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LRP-1 Pathway Targeted Inhibition of Vascular Abnormalities in the Retina of Diabetic Mice

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Abstract

Purpose—The cell surface LDL (low-density lipoprotein) receptor-related protein-1 (LRP-1) is important for lipid transport and several cell signaling processes. Human apolipoprotein E (apoE) is a ligand of LRP-1. We previously reported that a short peptide (apoEdp) mimicking the LRP-1 binding region of apoE prevents hyperglycemia-induced retinal endothelial cell dysfunction *in vitro*. The *in-vivo* outcome of apoE-based peptidomimetic inhibition of LRP-1 in the treatment of diabetic retinopathy is unknown.

Methods—Six months after streptozotocin induction of diabetes, male C57Bl/6 mice were intravitreally inoculated with apoEdp in a controlled release formulation. On the 15th day post-apoEdp treatment, mouse retinas were harvested to examine (1) blood–retinal–barrier (BRB) permeability by Evans blue dye, inflammatory leukostasis by concanavalin staining of leukocytes and LRP-1 pathway-related protein expression by Western blot analysis and gelatin zymography.

Results—Intravitreal apoEdp treatment of diabetic mice significantly reduced Evans blue extravasation and the number of adherent leukocytes in the diabetic mouse retinas. ApoEdp treatment inhibited the expression of extracellular matrix (ECM) degrading proteases heparanase and MMP-2, and restores the BRB tight junction proteins occludin and ZO-1. ApoEdp treatment also inhibited Wnt/β-catenin-related expression of pro-inflammatory molecules ICAM-1, HIF-1α, and VEGF through negative regulation by LRP-1.

Conclusion—Intravitreal apoEdp treatment of diabetic mice resulted a significant decrease in retinal vascular abnormalities through downregulation of LRP-1-related ECM protein degradation and Wnt/β-catenin-related pro-angiogenic molecules.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

Keywords

BRB; diabetic retinopathy; ECM; LRP-1; Wnt/ β -catenin

Introduction

Diabetic retinopathy (DR) is one of the most common causes of vision loss among the working-age population in the USA and most people develop DR after 20 years of diabetes.¹ DR is a slow-progressing microvascular complication. DR starts as the non-proliferative diabetic retinopathy (NPDR) when vision is normal and progress to the proliferative diabetic retinopathy (PDR) with non-reversible vision loss.² Current standard treatments of PDR are inadequate. Microvascular complication of DR starts at NPDR stage. Prevention of DR at its early NPDR stage may retard its progression to PDR. So understanding the mechanism of biological abnormalities characteristic to NPDR may result in the identification of a therapeutic target for the treatment of vision loss. Hyperglycemic injury to endothelial cells (ECs) lining the retinal vasculature results in extracellular matrix (ECM) degradation and loss of tight junction proteins in the blood–retinal–barrier (BRB).³ ECM degradation results in the release of pro-angiogenic molecules.⁴ The hyperglycemia-induced molecular events of ECM degradation and tight junction protein loss may progress to leaky retinas with increased BRB permeability. Increased BRB permeability results in the excess accumulation extracellular fluid and hard exudates in the macula leading to the development of diabetic macular edema and loss of central vision.^{5,6}

