High Glucose Increases Binding of Lysyl Oxidase to Extracellular Matrix Proteins: Implications for Diabetic Retinopathy

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Purpose. To determine whether high glucose (HG) compromises internalization of lysyl oxidase (LOX) through excess binding of LOX with extracellular matrix (ECM) proteins.

Methods. To determine whether HG promotes binding of LOX with ECM proteins, fibronectin (FN) and collagen IV (Coll IV), total or ECM-only proteins from rat retinal endothelial cells grown in normal (N; 5 mM) or HG (30 mM) medium were analyzed by coimmunoprecipitation and Western blot (WB). In parallel, coimmunostaining was performed to determine changes in LOX binding to FN or Coll IV. To determine the effect of HG on extracellular LOX levels, medium in which cells were grown for 1, 3, 5, and 7 days were assessed for LOX levels.

Results. WB analysis using total protein showed LOX overexpression and elevated levels of LOX bound to Coll IV or FN in HG condition. Similarly, a significant increase in LOX bound to FN or Coll IV was observed in ECM-only protein. These data were supported by Z-stack confocal microscopy images from coimmunostaining. Furthermore, immunostaining performed on ECM layer revealed increased presence of LOX bound to Coll IV or FN. Additionally, when media from cells grown in HG was monitored, a maximal increase in LOX level was observed by day 3, which declined by day 7.

Conclusions. Findings indicate that HG promotes binding of LOX to FN and Coll IV extracellularly that results in reduced LOX internalization, attenuation of negative feedback, and upregulation of LOX expression associated with diabetic retinopathy.

Keywords: lysyl oxidase, extracellular matrix, high glucose, diabetes, retinal endothelial cells

Diabetic retinopathy (DR) is a leading cause of blindness among working-age adults.[1,2] Although the incidence and prevalence of DR is increasing worldwide,[3] currently there is no cure for this ocular disease. Retinal capillary basement membrane thickening, a histological hallmark of DR,[4–7] has been implicated in the development of retinal vascular cell loss and vascular leakage.[8–12] Although studies have shown presence of elevated lysyl oxidase (LOX) levels in vitreous of patients with DR,[13] and increased LOX expression is closely associated with basement membrane thickening,[14,15] the mechanism by which high glucose (HG) upregulates LOX expression is unknown.

LOX is a crosslinking enzyme that plays a key role in the maturation and functionality of the retinal vascular basement membrane. Previous studies demonstrated LOX upregulation in rat retinal endothelial cells (RRECs) grown in HG medium, in retinas of diabetic rats,[16] and in vitreous of diabetic subjects.[13] Under diabetic conditions, LOX upregulation is known to compromise basement membrane ultrastructure and functionality, thereby promoting permeability due in part to increased interfibrillar space.[16–18] Moreover, excess LOX level has been shown to inhibit AKT activation in RRECs, thereby triggering apoptosis and promoting retinal vascular cell loss.[19] Importantly, our recent studies indicate that blocking HG-induced LOX upregulation could prevent apoptosis[19] and retinal vascular cell loss associated with DR.[20] Moreover, LOX has been reported to be present in the nucleus and regulates gene expression,[21,22] and studies have provided evidence for the internalization and presence of mature LOX in the cytoplasm.[23] However, little is known about internalization of extracellular LOX and whether this could be a key step in signaling feedback regulation for LOX expression.

LOX is synthesized as an inactive 50 kD proenzyme (pro-LOX) that is N-glycosylated in the endoplasmic reticulum and the Golgi complex and then secreted into the extracellular environment, in which it is cleaved into a 32 kD active enzyme (mature LOX) and a 18 kD propeptide (LOX-PP).[24,25] Although LOX has been considered to be an extracellular matrix (ECM) enzyme, studies also reported presence of LOX within the nuclei and cytoplasm.[21,26–29] Mature LOX has been shown to translocate from the extracellular environment into the cytosol and concentrate within the nucleus.[21,26] LOX has been identified within the nuclei of various cells and tissues in which it is known to retain its catalytic activity.[21,28,29] In the cytoplasm, LOX has been

identified in the cytoskeletal filaments and microtubule networks.[26,27] Because mature LOX can translocate from the extracellular into the intracellular environment, in this study, we examined whether HG compromises this process through excess binding of LOX with ECM proteins extracellularly, thereby decreasing LOX internalization, attenuating negative feedback, and promoting LOX overexpression.

