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Downregulation of lysyl oxidase protects retinal endothelial cells from high glucose–induced apoptosis

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Diabetic retinopathy, the leading cause of vision loss in the working-age population,1,2 is characterized by early vascular lesions such as the formation of acellular capillaries (AC) and pericyte loss (PL),3–8 which can contribute to retinal dysfunction. Studies suggest that retinal vasculature basement membrane (BM) thickening, a histologic hallmark of diabetic retinopathy,9–12 may promote apoptosis and thus lead to vascular cell loss.13–15 Our recent study suggests that lysyl oxidase (LOX), a cross-linking enzyme critical for the development and maturation of the BM, is upregulated in rat retinal endothelial cells from high glucose–induced apoptosis. Invest Ophtalmol Vis Sci. 2017;58:2725–2731. DOI: 10.1167/iovs.16-21340

**PURPOSE.** To investigate the effect of reducing high glucose (HG)-induced lysyl oxidase (LOX) overexpression and increased activity on retinal endothelial cell apoptosis.

**METHODS.** Rat retinal endothelial cells (RRECs) were grown in normal (N) or HG (30 mM glucose) medium for 7 days. In parallel, RRECs were grown in HG medium and transfected with LOX small interfering RNA (siRNA), scrambled siRNA as control, or exposed to β-aminopropionitrile (BAPN), a LOX inhibitor. LOX expression, AKT activation, and caspase-3 activity were determined by Western blot (WB) analysis and apoptosis by differential dye staining assay. Moreover, to determine whether diabetes-induced LOX overexpression alters AKT activation and promotes apoptosis, changes in LOX expression, AKT phosphorylation, caspase-3 activation, and Bax expression were assessed in retinas of streptozotocin (STZ)-induced diabetic mice and LOX heterozygous knockout (LOX+/−) mice.

**RESULTS.** WB analysis indicated significant LOX overexpression and reduced AKT activation under HG condition in RRECs. Interestingly, when cells grown in HG were transfected with LOX siRNA or exposed to BAPN, the number of apoptotic cells was significantly decreased concomitant with increased AKT phosphorylation. Diabetic mouse retinas exhibited LOX overexpression, decreased AKT phosphorylation, and increased Bax and caspase-3 activation compared to values in nondiabetic mice. In LOX+/− mice, reduced LOX levels were observed with increased AKT activity, and reduced Bax and caspase-3 activity. Furthermore, decreased levels of LOX in the LOX+/− mice was protective against diabetes-induced apoptosis.

**CONCLUSIONS.** Findings from this study indicate that preventing LOX overexpression may be protective against HG-induced apoptosis in retinal vascular cells associated with diabetic retinopathy.

Keywords: lysyl oxidase, apoptosis, high glucose, AKT, retinal endothelial cells
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+/− 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. 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 antymycotics 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/B6J albino male mice (Harlan Lab, Inc., Indianapolis, IN, USA) and 12 LOX+/− mice bred into the C57/B6J albino background kindly provided by Robert Mecham 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 of death. The diabetic group represented mice with a diabetic signal was performed at nonsaturating exposures and

to identify apoptotic cells, a differential dye staining method35 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-6014; 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 high glucose 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 upregulation and caspase-3

![Figure 1: Effects of HG and LOX downregulation on AKT activity in RRECs.](image1)

![Figure 2: LOX downregulation protects RRECs from HG-induced apoptosis.](image2)
the BM. Importantly, we have identified that HG-induced increase in LOX activity significantly increased the ratio of p-AKT to AKT (Fig. 3B). (B) Graphic illustration of cumulative data shows ratios of p-AKT to AKT significantly increased in cells grown in HG medium treated with BAPN compared to cells grown in HG medium alone. Data are expressed as mean ± SD. *N versus HG; P < 0.01. **HG versus HG+BAPN; P < 0.05. "HG versus HG+BAPN; P < 0.05. n = 4.