The ECM, composed of proteins and glycosaminoglycans, protects the plasma membrane and basement membrane of ECs and serves as a depot of ECM-degrading proteases, as well as pro-inflammatory molecules.⁷ ECM degradation results in the release of ECM-bound proteases such as heparanase,^{3,4} matrix metalloproteinases (MMPs)⁸ and vascular endothelial growth factor (VEGF).⁹ Understanding how these molecules are released by an injured EC may be an important insight into the development of DR. A cell surface receptor called LDL (low-density lipoprotein)-receptor-related protein-1 (LRP-1) is a large endocytic receptor that binds and endocytoses over 30 structurally and functionally different ligands including heparanase, MMPs and apolipoprotein E (apoE).¹⁰ LRP-1 also plays a pivotal role in regulating the expression of its other family (LDLR) members LRP-5/LRP-6 in regulating the Wnt/ β -catenin signaling pathway.¹¹ ApoE is an important protein involved in lipid transport through LRP-1.¹² In addition to lipid transport, reports suggest that apoE is also involved in diverse cell signaling processes.¹³ In the retina, apoE is produced by Müller glial cells, retinal pigmented epithelial cells and is secreted into the vitreous humour.¹⁴ ApoE also has a vascular protective function as evidenced by the finding that lack of apoE leads to blood–brain–barrier (BBB) breakdown.¹⁵ As such, we hypothesize that apoE may have a role in maintaining BRB integrity in the retina. We previously reported that one small peptide mimicking the receptor-binding domain of apoE prevents the loss of tight junction proteins of the BRB through inhibition of heparanase and VEGF in human retinal ECs *in vitro*.³ The N-terminal domain of apoE binds the receptor, while the C-terminus is required for its lipid delivery function.¹⁶ Within the N-terminal domain a particular nine amino acid sequence (141–149) of apoE (LRKLRKRL) is known to bind LRP-1.¹⁷ We previously

reported that a tandem repeat dimer peptide (apoEdp) of apoE 141–149, (LRKLRKRLLLRKLRKRL) was anti-angiogenic in a VEGF-induced rabbit corneal micro-pocket assay,¹⁸ prevents human retinal EC dysfunction through inhibition of the ECM-degrading enzyme heparanase and loss of tight junction proteins *in vitro*.³ The dimer form of apoEdp was chosen, because dimerization induces the adoption of an α -helical structure that is more stable than the monomer form.¹⁷ In this study, we sought to investigate whether a peptidomimetic inhibition of LRP-1 by apoEdp can be used as a therapeutic target for the treatment of DR through inhibition of retinal vascular abnormalities and related ECM degradation and angiogenic signaling.

Materials and methods

Streptozotocin (STZ)-induced diabetes

Male C57Bl/6 mice (Taconic Labs) were injected with 55 mg/kg of STZ (Sigma Aldrich, St. Louis, MO) diluted in sterile citrate buffer (0.05 M, pH 4.5) intraperitoneally (i/p) once per day for four consecutive days. Control mice received an i/p injection of vehicle (sterile citrate buffer). STZ-induced diabetic mice model reported to require ~6 months to develop measurable vascular remodeling lesions characteristic of DR.¹⁷

26 weeks after the injection of STZ or vehicle, all animals were tested for blood glucose level through tail vein puncture using the AlphaTRAK2 assay kit (Abbott Diabetes Care Inc., Alameda, CA). Animals with a blood glucose level greater than 350 mg/dL were considered diabetic. All experimental protocols were approved in advance by Xavier University of Louisiana's Animal Care and Use Committee and were conducted in accordance with the ARVO statement for the use of Animals in Ophthalmic and Vision Research.

Peptide production and preparation of alginate *in-situ* gel for ophthalmic controlled release formulation

ApoEdp (Ac-LRKLRKRLLLRKLRKRL-amide) was synthesized by Genemed, Arlington, TX with >95% purity.^{3,18} An alginate *in-situ* gel formulation was used as controlled release formulation.^{19–21} Alginate *in situ* gel formulation was reported to have the ability of sustained release for 21²² to 30 days.²³ The following protocol was used to prepare the alginate *in-situ* gel formulation. To a final volume of 10 mL, the following were added to and dissolved in sterile water: 0.4 g of sodium alginate (Sigma), 45 mg of sodium chloride (Sigma), and 2 μ L of a 100 mM apoEdp solution. The sample was then brought to the dilution of 100 μ M/5 μ L with deionized water. For intravitreal injection, each eye received 5 μ L of a 100 μ M solution of this formulation.