Methods

Cell Culture

Capillary endothelial cells derived from rat retinas of 6 to 8 weeks old Sprague-Dawley rats confirmed positive for von Willebrand factor, and isolated as previously described[30] were used in this study. All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. To determine levels of intracellular LOX or LOX binding with ECM protein, RRECs were grown in normal (N; 5 mM glucose) or HG (30 mM glucose) Dulbecco's modified Eagle's medium (Gibco/Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (Sigma-Aldrich Corp., St. Louis, MO, USA), antibiotics, and antimycotics for 7 days. In parallel, to determine whether HG alters extracellular LOX expression over time, RRECS were grown in N or HG medium for 1, 3, 5, and 7 days. Cells from the experimental groups were then subjected to immunostaining, assayed for LOX expression in LOX media, and LOX binding with fibronectin (FN) or collagen IV (Coll IV) in total protein and ECM-only protein.

Cell Removal with Ammonium Hydroxide (NH_4OH)

RRECs were cultured in N or HG media for 7 days. Cells were washed with PBS and exposed to 20 mM ammonium hydroxide (1 mL of 20 mM NH_4OH per 60-mm dish). Cells were then incubated at room temperature for 5 minutes and gently agitated every minute. After 5 minutes, copious amounts of deionized H_2O were added, and any material solubilized by ammonium hydroxide, including lysed cells and deionized H_2O, were removed. The insoluble ECM layer was then washed with copious amounts of deionized H_2O four more times.

Coimmunostaining

To determine whether HG alters binding of LOX with FN or Coll IV in total protein extracts, RRECs were grown in cover slips in N or HG medium for 7 days. These cover slips were coimmunostained using mouse anti-LOX antibody (1:50, Catalog No. 373995; Santa Cruz, Dallas, TX, USA), and rabbit anti-Coll IV antibody (1:50, Catalog No. ab6586; Abcam, Cambridge, MA, USA), or rabbit anti-FN antibody (1:50, Catalog No. ab2413; Abcam). Confocal microscopy was then used to take the images. In parallel, to determine whether HG alters binding of LOX with FN or Coll IV in ECM-only protein, RRECs were grown in cover slips in N or HG medium for 7 days. The cells were then removed with 20 mM ammonium hydroxide as previously described. Following removal of cells, the cover slips containing only the ECM layer were coimmunostained using mouse anti-LOX antibody (1:50, Catalog No. 373995; Santa Cruz), and rabbit anti-Coll IV antibody (1:50, Catalog No. ab6586; Abcam), or rabbit anti-FN antibody (1:50, Catalog No. ab2413; Abcam). Digital microscopy was then used to take the images.

Coimmunoprecipitation

Bicinchoninic acid assay (Pierce Chemical, Rockford, IL, USA) was used to determine the protein concentrations. An equal amount of total protein extracts including ECM layer (200 µg) from RRECs grown in N or HG medium was immunoprecipitated by rabbit polyclonal LOX antibody (1:100, Catalog No. NB110; Novus, Littleton, CO, USA) and subjected to Western blot (WB) analysis using anti-Coll IV antibody or anti-FN antibody. In parallel, RRECs grown in N or HG medium for 7 days were removed using 20 mM ammonium hydroxide to obtain only the ECM layer. Following cell removal, an equal amount of protein (200 µg) was extracted from the ECM layer and immunoprecipitated by rabbit polyclonal LOX antibody (1:100, Catalog No. NB110; Novus) followed by WB analysis using anti-Coll IV antibody or anti-FN antibody. Moreover, to determine whether HG alters extracellular LOX expression over time, RRECs was grown in N or HG medium for 1, 3, 5 or 7 days. On the day of harvest in respective time points, cell media was replaced with serum- and phenol red-free media containing 0.1% BSA. An equal volume of media collected (1 mL) was immunoprecipitated with rabbit polyclonal LOX antibody (1:100, Catalog No. NB110; Novus) and subjected to WB analysis using rabbit polyclonal LOX antibody (1:2000, Catalog No. NB110; Novus). Additionally, supernatant extract containing nonspecific protein was collected and subjected to WB analysis using anti-β-actin antibody (1:1000, Catalog no. 4967; Cell Signaling, Danvers, MA, USA).

