HHS Public Access

Author manuscript

Microvasc Res. Author manuscript; available in PMC 2024 July 01.

Published in final edited form as:

Microvasc Res. 2023 July ; 148: 104510. doi:10.1016/j.mvr.2023.104510.

Loss of Cystatin C regulates permeability and inflammatory pathways in retina

Li Liu,

Youde Jiang,

Jena J. Steinle

Department of Ophthalmology, Visual, and Anatomical Sciences, Wayne State University School of Medicine, Detroit, MI 48201, USA

Abstract

Cystatin C has been linked to inflammation in other diseases, such as epilepsy and Alzheimer's disease. These studies were designed to investigate whether Cystatin C regulates retinal inflammation and permeability. To address this question, we used Cystatin C knockout mice in a retinal ischemia/reperfusion model to determine whether Cystatin C regulated retinal damage, as well as inflammatory mediators and retinal permeability. To support the mouse work, we also used primary retinal endothelial cells cultured in normal and high glucose. Ischemia/reperfusion in Cystatin C knockout mice caused increased formation of degenerate capillaries. Loss of Cystatin C increased fluorescein leakage in the retina, which was accompanied by reduced levels of zonula occludin 1 (ZO-1) and occludin proteins. When REC were grown in high glucose, recombinant Cystatin C decreased retinal permeability, while Cystatin C siRNA increased dextran flux compared to high glucose alone. Recombinant Cystatin C decreased levels of interleukin-1-beta (IL-1β) and high mobility group box 1 (HMGB1) levels. In conclusion, loss of Cystatin C increased vascular damage in response to ischemia/reperfusion. Cystatin C regulated permeability and inflammatory mediators in the retina in response to stressors. Cystatin C offers a new target for retinal disease therapeutic development.

Keywords

Cystatin C; ischemia/reperfusion; permeability; inflammation

Introduction.

Inflammation in the retina is a component in several diseases, including diabetic retinopathy, macular degeneration, and glaucoma. One potential player in regulating retinal inflammation

Contributions: Liu performed the experiments, analyzed the data, wrote the methods, edited the final version; Jiang performed the experiments, edited the final version; Steinle designed the experiments, received the funding and wrote the text.

Conflicts of Interest: No authors have any conflicts of interest with this work.

is cystatin C (Kaur et al., 2010; Mi et al., 2007). Cystatin C is a member of the type 2 cystatin superfamily of cysteine proteases that can be found both inside and outside of cells (Paraoan et al., 2010). Cystatin C has been shown to be involved in protein turnover, innate immunity, activation of precursor proteins, MHC-II antigen presentation, and apoptosis (Paraoan et al., 2010; Zi and Xu, 2018). One of the key functions of cystatin C is the regulation of the cathepsins, specifically cathepsin B, D, H, L, and S (Paraoan et al., 2010).

Alterations in cystatin C levels are associated with numerous diseases. Increased cystatin C is a marker of kidney disease (Shimizu et al., 2003), and elevated cystatin C levels have been noted in models of patients with heart disease (Vigil et al., 2009), breast cancer (Zavrsnik et al., 2017), Alzheimer's (Duan et al., 2018), epilepsy (Pirttila et al., 2005), and aortic aneurysms (Liu et al., 2019). Mutations in cystatin C are responsible for the Icelandic form of cerebral amyloid angiography (Levy et al., 1989). Work in an Alzheimer's model found that overexpression of cystatin C was able to cause microtubule instability (Duan et al., 2018). Others have shown that cystatin C is increased in response to injury, such as ischemia, seizure, or epilepsy (Kaur et al., 2010), suggesting that Cystatin C levels may rise in response to injury to inhibit negative cathepsin actions. Work has also demonstrated a role for cystatin C in metabolic syndrome (Vigil et al., 2009) and insulin resistance (Lee et al., 2010; Uruska et al., 2014).

Focusing on the eye, a variant form of cystatin C has been linked to age-related macular degeneration (Butler et al., 2015; Paraoan et al., 2004). Others have also shown that Cystatin C mRNA occurs with a reasonably high abundance in retinal pigmented epithelial (RPE) cells (Paraoan et al., 2000), potentially functioning in secretory proteostasis in the RPE (Paraoan et al., 2020). Others showed that the addition of advanced glycation end-products to human RPE or increased donor age significantly decreased cystatin C levels, suggesting age-related changes in cystatin C actions in the RPE (Kay et al., 2014). Other studies have shown a link between elevated cystatin C levels and the diabetic retina in later stages of the disease (He et al., 2013; Wong et al., 2015)

Since Cystatin C has been associated with several ocular diseases, our goal was to explore Cystatin C actions in the retina. We used Cystatin C knockout mice to investigate Cystatin C in the retina, as well as human primary retinal endothelial cells (REC) grown in normal and high glucose to examine Cystatin C actions on retinal inflammation and permeability.

Materials and Methods.

Mice.

Drs. Efrat Levy and Monika Pawlik generously provided the Cystatin C knockout mice (Kaur et al., 2010). Both male and female mice were used at approximately 8 weeks of age. C57BL/6 mice were purchased from Jackson laboratories and used as controls since the Cystatin C KO mice were generated on a C57BL/6-129Sv background (Kaur et al., 2010). All animal procedures meet the Association for Research in Vision and Ophthalmology requirements and were approved by the Institutional Animal Care and Use Committee of Wayne State University and conform to NIH guidelines.

Genotyping.