Treatment regimen

Mice were divided into three test groups of five mice each including group 1 (wild type, non-diabetic), group 2 (diabetic mock-treated with vehicle), and group 3 (diabetic treated with apoEdp in a vehicle of controlled release formulation). Treatment began at 26 weeks post-STZ induction of diabetes. Intravitreal injection of 100 μ M apoEdp in 5 μ L of vehicle (controlled release formulation) was used. Animals were sacrificed at 15th day of post-

apoEdp treatment. Before starting the treatment, groups of mice were intravitreally inoculated in their right eyes with apoEdp in sustained release formulation and the left eye with apoEdp in phosphate buffered saline (PBS) suspension. At 15th day of intravitreal post-apoEdp treatment, vitreal fluid samples were analyzed by ultra-high-pressure liquid chromatography on a Waters Acquity system coupled with a single quadrupole mass spectrometer. Vitreal samples from right eye inoculated with apoEdp in sustained release formulation resulted 98% recovery compared to 58% recovery from vitreal samples of left eye inoculated with apoEdp in PBS suspension only.

Evans blue dye assay

Each mouse was anesthetized and received an i/v injection (tail vein) of an Evans blue dye at 45 mg/kg. Two hours later, a 0.1–0.2 mL blood sample was obtained from re-anesthetized mice and the animal was perfused via the left ventricle with a solution of PBS followed by 1% paraformaldehyde. Eyes were enucleated and the retinas were removed by manual dissection. Retinas were treated with dimethylformamide (Sigma Aldrich, St. Louis, MO, USA) for 16 h at 78°C, centrifuged at 12,000 g for 15 min. Retinal extract supernatants were tested spectrophotometrically for absorbance at 620 nm (detection of blue dye) and 740 nm (background). Blood samples were not treated with dimethylformamide and centrifuged at 3,500 g at 25°C for 15 min. The resulting supernatant was diluted 1:1000 before testing. BRB breakdown was measured by calculating a ratio of the spectrophotometric data as follows:

$$\frac{\text{Retinal Evan's blue concentration (mg/mL) / retinal weight (mg)}}{\text{Blood Evan's blue concentration (mg/mL) } \times \text{ circulation time (hours)}}$$

Retinal leukostasis assay

Fourteen days after apoEdp treatment, mice retinas from each group were examined for leukostasis. Following an anesthetic overdose, each mouse received 250 mL/kg of PBS via an intracardiac perfusion for 2 min to remove RBCs and non-adherent leukocytes, immediately followed by perfusion with a 40 µg/mL of FITC-conjugated concanavalin lectin A (5 mg/kg) to label adherent leukocytes. An additional perfusion with PBS was performed to remove excess fluorescent dye and decrease background. Following euthanasia, enucleated eyeballs were collected to generate flat-mount retinas. Fluorescence images of FITC-labeled leukocytes in flat-mount retinas were captured using an Olympus IX71 inverted fluorescence microscope at 20× magnification equipped with a Coolsnap CCD camera. Numbers of observed leukocytes per eye were compared among groups of mice.

Western blot analysis

Fourteen days after apoEdp treatment, enucleated eyeballs from three different groups of mice were processed for collection of retinal extract. Retinal tissue homogenates and nuclear fraction of retinal tissue (isolated using NE-PER Nuclear and Cytoplasmic Extraction Reagents—Cat# 78833—Thermofisher Scientific) concentration was determined using a BCA protein assay kit (Pierce, Rockford, IL, USA). An equal amount (50 µg/lane) of protein was loaded on a 5–20% SDS-polyacrylamide gel and transferred electrophoretically onto