Western Blot

Protein samples collected from day 2 of immunoprecipitation were subjected to electrophoresis in polyacrylamide gel and transferred to polyvinyl difluoride membrane using a semidry apparatus as described previously.[31] The membrane was then blocked with 5% nonfat dry milk for 1 hour at room temperature. To determine LOX binding with FN or Coll IV in total protein extracts or ECM-only protein, membranes were subsequently incubated overnight at 4°C with rabbit anti-Coll IV antibody (1:1000, Catalog No. ab6586; Abcam) and rabbit anti-FN antibody (1:1000, Catalog No. ab2413; Abcam) followed by alkaline phosphatase (AP)-conjugated anti-rabbit IgG secondary antibody (1:1000, catalog no. 7054; Cell Signaling). Similarly, to assess extracellular LOX expression, membranes were incubated overnight at 4°C with rabbit polyclonal LOX antibody (1:2000, Catalog No. NB110; Novus) followed by AP-conjugated anti-rabbit IgG secondary antibody (1:1000, catalog no. 7054; Cell Signaling). The amount of protein loaded in the gel lanes was confirmed by β-actin antibody (1:1000, Catalog No. 4967; Cell Signaling). Densitometric values were determined to ascertain differences in protein levels using ImageJ software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD, USA).

Statistical Analyses

All data are expressed as mean ± SD. Values from the normal group were considered as 100%, and values from the HG group are expressed as percentages of the normal group. Comparisons between groups were performed using the Student's *t*-test. A level of $P < 0.05$ was considered statistically significant.

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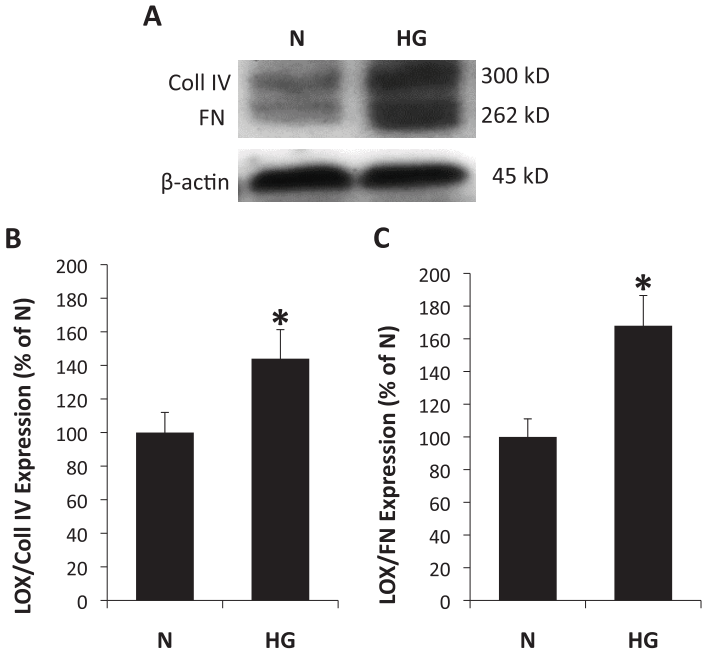

Figure 1. Effect of HG on LOX binding with Coll IV and FN in total protein extracts. Representative (A) WB image and graphic illustration of cumulative data show that the expression levels of (B) LOX bound to Coll IV (LOX/Coll IV) and (C) LOX bound to FN (LOX/FN) from total protein extracts are significantly increased by HG. All data have been normalized using β-actin correction. Data are expressed as mean ± SD. *N versus HG; P <0.05.

