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# Caveolin-1 regulates inflammatory mediators in retinal endothelial cells

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**Purpose:** We previously reported that the high mobility group box 1 (HMGB1) and NLR family pyrin domain-containing 3 (NLRP3) inflammatory pathways are involved in the retinal complications of diabetes. Caveolin-1 (Cav1) has been shown to regulate inflammatory pathways in other targets, inspiring us to explore them in the retinal vasculature.

**Methods:** For these studies, we hypothesized that the blockade of Cav1 would reduce inflammatory pathways in primary human retinal endothelial cells (RECs). To test our hypothesis, we first measured Cav1 protein levels in retinal lysates from humans with and without diabetes. We also measured Cav1 in control and streptozotocin-treated diabetic mice. We grew REC in normal glucose (5 mM) and high glucose (25 mM) media. Some cells in the high glucose condition were treated with Cav1 siRNA or a scrambled siRNA. We used Western blotting to measure Cav1 protein levels as well as HMGB1 and NLRP3 pathway proteins.

**Results:** Our data show that diabetes in both humans and mice led to increased levels of Cav1. The Cav1 siRNA reduced Cav1 levels in RECs, and RECs grown in high glucose had increased levels of HMGB1, tumor necrosis factor alpha (TNFα), and NLRP3 pathway proteins. All the inflammatory proteins were reduced by Cav1 siRNA.

**Conclusions:** These data suggest that Cav1 can alter inflammatory mediators in RECs. The inhibition of Cav1 may offer a new avenue for therapeutic development.

The past two decades have seen the emergence of increased awareness of inflammation's role in the diabetic retina [1,2]. While the role of inflammation is clear, the regulation of these inflammatory mediators has remained elusive. One reported factor that potentially regulates inflammatory mediators in other targets is caveolin-1 (Cav1). The literature reveals that patients with proliferative diabetic retinopathy had significantly increased levels of Cav1 [3], and a type 2 diabetic rat model, the Goto-Kakizaki model, showed increased Cav1 levels at 6 months of diabetes [4]. In a laser-induced retinal damage model, Jiang et al. found increased ocular neovascularization, which was further exacerbated by the loss of Cav1 [5]. However, other studies have shown that loss of Cav1 reduced vascular endothelial growth factor (VEGF) in retinal pigmented epithelial (RPE) cells [6]. Thus, Cav1's exact actions seem to depend on the model used for investigation.

Cav1 is a member of the caveolin family (Cav1, Cav2, Cav3), the primary protein components of caveolae [7], which are 50–100 nm vesicles that form invaginations in the plasma membrane and play a significant role in cellular signaling [7]. Caveolins are the structural family that form caveolae, with Cav1 recruiting the cavins (1/2/3/4) to the caveolae [8]. Cav1 has been linked to a plethora of cellular effects, including lipid droplet formation [9], oxidative stress modulation [10], permeability changes [11], and inflammation [12]. In many of these studies, Cav1 is enriched in endothelial cells [13].

Cav1 is important to endothelial-mediated inflammation in multiple tissues [14]. Cav1 knockout mice are characterized as having a low-grade inflammatory state as evidenced by increased levels of IL-6 and tumor necrosis factor alpha (TNFα) in their plasma [14] as well as increased numbers of lymphocytes. A study of the mechanism by which Cav1 induces inflammation shows that blocking Cav1 reduces morphine-induced inflammation through inhibition of the NLRP3 inflammasome [15]. Cav1 has been shown to repress or promote tumor growth depending upon the cellular milieu. In breast cancer cells, the knockdown of Cav1 or high mobility group box 1 (HMGB1) reduced estradiol (E2)-mediated cell growth and inflammation [16], suggesting that HMGB1 mediates cancer growth. Similarly, others report

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Table 1. Body weights (BW) and blood glucose (BG) of diabetic (STZ) mice.

| C57BL/6 | | | | |
|---|---|---|---|---|
| Time | Ctrl | | STZ | |
| | BW (g) | BG | BW (g) | BG |
| 8 weeks | 25.6±2.2 | 113±12 | 25.2±2.0 | 110±8.1 |
| 6 months after STZ | 35±2* | 119±8 | 26±1.4 | 397±119# |

Data are mean ± Standard deviation. Streptozotocin (STZ) BW (bodyweight); BG (blood glucose). *p<0.05 # p<0.05.

that Cav1 contributed to HMGB1 secretion and increased breast cancer metastasis via toll-like receptor 4 (TLR4) [17]. In addition to cancer, research in lung injury found a role for the Cav1-induced HMGB1 pathway, with the authors using glycyrrhizin to inhibit HMGB1 to reduce lung injury [18]. We have previously shown that inhibiting both HMGB1 and NLRP3 plays a role in protecting the diabetic retina [19].