PVDF membranes (Amersham, Little Chalfont, UK). Membranes were blocked in a 5% nonfat milk solution for 1 h, and then incubated at 4°C overnight with anti-heparanase (Sc-25825, Santa Cruz Biotechnology, CA, USA), anti-MMP-2 (Sc-6841, Santa Cruz Biotechnology), anti-ICAM-1 (Sc-1511, Santa Cruz Biotechnology, CA), anti-VEGF (Sc-1836, Santa Cruz Biotechnology), anti- β -catenin (Sc-7199, Santa Cruz Biotechnology), anti-LRP-6 (Sc-25317, Santa Cruz Biotechnology, CA), anti-p-LRP-1 (Sc-33049, Santa Cruz Biotechnology, CA), anti-LRP-1 (Sc-25469, Santa Cruz Biotechnology), anti-HIF-1 α (Sc-10700, Santa Cruz Biotechnology), anti- β -actin (Sc-81178, Santa Cruz Biotechnology) and anti-histone H3 (Cat# 05-499, EMD Millipore, MA, USA) antibodies. Following overnight incubation with primary antibody, membrane was washed with PBS-Tween20, and incubated with HRP-conjugated respective secondary antibody (1:2500) for 1 h at room temperature in PBS-Tween20 and 1% nonfat milk. Same blots were then stripped and re-probed with an antibody against β -actin to ensure the equal loading of protein in each lane. Band intensities were analyzed using ImageJ (NIH).

Gelatin zymography

MMP-2 gelatinase activity was measured using Novex 10% zymogram (gelatin) gel (Invitrogen) electrophoresis. Gels were gently washed with Novex zymogen renaturing buffer (Invitrogen) for 30 min and treated with Novex zymogen developing buffer (Invitrogen) overnight at 37°C and finally stained with Simply blue safestain (Invitrogen). Gelatinase activity was detected by the appearance of white lytic bands and relative intensities were calculated using ImageJ (NIH).

Statistical analysis

Kruskal–Wallis one-way ANOVA was used to determine the significant differences between groups. Results are expressed as mean $\pm$ SEM. A *P*-value less than 0.05 were considered significant and is highlighted in all values with an asterisk (*).

Results

Intravitreal apoEdp treatment significantly reduced the extravasation of Evans blue dye in diabetic mice retina

Chronic hyperglycemia causes increased retinal vascular permeability and leaky retina. After 26 weeks of diabetic onset and 14 days after a single intravitreal injection of apoEdp, tail vein injection of Evans blue dye was performed to determine the extent of retinal leakiness. The inherent property of Evans blue dye binding with plasma albumin allows the albumin-bound Evan's blue permeation in the tissue surrounding retinal capillaries. A non-leaky capillary is expected to have little or no extravasation of albumin-bound Evans blue. Figure 1 shows that intravitreal apoEdp treatment significantly ($p < 0.05$) reduced retinal permeability as measured by Evans blue concentration in the formamide extract of retina compared to vehicle treated retinas of diabetic mice. Retinas of control mice did yield significantly ($p < 0.05$) lower concentrations of Evans blue dye compared to diabetic mice retina.

Intravitreal apoEdp treatment significantly reduced adherent leukocytes in diabetic mice retinas

At 15th day of post-apoEdp treatment, intracardiac perfusion of FITC-conjugated concanavalin lectin was used to stain adherent leukocytes inside the retinal vasculature. Flat-mount retinas were observed for the number of adherent leukocytes. Figure 2a is a representative image of a flat-mount retina from a diabetic mouse with adherent leukocytes indicated by white arrowheads. As shown in Figure 2b, diabetic mice had significantly ($p < 0.05$) higher numbers of leukocytes compared to non-diabetic mice. Leukocyte counts in the flat-mount retinas of apoEdp-treated diabetic mice were significantly ($p < 0.05$) lower than vehicle-treated diabetic mice.