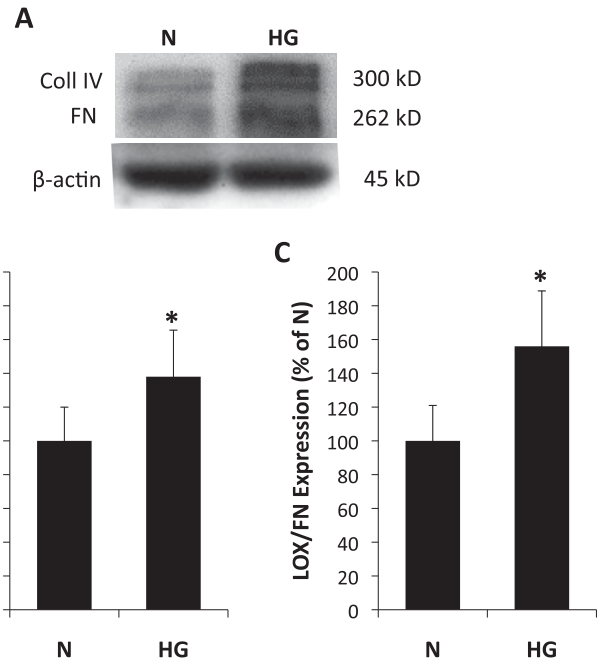

Figure 2. Effect of HG on LOX binding with ECM proteins in ECM-only extracts. Representative (A) WB image and graphic illustration of cumulative data show that the expression levels of (B) LOX bound to Coll IV and (C) LOX bound to FN from ECM-only extracts are significantly increased by HG. All data have been normalized using β-actin correction. Data are expressed as mean ± SD. *N versus HG; P <0.05.

Results

HG Promotes Excess LOX Binding to FN and Coll IV in Total Protein Extracts

Results from coimmunoprecipitation (co-IP) followed by WB analysis showed significant increase in LOX bound to Coll IV and FN in total protein extracts of cells grown in HG medium (144 ± 12% of control, $P < 0.05$, $n = 6$; 168 ± 11% of control, $P < 0.05$, $n = 6$, respectively, Figs. 1A–C) compared with those of cells grown in N medium.

HG Accelerates LOX Binding to FN and Coll IV in ECM Laid Down by RRECs

Following cell removal using ammonium hydroxide, co-IP/WB data showed a significant increase in LOX bound to Coll IV, and FN was observed in ECM-only protein of cells grown in HG medium (138 ± 20% of control, $P < 0.05$, $n = 6$; 156 ± 21% of control, $P < 0.05$, $n = 6$, respectively; Figs. 2A–C) compared with those of cells grown in N medium.

