; Figs. 1A, 1C). Interestingly, reducing LOX overexpression improved the ratio of p-AKT to AKT, indicating that AKT activity was restored. Cells grown in HG medium and transfected with LOX siRNA showed a significantly improved ratio of p-AKT to AKT compared to cells grown in HG alone (90.1 ± 12.9% of N versus 65.8 ± 12.3% of N; P < 0.05; n = 4; Figs. 1A, 1C). As expected, cells grown in HG medium transfected with Scram siRNA did not show a significant difference in the ratio of p-AKT to AKT compared to cells grown in HG medium alone (68.9 ± 11.4% of N versus 65.8 ± 12.3% of N; P > 0.05; n = 4; Figs. 1A, 1C). Moreover, cells grown in HG medium exhibited significantly increased caspase-3 activation (153.0 ± 27.1% of N; P < 0.05; n = 4; Figs. 1A, 1D). Importantly, cells grown in HG medium and transfected with LOX siRNA showed reduced caspase-3 activation compared to cells grown in HG medium (102.3 ± 11.4% of N; P < 0.05; n = 4; Figs. 1A, 1D).

**Reduced LOX Expression and Activity Protect Against HG-Induced Apoptosis in RRECs**

Differential dye staining indicated that the cells grown in HG medium showed significantly increased number of apoptotic cells compared to those grown in N medium (4.10 ± 0.53 cells per 100 cells versus 1.85 ± 0.14 cells per 100 cells; P < 0.05; n = 4; Figs. 2A, 2B, 2E). Interestingly, cells grown in HG medium transfected with LOX siRNA exhibited a significantly reduced number of apoptotic cells compared to cells grown in HG medium alone (2.74 ± 0.26 cells per 100 cells versus 4.10 ± 0.53 cells per 100 cells; P < 0.05; n = 4; Figs. 2B, 2C, 2E). RRECs grown in HG medium transfected with Scram siRNA did not show a significant difference in the number of apoptotic cells compared to cells grown in HG medium alone (4.15 ± 0.16 cells per 100 cells versus 4.10 ± 0.53 cells per 100 cells; P > 0.05; n = 4; Figs. 2C, 2D, 2E).

**Reduced LOX Activity Rescues AKT Activity and Protects Against HG-Induced Apoptosis in RRECs**

To determine whether reduced LOX activity alters AKT activity and influences cell survival, WB analysis and differential dye staining assay were performed. Interestingly, cells grown in HG medium and exposed to BAPN exhibited a significant increase in the ratio of p-AKT to total AKT compared to cells grown in HG medium alone (65.0 ± 10.9% of N versus 44.1 ± 8.5% of N; P < 0.05; n = 4; Figs. 3A, 3B). Differential dye staining data indicated that cells grown in HG medium showed a significantly increased number of apoptotic cells compared to those grown in N medium (3.16 ± 0.39 cells per 100 cells versus 1.38 ± 0.40 cells per 100 cells; P < 0.05; n = 4; Figs. 4A, 4B, 4D). Furthermore, cells grown in HG medium and exposed to BAPN exhibited significantly reduced number of apoptotic cells compared to cells grown in HG medium alone (2.12 ± 0.52 cells per 100 cells versus 3.16 ± 0.39 cells per 100 cells; P < 0.05; n = 4; Figs. 4B, 4C, 4D).