Inhibition of ECM-degrading proteases by intravitreal apoEdp treatment is mediated through a negative regulation of LRP-1

ApoE protein is a ligand for the cell surface receptor LRP-1 expressed in the retina. Images (Figure 3a) of Western blots of retinal protein extracts were captured and densitometry analysis was performed to quantitatively analyze protein bands with the relative intensity values normalized to non-diabetic control value set to 1.0. As shown in Figure 3b, apoEdp treatment resulted in a significant ($p < 0.05$) downregulation of both total LRP-1 and phosphorylated LRP-1 (p-LRP-1) in diabetic mice compared to non-diabetic mice. In apoEdp-treated diabetic mice, a significant ($p < 0.05$) upregulation of both total LRP-1 and p-LRP-1 was seen and suggests a negative role of LRP-1 is related to expression of ECM-degrading proteases heparanase and MMP-2. We found a significant ($p < 0.05$) inhibition of heparanase and MMP-2 by apoEdp treatment of diabetic mice compared to vehicle-treated diabetic mice. Figure 4a is representative gelatin zymogram of retinal tissue homogenates and densitometry analysis of white lytic bands indicative of gelatinase activity of MMP 2 (~70 kDa) as shown in Figure 4b suggests a significant inhibition of gelatinase activity in apoEdp-treated diabetic mice compared to control diabetic mice ($p < 0.05$).

Inhibition of ECM-degrading enzymes by apoEdp treatment significantly ($p < 0.05$) increased tight junction proteins occludin and ZO-1 expression. Thus, our results suggest that LRP-1 upregulation has a positive correlation to tight junction protein retention in apoEdp-treated diabetic mice.

ApoEdp treatment downregulated the Wnt/ β -catenin pathway in diabetic retinas

ApoEdp treatment resulted downregulation in the expression of its other family (LDLR) members LRP-5/LRP-6. Figure 5a shows the expression level of specific proteins related to Wnt/ β -catenin pathway in mice retinas by Western blot. Figure 5b shows the result of quantitative densitometry analysis of retinal protein expression. As seen in Figure 4b, apoEdp treatment significantly ($p < 0.05$) reduced the pro-inflammatory molecules HIF-1 α , VEGF, and ICAM-1 through a downregulation of LRP-6. Figure 6a shows the nuclear β -catenin accumulation in experimental retinal tissues. Densitometry analysis (Figure 6b) suggests a significant ($p < 0.05$) increase in nuclear β -catenin accumulation was seen in retinas of diabetic mice compared to non-diabetic. ApoEdp treatment resulted a significant ($p < 0.05$) drop of nuclear β -catenin compared to diabetic control.

Discussion

We used a mouse eye model of chronically developed (26 weeks of diabetes) DR with measurable retinal permeability and inflammatory leukostasis.

ECM degradation is important to facilitate endothelial basement membrane breakdown and the loss of tight junction proteins occludin and ZO-1, leading to the development of porous retina with increased BRB permeability.³ ECM-degrading enzymes heparanase⁴ and MMP-2⁸ are related to vascular remodeling. Both heparanase and MMP-2 are the ligands of LRP-1.^{4,10} Both of these ligands normally present extracellularly in their inactive form and become functionally active following internalization through LRP-1 in clathrin coated pits, lysosomal processing, and recycling back to the cell surface. For example, heparanase—an ECM-degrading proteinase—is initially produced as pro-heparanase (inactive) and uses this LRP-1 mediated processing to derive the mature and active form of heparanase.⁴ Similarly, another ECM-degrading proteinase pro-MMP-2 (inactive) uses LRP-1 as a port of entry for internalization and subsequent endocytic processing and peripheral localization in its active form of MMP-2.⁸ Our study suggests apoE-derived peptide inhibits both of these proteinases (heparanase and MMP-2) in diabetic retinas.