Based on these findings in the retina and other targets, and given our focus on retinal inflammation, our goal was to evaluate whether Cav1 regulates the HMGB1 and NLRP3 inflammatory pathways (including HMGB1, TNFα, cleaved caspase 1, and interleukin-1 beta [IL-1β]) in primary human retinal endothelial cells (RECs) exposed to high glucose. We hypothesized that inhibiting Cav1 would protect RECs against high glucose–induced inflammation.

## METHODS

*Human retinal samples:* Dr. Mohamed Al-Shabrawey (Oakland University) provided retinal samples from seven healthy control patients and seven patients with diabetic retinopathy (both type 1 and type 2), having received approval for these samples from Oakland University. This study followed the principles of the Declaration of Helsinki and was approved by the Ethics Committee of Oakland University (5/9/23; IRB-FY2023–292). Oakland University's Institutional Review Board (IRB) evaluated the samples and information and on May 9, 2023 deemed that the study entitled "Molecular and Cellular Mechanisms of Diabetic Retinopathy" did not constitute human research, as the samples were de-identified and collected postmortem. All diabetic patients had had the disease for 10+ years. The samples were processed for protein detection by western blotting [20].

*Mice:* Eight-week-old male C57BL/6 mice (Strain# 000664) were purchased from Jackson Laboratories (Bar harbor, ME), and some were injected with 60 mg/kg streptozotocin (STZ) to render them type 1 diabetic. Table 1 shows the mice's body weights and glucose levels. At 6 months of diabetes, the mice were sacrificed to measure the protein levels. All animal procedures followed the requirements of the Association for Research in Vision and Ophthalmology, conformed to National Institute of Health (NIH) guidelines, and were approved by the Institutional Animal Care and Use Committee of Wayne State University.

*Retinal endothelial cells :* Primary human RECs were purchased from Cell Systems Corporation (Kirkland, WA). The cells were grown in Cell Systems medium (5 mM glucose) supplemented with microvascular growth supplement (MVGS), 10 ug/ml gentamycin, and 0.25 ug/ml amphotericin B (Invitrogen, Carlsbad, CA) on attachment factor coated dishes (Cell Systems). Once the cells reached confluence, some dishes were switched to Cell Systems medium with high glucose (25 mM glucose). Only cells before passage 6 were used. The cells were quiesced by incubating in high or normal glucose medium without MVGS for 24 h before experimental use. The cells were in normal or high glucose conditions for a minimum of 3 days before any experiments.

*Cell treatments:* The RECs in high glucose were transfected with Cav1 siRNA or scrambled siRNA (Origene, Rockville, MD) using RNAiMax following the manufacturer's instructions. We have previously reported that osmotic actions are not key to inflammatory changes in these cells [21].

*Western blotting:* Whole retinal lysates from the control and diabetic humans as well as mice and cell culture lysates were collected in lysis buffer containing protease and phosphatase inhibitors. Equal amounts of protein were separated onto a precast tris-glycine gel (Invitrogen, Carlsbad, CA) and blotted onto a nitrocellulose membrane. After blocking in Tris-buffered saline with 0.1% Tween® 20 detergent (TBST; 10 mM Tris-HCl buffer, pH 8.0; 150 mM NaCl; 0.1% Tween-20) and 5% (w/v) bovine serum albumin (BSA), the membranes were treated with Cav1, HMGB1, NLRP3, cleaved caspase 1, IL-1β, TNFα (Abcam), or beta actin (Santa Cruz Biotechnology, Santa Cruz, CA) primary antibodies, followed by incubation with secondary antibodies labeled with horseradish peroxidase. Antigen-antibody complexes were detected by a chemiluminescence reagent kit (Thermo Scientific, Pittsburgh, PA), and data were acquired using an Azure C500 (Azure Biosystems, Dublin, CA). Western blot data were assessed using Image Studio Lite software.