FIGURE 4. BAPN treatment prevents HG-induced apoptosis. (A-C) Representative images of cells undergoing apoptosis (arrows): (A) Normal. (B) HG, (C) HG+BAPN. Scale bar: 50 μm. Differential staining assay showing that inhibition of HG-induced increased LOX activity via BAPN treatment reduced the number of cells undergoing apoptosis. (D) Graphic illustration of cumulative data shows that inhibiting HG-induced increased LOX activity rescues RRECs from HG-induced apoptosis. Data are expressed as mean ± SD. N versus HG; P < 0.05. **HG versus HG+BAPN; P < 0.05. n = 4.

Inhibiting increased LOX activity rescues AKT signaling in RRECs. (A) Representative WB image shows that the ratio of p-AKT to AKT was significantly decreased in cells grown in HG medium. Interestingly, inhibiting HG-induced increased LOX activity significantly increased the ratio of p-AKT to AKT. (B) Graphic illustration of cumulative data shows ratios of p-AKT to AKT significantly increased in cells grown in HG medium treated with BAPN compared to cells grown in HG medium alone. Data are expressed as mean ± SD. *N versus HG; P < 0.01. **HG versus HG+BAPN; P < 0.05. "HG versus HG+BAPN; P < 0.05. n = 4.

LOX and Apoptosis in Retinal Endothelial Cells

Decreased Akt activity under HG or diabetic conditions leads to apoptosis, which can inhibit proapoptotic NF-κB. In the current study, we observed restoration of AKT activity, suggesting a direct link between LOX expression and AKT activation. In addition, when HG-induced increased LOX activity was reduced using BAPN, AKT activity was improved. To our knowledge, this is the first study showing that HG-mediated increased LOX expression and activity activate AKT signaling, which triggers caspase-3 activation, thereby promoting apoptosis in RRECs, and that reducing abnormal LOX overexpression and increased activity improves AKT activation, protecting RRECs from HG-induced apoptosis. Moreover, this finding is supported by previous studies indicating that LOX upregulation impairs AKT activity contributing to apoptosis. These results derived from an in vitro model of hyperglycemia, a STZ-induced diabetes animal model, and a LOX−/− animal model, suggest that abnormal LOX upregulation may play an important role in promoting the development of retinal vascular lesions associated with diabetic retinopathy.

Previously, we have shown that LOX expression is significantly increased in retinal endothelial cells under HG condition and in retinas of diabetic rats. Furthermore, our previous data suggest that increased LOX activity may lead to excessive cross-linking resulting in compact collagen fibrils, which in turn may compromise the ultrastructural integrity of the BM. Importantly, we have identified that HG-induced increase in LOX activity promotes excess cell monolayer permeability in retinal endothelial cells, providing a potential mechanistic insight into vascular leakage associated with thickened BM seen in diabetic retinopathy. The association between LOX and formation of the BM is well established, the relationship between LOX overexpression and retinal vascular cell loss is unclear. The current study shows for the first time that excess LOX levels play a critical role in triggering apoptosis and thereby promote retinal vascular cell loss.

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. 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. Furthermore, HG-induced AKT inactivation can lead to reduced nitric oxide production, which can inhibit prosapoptotic NF-κB signaling. 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. 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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References

Figure 5. LOX downregulation restores AKT activity, and inhibits Bax and caspase-3 activation in diabetic LOX−/− mouse retinas. (A) Representative WB image shows LOX, p-AKT, AKT, cleaved caspase-3, and β-actin expression in the retinas of WT, diabetic (DM), LOX−/−, and diabetic LOX−/− (DLOX−/−) mice. (B) Graphic illustration of cumulative data shows that diabetes significantly increases LOX expression. (C) Graphic illustration of cumulative data shows that AKT activity is significantly decreased in diabetic mouse retinas. Interestingly, AKT activity is increased in DLOX−/− mouse retinas compared to diabetic mouse retinas. Graphic illustration of cumulative data shows that (D) caspase-3 and (E) Bax activity are significantly reduced in DLOX−/− mouse retinas compared to diabetic mouse retinas. Data are expressed as mean ± SD. *WT versus DM; P < 0.05. **DM versus DLOX−/−; P < 0.05. ***WT versus LOX−/−; P < 0.05. n = 6.