This is the first report that intravitreal administration of apoEdp significantly inhibited retinal permeability as evidenced by this study of Evans blue extravasation assay. Our *in vivo* results of the inhibitory function of apoEdp against retinal permeability in diabetic mice is correlated to (1) significant downregulation of heparanase and MMP-2, and (2) upregulation of tight junction proteins occludin and ZO-1. LRP-1 is found to play a negative role in the expression of ECM-degrading proteinases, as significantly ($p < 0.05$) higher expression of heparanase and MMP-2 is observed in diabetic mice while LRP-1 expression is significantly ($p < 0.05$) lower. Higher LRP-1 expression was correlated with a significant ($p < 0.05$) increase in the amount of the tight junction proteins occludin and ZO-1.

In a mouse model of oxygen-induced retinopathy, LRP-1 functions as negative regulator of retinal angiogenesis.²⁴ No reports available about the role of LRP-1 in hyperglycemia-induced retinopathy. This is the first report to suggest that LRP-1 also negatively regulate vascular abnormalities related to DR. In cancer biology, conflicting reports of negative and positive regulation of pathogenesis are available. LRP-1 also reported to have negative relationship to metastatic potential of many different types of cancer cells.^{25–28} Low expression of LRP-1 reported to promote ECM degradation and enhance invasion and migration of tumor cells. Increased expression of LRP-1 found to have a negative and protective role in the progression of hepatic carcinoma through downregulated expression of MMP-9.²⁹ However, in an *in-vitro* study, LRP-1 is found to have positive regulation in the invasion and migration of glioblastoma cell.³⁰ Therefore, all these data suggest that a cell-specific microenvironment may be responsible for LRP-1 regulated pathology. Further study is needed to decipher the precise cell-specific role of LRP-1 in the pathogenesis of retinal diseases.

RP-1 is also involved in many signal transduction pathways including the Wnt/ β -catenin pathway. Wnt/ β -catenin signaling pathway is reported to play key roles in the production of

inflammatory and angiogenic molecules in the diabetic retina.³¹ In retinal vascular complications, the Wnt ligand binds to its receptor Fz (frizzled) and co-receptor LRP-5 or LRP-6 to inactivate GSK-3 β , resulting in the stabilization and cytosolic accumulation β -catenin that leads to its nuclear translocation and subsequent upregulation in the transcription of the pro-inflammatory genes VEGF, HIF-1 α , and ICAM-1³². In our study, diabetic mice had significantly ($p < 0.05$) lower expression of LRP-1, but higher ($p < 0.05$) LRP-6, β -catenin, and related pro-inflammatory molecules compared to non-diabetic mice. This suggests a negative role of LRP-1 in the Wnt/ β -catenin pathway in diabetic retinas. ApoEdp treatment of diabetic mice reversed the negative role of LRP-1. Others¹² also reported a similar negative role of LRP-1 in the regulation of the Wnt/ β -catenin pathway. LRP-1 is reported to sequester Fz and inhibits the receptor-co-receptor complex formation causing subsequent suppression of the Wnt/ β -catenin pathway.¹² Both LRP-1 and LRP-6 are known to interact with Fz, and like others^{12,31}, we also conclude that LRP-1 negatively regulates the Wnt/ β -catenin pathway.

In our study, we found that apoEdp inhibition of LRP-1 causes multitargeted effects in the inhibition of retinal vascular abnormalities in diabetic mice rather than one specific target. We found, inhibiting LRP-1 by apoEdp results in the prevention of ECM degradation and BRB integrity as well as downregulation of angiogenic and anti-inflammatory mediators of DR. Such a multi-potent therapeutic agent like apoEdp may be a suitable candidate for the treatment of DR.

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Figure 1.

Six months after the onset of STZ-induced diabetes, retina permeability was tested by Evan's blue dye permeation between apoEdp-treated versus vehicle treated mice. Dimethylformamide extract of diabetic retina had significantly ($*p < 0.05$) higher yield of exudated Evan's blue dye concentration compared to non-diabetic control mice. ApoEdp-treated mice had significant ($*p < 0.05$) reduction in the Evan's blue dye permeation compared to vehicle treated diabetic mice. Each value represents the mean $\pm$ SEM ($n = 5$).