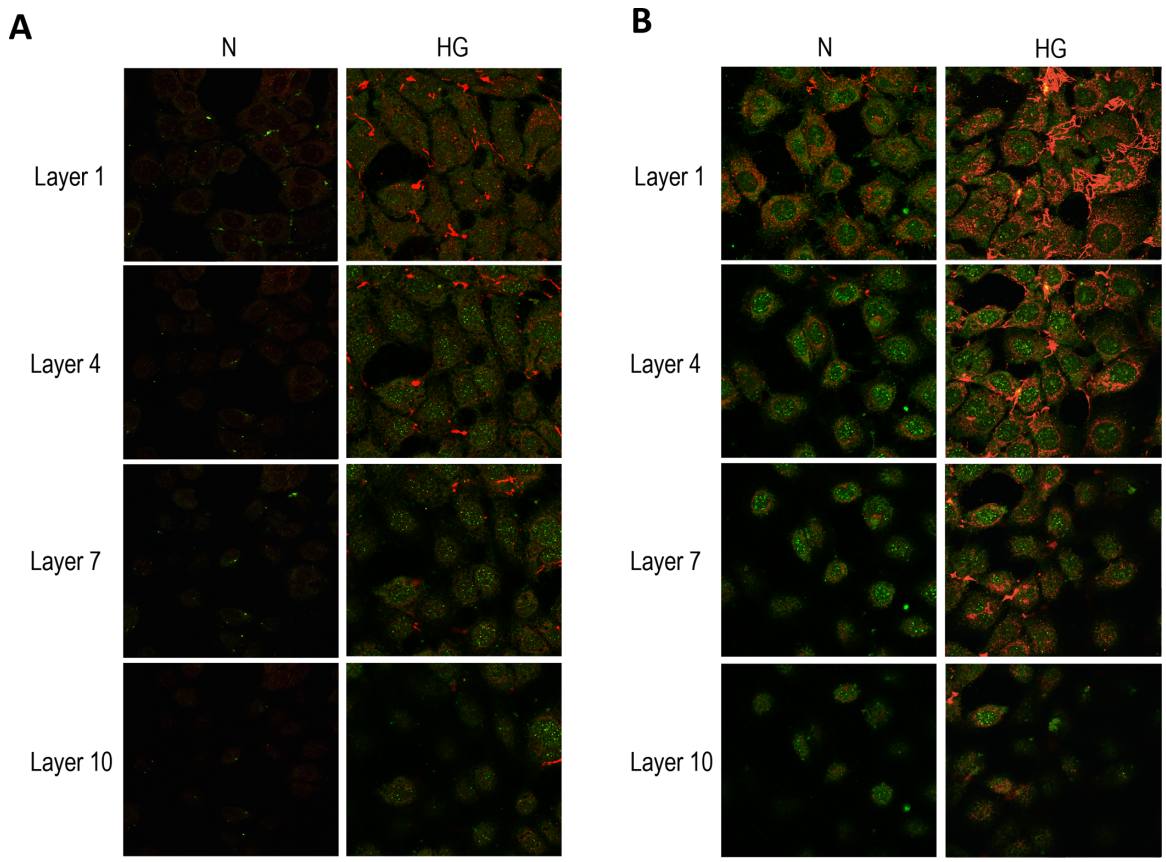

Figure 3. HG promotes excess LOX binding to Coll IV and FN in RRECs. Representative Z-stack images of coimmunostaining with cells grown in N or HG medium show that (A) LOX bound to Coll IV and (B) LOX bound to FN increases significantly in HG media. Z-stack images show representative layers 1 to 10, with layer 1 at the basal position and layer 10 at the apical position. Left panel: green = LOX, red = Coll IV, orange = LOX bound to Coll IV. Right panel: green = LOX, red = FN, orange = LOX bound to FN.

HG Induces Increased Binding of LOX to FN and Coll IV in RRECs

Coimmunostaining of LOX and FN or LOX and Coll IV analyzed by Z-stack imaging revealed an increase in LOX bound to FN and LOX bound to Coll IV in cells grown in HG medium compared with those of cells grown in N medium (Figs. 3A–B). Importantly, after removal of cells, coimmunostaining data showed LOX bound to Coll IV or FN was found to be increased in ECM-only layer laid down by cells grown in HG medium (Figs. 4A, 4B) compared with those of cells grown in N medium. A schematic representation illustrating excess binding of LOX to ECM of cells grown in HG medium is indicated in Figure 5.

Extracellular LOX Expression is Differentially Regulated Over Time by HG

WB analysis of total protein isolated from cell medium indicated that extracellular LOX expression slightly increased after 1 day of HG media (124 ± 8% of control, $P < 0.05$, $n = 6$; Figs. 6A, 6E), reached the peak after 3 days of HG media (158% ± 11 of control, $P < 0.05$, $n = 6$; Figs. 6B, 6E), then decreased to 117 ± 6% of control ($P > 0.05$, $n = 6$; Figs. 6C, 6E) after 5 days of HG media, and ultimately dropped significantly to 78 ± 5% of control ($P < 0.05$, $n = 6$; Figs. 6D, 6E) after 7 days of HG media. Data from the latter two time points indicate that there is an increase in LOX binding to the ECM, specifically to FN and Coll IV.