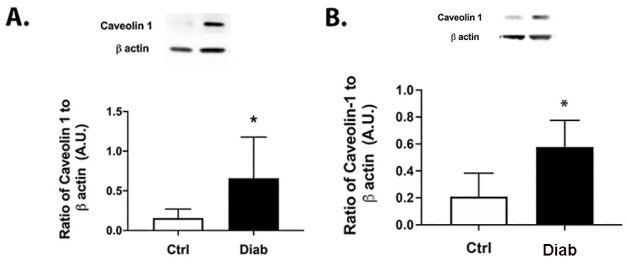


Figure 1. Cav1 is increased in the retina of diabetic humans and mice. Panels **A** and **B** show protein data from whole retinal lysates from control and diabetic patients (A) and control and diabetic (STZ-treated) mice (B). Arbitrary units (A.U.); *p <.05 versus ctrl.; n = 7 for human retinae and 6 for STZ-treated mice retinae.

*Statistics:* Statistical analyses were conducted by one-way analysis of variance (ANOVA) with Tukey's post-hoc test on Prism software 9.0 (GraphPad, La Jolla, CA). For work with human and mice samples, an unpaired *t*-test was used, with $p < 0.05$ deemed significant. A representative blot is provided for western blot data.

## RESULTS

*Cav1 Is increased in diabetic human and mouse retinas:* To explore the actions of caveolin in the retina, we first measured protein levels in the diabetic retina. Figure 1A shows that diabetes significantly increased Cav1 in the retina when compared to samples from nondiabetic patients. In the diabetic mouse samples, 6 months of diabetes significantly increased Cav1 levels (Figure 1B) compared to the control mice.

*Exposure to high glucose increases Cav1, which can be blocked by siRNA:* To explore Cav1's potential mechanisms in the retinal vasculature, we investigated whether high glucose culturing conditions increased Cav1. Figure 2A shows significantly higher Cav1 levels in RECs grown in high glucose. To support these findings and inform future work, we also grew RECs in normal and high glucose media and treated with Cav1 or scrambled siRNA. Cav1 siRNA effectively reduced Cav1 levels (Figure 2B).

*Reduced levels of Cav1 lead to reduced HMGB1 levels:* RECs grown in high glucose had significantly higher levels of HMGB1 (Figure 3A) and TNFα (Figure 3B) than those grown in normal glucose. For each of these proteins, Cav1 siRNA significantly reduced the levels of inflammatory mediators.

*Cav1 regulates NLRP3 pathway proteins:* RECs were grown to confluence in normal and high glucose. Those grown in high glucose had significantly increased levels of NLRP3 (Figure 4A), cleaved caspase 1 (Figure 4C), and IL-1β (Figure 4D). Cav1 siRNA significantly reduced levels of the NLRP3 pathway proteins.

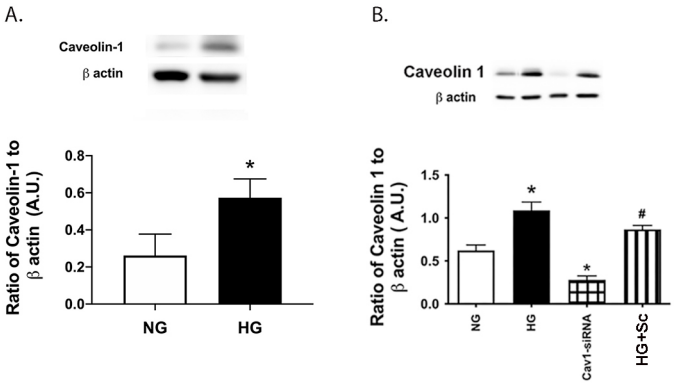


Figure 2. High glucose increased Cav1 in retinal endothelial cells (RECs). Panel **A** shows Cav1 levels in RECs grown in normal glucose (NG) or high glucose (HG). Panel **B** shows Cav1 levels in RECs grown in NG, HG, HG+Cav1 siRNA (Cav1 siRNA), or HG+scrambled siRNA (HG+Sc). Arbitrary units (A.U.); *p <.05 versus NG; #p <.05 versus HG; n = 5. Data are mean±SEM.

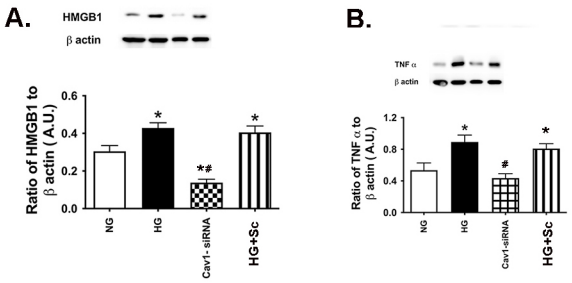


Figure 3. Cav1 regulates HMGB1 and TNFα. Retinal endothelial cells (REC) grown in normal glucose (NG), high glucose (HG), HG+Cav1 siRNA (Cav1 siRNA), and HG+scrambled siRNA (HG+Sc). Panel **A** shows HMGB1, and Panel **B** shows TNFα protein levels. Arbitrary units (A.U.); *p <.05 versus NG; #p <.05 versus HG; n = 5. Data are mean±SEM.