Figure 2.

Hyperglycemic injury-led EC dysfunction results in the secretion of ICAM-1 and attracts leukocytes to adhere along the intima of blood vessels resulting inflammatory leukostasis characteristic to NPDR. Following intracardiac perfusion of Leukocyte staining dye concanavalin A lectin conjugated FITC, counts of leukocytes in the blood vessels of flat-mount retinas were compared between vehicle treated diabetic mice versus apoEdp-treated diabetic mice retinas. Figure 4a is a representative picture of flat-mount retina from a diabetic mouse showing adherent leukocytes indicated by white arrows. As shown in Figure 4b, diabetic mouse retina had significantly ($p < 0.05$) higher count of leukocytes compared to non-diabetic mice. ApoEdp-treated diabetic mice retinas had significantly ($p < 0.05$) lower count of adherent leukocytes compared to vehicle treated diabetic mice. Each value represents the mean $\pm$ SEM results in three independent experiments.

Figure 3.

LRP-1 is an endocytic receptor for many ligands including apoE and proteinases. Retinal protein extracts were analyzed to determine the effect of blocking LRP-1 by apoEdp in the expression of ECM-degrading enzymes and tight junction proteins. (a) Western blot detection of LRP-1 mediated regulation of ECM-degrading enzymes heparanase, MMP-2 and tight junction proteins occludin and ZO-1. (b) Densitometry analysis of Western blot suggests a significant ($p < 0.05$) decrease in the expression of both total LRP-1 and p-LRP-1 in diabetic mice compared to non-diabetic mice. ApoEdp treatment showed a significant ($p < 0.05$) upregulation of LRP-1 and p-LRP-1 compared to vehicle treated diabetic mice. Increased LRP-1 suggests a positive correlation to protect tight junction proteins occludin and ZO-1, however, a negative correlation to ECM-degrading enzymes heparanase and MMP-2. Each value represents the mean $\pm$ SEM, $p < 0.05$.

Figure 4.

Gelatinase activity of MMP-2 was detected using commercial gelatin zymography gels. (a) Appearance of white lytic bands at ~70 kDa mark suggests gelatinase activity of MMP-2. (b) A densitometry analysis of gelatinase activity of MMP-2 suggests a significant increase in the intensity of lytic band in diabetic mice compared to non-diabetic. Gelatinase activity of MMP-2 was found to have a significant drop in apoEdp-treated samples compared to diabetic control. The relative intensity values were normalized to control value set at 1.0. Each value represents the mean $\pm$ SEM, $p < 0.05$.

Figure 5.

Western blot analysis of retinal protein extract was done to determine the effect of apoEdp treatment in the expression Wnt/ β -catenin signaling pathway. (a) Western blot detection of the effect of apoEdp treatment on LRP-6 regulated expression of pro-inflammatory molecules HIF-1 α , VEGF and ICAM-1. (b) Densitometry analysis suggests that Wnt/ β -catenin pathway molecules LRP-6, HIF-1 α , VEGF and ICAM-1 are significantly increased in diabetic mice retinas compared to non-diabetic mice. ApoEdp treatment significantly decreased all these Wnt/ β -catenin pathway molecules. The relative intensity values were normalized to control value set at 1.0. Each value represents the mean $\pm$ SEM, $p < 0.05$.

Figure 6.

Nuclear fraction of protein was extracted from retinal tissues and Western blot analyzed to detect β -catenin mobilization and accumulation in the nucleus. (a) Representative documentation of β -catenin bands in the nuclear fraction of retinal tissues. (b) Densitometry analysis suggests a significant increase of nuclear β -catenin in the diabetic mice compared to non-diabetic. However, apoEdp treatment resulted a significant decrease in nuclear β -catenin accumulation compared to diabetic control mice. The relative intensity values were normalized to control value set at 1.0. Each value represents the mean $\pm$ SEM, $p < 0.05$.