Discussion

Findings from this study indicate that HG promotes binding of LOX with ECM proteins, FN and Coll IV, extracellularly and this could interfere in LOX internalization resulting in reduced intracellular LOX level, creating negative feedback signaling and contributing at least in part to LOX overexpression associated with DR. To our knowledge, this is the first study that shows increased LOX bound to FN and Coll IV proteins could decrease LOX internalization under HG condition in RRECs.

Traditionally, LOX that is processed extracellularly has been exclusively regarded as an extracellular enzyme,[32] but studies revealed that mature LOX is also present intracellularly, both in the cytoplasm[23,33–35] and in the nucleus.[33,36] Interestingly, even in the presence of β-aminopropionitrile, an irreversible inhibitor of LOX and all its isoforms,[36] catalytic activity of LOX was present in the nucleus, suggesting that nuclear LOX is protected and remains functionally active, which is of great interest. A study has shown

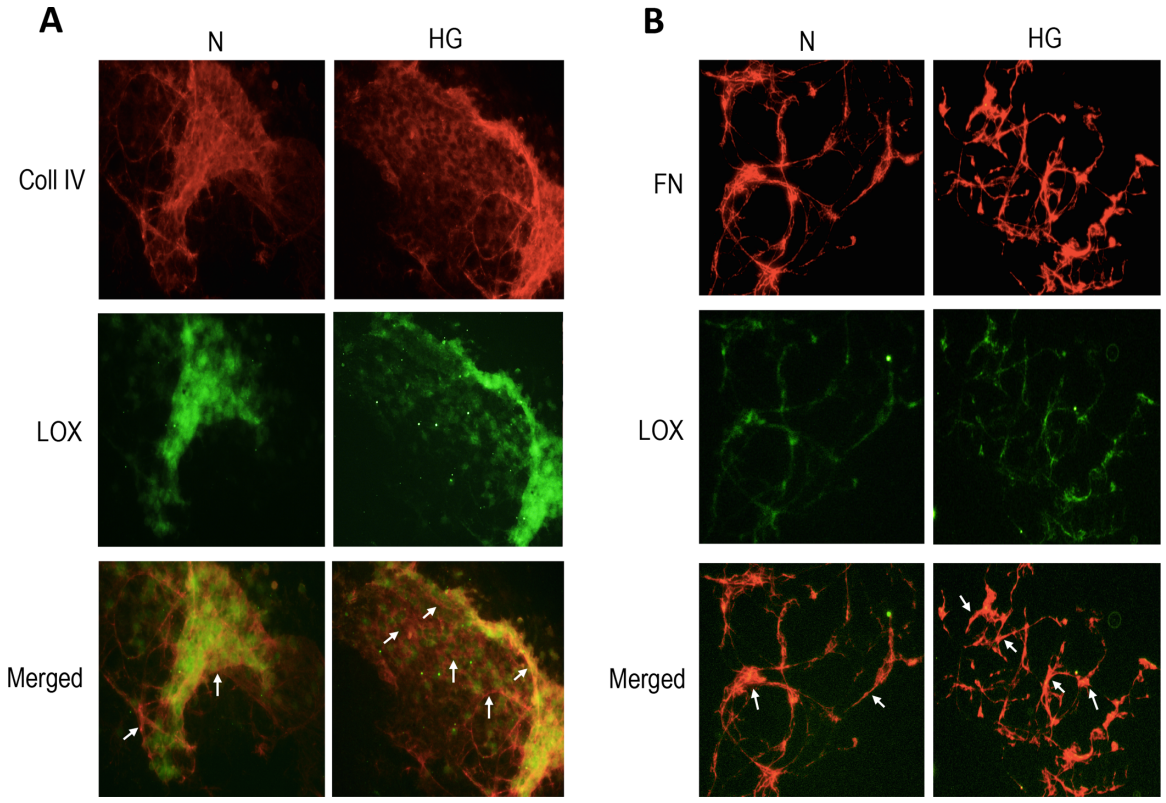

Figure 4. HG elevates LOX binding to Coll IV and FN in ECM layer. Representative images of coimmunostaining of the ECM layer laid down by cells grown in N or HG medium show that (A) LOX bound to Coll IV and (B) LOX bound to FN increases significantly in HG condition. Arrows: (A) Areas of Coll IV and LOX colocalization. (B) Areas of FN and LOX colocalization.