## DISCUSSION

We found increased Cav1 levels in protein samples from whole retinal lysates of human diabetic patients and diabetic mice. We also show that high glucose culturing conditions significantly increased Cav1 levels. The high glucose–induced increase in Cav1 was associated with increased inflammatory mediators, including the HMGB1, TNFα, and NLRP3 signaling proteins. RECs transfected with siRNA against Cav1 had significantly reduced levels of the inflammatory mediators.

The literature suggests that humans with diabetic retinopathy have higher levels of Cav1 [3], and one study found an increased expression of Cav1 in the retina of STZ-treated mice at 6 and 12 weeks of diabetes [11]. Studies conducted with Goto-Kakizaki mice at 6 months of diabetes found increased Cav1 levels [4]. Our findings in 6-month STZ-treated diabetic mice support these findings.

Since Cav1 was increased in the diabetic mice, we next wanted to explore Cav1's effects on RECs, as others have reported Cav1 localization on RECs [22]. We used primary human RECs grown in normal and high glucose to show that Cav1 was increased in high glucose conditions and was blocked by Cav1 siRNA. We have previously reported that high glucose culturing conditions increased inflammatory mediators, including HMGB1 and NLRP3 inflammasome proteins [23,24]. We explored whether Cav1 was involved in these actions, finding that the inhibition of Cav1 led to significantly decreased levels of HMGB1 and NLRP3 inflammasome proteins in RECs grown in high glucose. These findings agree with other studies showing that HMGB1 regulates Cav1 activities in RPE cells [25]. In immune cells, Cav1 regulates HMGB1 to mediate inflammation in breast cancer [17]. Similarly, inhibition of HMGB1 with glycyrrhizic acid protected against acute lung injury through the modulation of Cav1 activities [18]. Cav1 may regulate HMGB1 and NLRP3 in RECs through actions on TLR4, which has been reported in blood-brain barrier permeability [26]. We have previously reported TLR4's actions in the retinal vasculature. Other studies report that blocked Cav1 actions significantly reduced NLRP3 inflammasome–induced injury in rats [15]. Thus, much of the literature agrees that Cav1 can modulate inflammation through HMGB1 and NLRP3 inflammasome actions, which agrees with our findings in RECs.

Most of the studies conducted for the present research were done in RECs grown in normal or high glucose. Future studies should be conducted in the diabetic retina and in diabetic Cav1 knockout mice, as this will be critical to determining the role of Cav1 in inflammation in the diabetic retina. We can also explore the role of Cav1 in mitochondrial

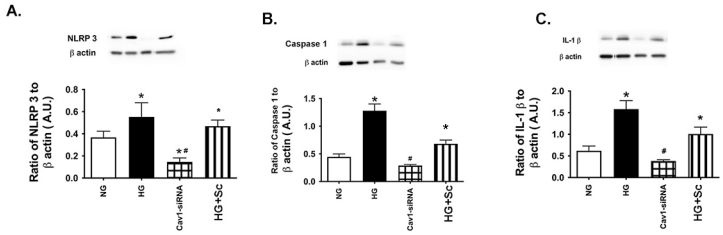


Figure 4. Cav1 regulates the NLRP3 pathway. Retinal endothelial cells (RECs) grown in normal glucose (NG), high glucose (HG), HG+Cav1 siRNA (Cav1 siRNA), and HG+scrambled siRNA (HG+Sc). Panel **A** shows NLRP3, Panel **B** shows cleaved caspase 1, and Panel **C** shows IL-1β protein levels. Arbitrary units (A.U.); *p <.05 versus NG; #p <.05 versus HG; n = 5. Data are mean±SEM.

function in RECs, as Cav1 reportedly regulates mitochondrial actions in other targets [27].

In conclusion, our data agree with the existing literature, suggesting that Cav1 is increased in the diabetic retina. Cav1 siRNA reduced key inflammatory pathways in RECs grown in diabetic-like conditions. These studies lay the groundwork for more in vivo studies.

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