**Reducing Diabetes-Induced LOX Overexpression Restores AKT Activity and Inhibits Apoptosis in Mouse Retinas**

To determine the effect of diabetes on LOX expression and AKT activity in diabetic mouse retinas, and also whether reducing diabetes-induced LOX overexpression alters AKT activity and caspase-3 activation in diabetic LOX+/− mouse retinas, WB was performed. As expected, WB analysis revealed that the diabetic mouse retinas showed a significant increase in LOX expression compared to nondiabetic mouse retinas (145 ± 21% of WT versus 100 ± 30% of WT, P < 0.05; n = 6; Figs. 5A, 5B). Additionally, the LOX−/− mice showed a decrease in retinal LOX levels compared with those of control mice (64 ± 13% of WT versus 100 ± 30% of WT, P < 0.05; n = 6; Figs. 5A, 5B). Importantly, diabetic mouse retinas exhibited a significant decrease in the ratio of p-AKT to total AKT (56.5 ± 19% of WT versus 100 ± 9.7% of WT, P < 0.05; n = 6; Figs. 5A, 5C) as well as a significant increase in cleaved caspase-3 expression (181 ± 31% of WT versus 100 ± 20% of WT, P < 0.05; n = 6; Figs. 5A, 5D) and Bax expression (150 ± 14% of WT versus 100 ± 23% of WT, P < 0.05; n = 6; Figs. 5A, 5E) compared to nondiabetic mouse retinas. Interestingly, reducing LOX overexpression improved the ratio of p-AKT to AKT,
indicating that AKT activity was restored. Retinas of diabetic LOX−/− mice showed a significantly improved ratio of p-AKT to AKT (77.5 ± 12% of WT versus 56.5 ± 19% of WT; P < 0.05; n = 6; Figs. 5A, 5C) as well as decreased cleaved caspase-3 expression (115 ± 6% of WT versus 181 ± 31% of WT; P < 0.05; n = 6; Figs. 5A, 5D) and reduced Bax expression (114 ± 18% of WT versus 150 ± 14% of WT; P < 0.05; n = 6; Figs. 5A, 5E) compared to diabetic mouse retinas.

**DISCUSSION**

The present study demonstrates that retinal endothelial cells grown in HG medium exhibit abnormal LOX overexpression and decreased AKT phosphorylation concomitant with caspase-3 activation. Similarly, retinas of diabetic mice show LOX upregulation, compromised AKT phosphorylation, and caspase-3 and Bax activation. Interestingly, when LOX upregu-
Importantly, we have identified that HG-induced excessive cross-linking resulting in compact collagen fibrils, which in turn may compromise the ultrastructural integrity of the BM.16 Importantly, inhibiting HG-induced increased LOX activity significantly increased the ratio of p-AKT to AKT in retinal endothelial cells under HG conditions and in retinas of diabetic rats.16 Furthermore, our previous data suggest that increased LOX activity may lead to increased nitric oxide production, 37 which can inhibit proapoptotic NF-κB activation.37 These findings indicate that HG-induced LOX upregulation may contribute to retinal vascular cell apoptosis, at least in part, by impairing AKT activation necessary for cell survival.

The role of LOX in maintaining retinal homeostasis and cell survival is only beginning to be understood. Interestingly, a recent study by Yang et al.43 showed that LOX inhibition was associated with reduced nuclear factor-κB (NF-κB) activation. Importantly, NF-κB activation has been shown to promote apoptosis in the context of diabetic retinopathy.44–46 Furthermore, HG-induced AKT inactivation can lead to reduced nitric oxide production, 57 which can inhibit proapoptotic NF-κB signaling.57 These findings indicate that HG can lead to sustained NF-κB activation and reduce AKT signaling in endothelial cells, thereby contributing to vascular cell loss.57

These cellular events may provide insight into a potential mechanism by which normalizing LOX expression may restore AKT activity, and thereby promote cell survival by inhibiting the proapoptotic NF-κB signaling in retinal endothelial cells.
Findings from the current study suggest that HG-induced LOX overexpression contributes to accelerated cell loss associated with early-stage diabetic retinopathy, at least in part by AKT inactivation. These findings may offer an insight into potential mechanisms by which HG promotes apoptosis. The present study demonstrated that blocking HG-induced LOX upregulation might have beneficial effects including prevention of retinal vascular cell loss associated with diabetic retinopathy. Therefore, targeting LOX overexpression and/or increased activity could be a useful strategy for preventing vascular cell loss in diabetic retinopathy.

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