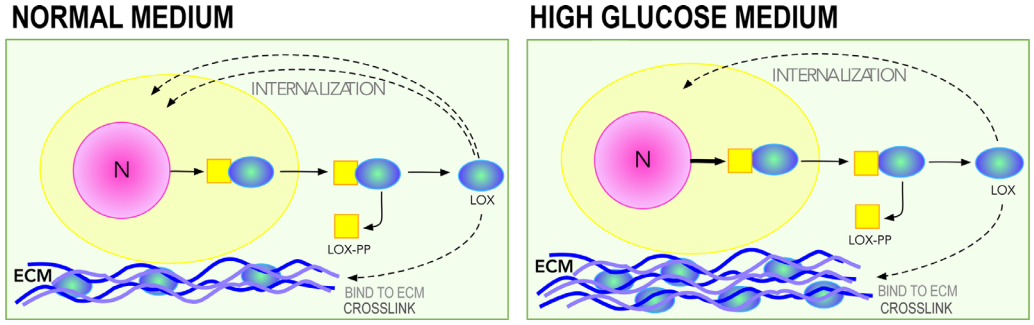

Figure 5. Schematic illustration shows compromised LOX internalization under HG condition. HG-induced ECM accumulation provides target ECM components for excess LOX binding, thus reducing LOX internalization, and attenuating feedback from reduced presence of LOX inside the cell, resulting in upregulation of LOX expression. N = nucleus.

that mature LOX is able to translocate from the extracellular space into the cytosol, then cluster in the nucleus,[37] potentially mediated by interaction of the catalytic domain of LOX with p66β, a transcription repressor.[22] Taken together, these studies indicate that LOX internalization into the intracellular and nuclear space is documented; however, its purpose or functionality remains unknown at this time.

Although the biological function of nuclear LOX remains unclear, studies have demonstrated that nuclear LOX can modulate the expression of collagen,[38] affect chromatin organization,[39] and suppress the promoter of cyclin D1[40] and E-cadherin.[41] Studies also suggest that LOX is able to regulate gene expression levels through its interaction with histones H1 and H2[39,42,43] in the nucleus. Importantly, LOX has been reported to carry out proapoptotic effects. A study has demonstrated that nuclear LOX can directly inhibit the rate of nuclear growth, which is indicative of cell proliferation, suggesting that LOX possesses anti-proliferative activity.[44] Moreover, lentivirus-mediated LOX overexpression was shown to promote apoptosis in human vascular smooth muscle cells.[45] Overall, these findings suggest that abnormal LOX overexpression can negatively influence cell viability and ultimately promote apoptosis.

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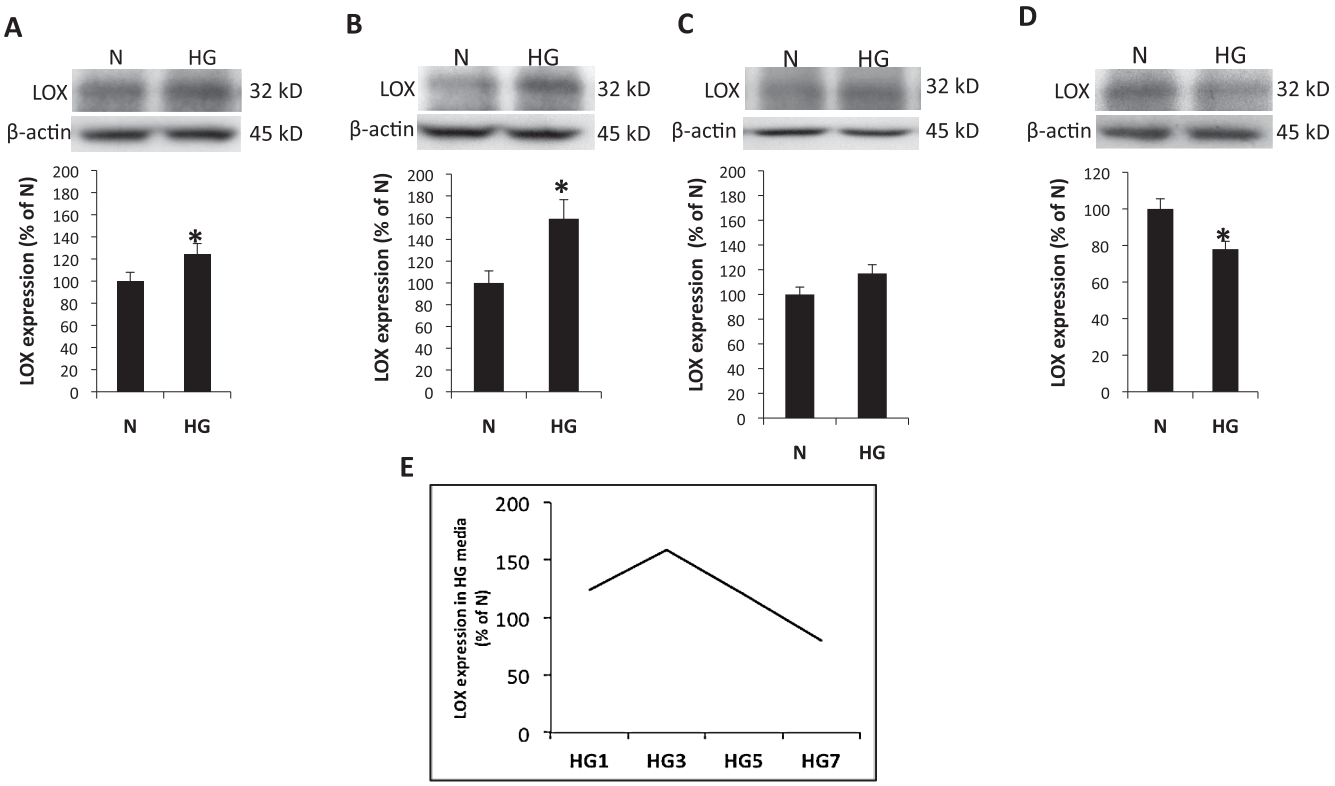

Figure 6. Effect of HG on extracellular LOX expression. Representative WB images and graphic illustration of cumulative data show that LOX expression in the media significantly increased after (A) 1 day and (B) 3 days of HG media, with slight increase at (C) 5 days of HG media and significant decrease at (D) 7 days of HG exposure. (E) Graphic chart indicates extracellular LOX expression slightly increased at 1 day of HG media (HG1), reached the peak at 3 days of HG media (HG3), then decreased at 5 days (HG5), and 7 days of HG media (HG7). Data have been normalized using β-actin correction. Data are expressed as mean ± SD; *N versus HG; $P < 0.05$.

Conclusions

The possibility that LOX bound to FN or Coll IV is higher in HG condition is facilitated by the presence of excess FN and Coll IV, which are known to be overexpressed in HG[7,12,17,46,47] or diabetes.[8,9,11,48,49] In this study, our data show that extracellular LOX level increases and reaches a peak in 3 days of HG media, then starts to decrease by 5 days of HG media. This reduced extracellular LOX level is likely attributed to increased binding of LOX with FN and Coll IV proteins as determined by WB analysis, coprecipitation, and coimmunostaining in our study. Combined results of this finding and previous studies support the hypothesis that excess synthesis of basement membrane (BM) components, FN or Coll IV, over time serves as additional targets for extracellular LOX to bind, thereby resulting in decreased LOX level in the culture media concomitant with reduced LOX internalization under HG condition. Although studies indicate that decreasing HG-induced Coll IV and FN overexpression improves vascular permeability in diabetic animals,[12] it is unclear whether downregulation of FN or Coll IV directly prevents LOX overexpression in DR. Further studies are necessary to understand the mechanistic relationship between basement membrane components and LOX and their influence over vascular permeability associated with blood–retinal-barrier breakdown in DR.

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