We did genotyping using methods similar to our previous work (Liu et al., 2017c). The primer pairs in this study were: 5'→3' mutant forward: CGG CGA GTA CAA CAA AGC CA and 5'->3' mutant reverse: GGA GGT GTG CAT AAG AGG TG. For the intact Cystatin C 5'→3' forward: AAA ACA AGG GCA TTC TCT ACA TAC and reverse: ACC AAA ACA GCA AGG CAG CAC AGG.

Ischemia/reperfusion model (I/R).

Ischemia/reperfusion was done as we have previously published (Abcouwer et al., 2013; Liu et al., 2016, 2018a).

Vascular Analyses.

Ten days after I/R exposure, additional mice were sacrificed to measure degenerate capillaries, as we have done previously (Liu et al., 2016; Veenstra et al., 2015).

Fluorescein Angiography.

We performed fluorescein angiography on control and cystatin C mice as we have done in the past (Liu et al., 2017c; Seidel et al., 2021).

Cell Culture.

Primary human retinal endothelial cells (REC) were cultured as we have done previously (Liu et al., 2017a). Cells were used prior to passage 6. Cells were cultured in normal (5mM) or high (25mM) glucose.

Some cells in normal and high glucose were treated with recombinant Cystatin C (100nM, Sigma) or Cystatin C siRNA and scrambled siRNA (Origene, Rockville, MD).

Permeability assay.

REC grown in normal and high glucose medium in transwell chambers. On the day of the experiments, 70kD rhodamine isothiocyanate (RITC) dextran was added to the upper chamber of the transwell insert. Cells were near 100% confluence after hydrocortisone treatment before any treatments. Analyses were done as we have reported previously (Liu et al., 2021).

Western blotting.

Cell culture lysates or whole retinal lysates were collected into lysis buffer with protease and phosphatase inhibitors for Western blotting as we have done previously (Liu et al., 2022; Zhang et al., 2012). Primary antibodies to ZO-1, occludin, HMGB1, IL-1β, TNFα (Abcam, Cambridge, MA) or beta actin (Santa Cruz Biotechnology, Santa Cruz, CA) were used. Data was acquired using an Azure C500 (Azure Biosystems, Dublin, CA) and blot data measured using Image Studio Lite software, as we have done previously (Liu et al., 2018b).

Statistics.

Prism software 9.0 (GraphPad, La Jolla, CA) was used for statistical analyses. A one-way ANOVA with Tukey's post-hoc test was used for analyses. For work with mice, an unpaired T test was used. $P<0.05$ was taken as significant. A representative blot is provided for Western blot data.

Results.

Validation of the Cystatin C knockout mice.

We performed genotyping and Western blotting to verify successful knockdown of cystatin C in the mice. Results for genotyping show that Cystatin C bands are at the appropriate molecular weight for both control and Cystatin C knockout mice (Figure 1A). To verify protein levels, we did Western blots for cystatin C in whole retinal lysates from knockout and control mice showing significantly lower Cystatin C in the retina of the mice (Figure 1B).

Loss of Cystatin C exacerbates the formation of degenerate capillaries in ischemia/reperfusion (I/R).

We measured degenerate capillaries in the Cystatin C and controls mice 10 days after exposure to I/R. We found that I/R significantly increased numbers of degenerate capillaries in control mice, which were further increased with in the Cystatin C knockout mice exposed to I/R (Figure 2), suggesting that loss of Cystatin C induces vascular damage to the mouse retina.

Loss of Cystatin C increases inflammatory mediators.

Literature suggested that Cystatin C regulated inflammatory mediators in other targets (Zi and Xu, 2018). Therefore, we wanted to explore Cystatin C actions on inflammatory mediators in the mouse retina. Using whole retinal lysates from 2-month-old Cystatin C knockout mice and C57BL/6 mice, we measured protein levels of HMGB1 (A), IL-1β (B) and TNFα (C). Loss of Cystatin C significantly increased all 3 inflammatory mediators compared to retinal levels in control mice (Figure 3).

Cystatin C regulates permeability in mouse retina.

Retinal stressors often alter permeability (Abcouwer et al., 2010), so we investigated whether vascular leakage was increased in the Cystatin C knockout mice. Fluorescein angiography leakage was increased in the Cystatin C knockout mice vs. control mice (Figure 4A). Since both ZO-1 and occluding have been linked to retinal permeability changes (Murakami et al., 2009), we wanted to test these proteins in our mice. We found that occludin (B) and ZO-1 (C) levels were significantly decreased in whole retinal lysates from the Cystatin C knockout mice compared to control mice (Figure 4).

Recombinant Cystatin C decreases inflammatory mediators in REC.

To support our work in mouse retina, we grew human REC in normal (5mM) and high (25mM) glucose and treated them with 100nM cystatin C. Culturing in high glucose alone

significantly increased both IL-1β (A) and HMBG1 (B), which were significantly reduced by treatment with recombinant Cystatin C (Figure 5).

Cystatin C alters retinal endothelial cell permeability.

Since we found altered permeability proteins in the Cystatin C knockout mice, we performed a permeability assay on REC grown in normal or high glucose or cells cultured in high glucose treated with Cystatin C siRNA, scrambled siRNA or recombinant Cystatin C (Figure 6). All cells were at greater than 90% confluency before the experiment was initiated. Data shows that high glucose increased permeability, which was further increased by Cystatin C siRNA. Cells in high glucose treated with recombinant Cystatin C had reduced permeability compared to high glucose only (A). To further explore this, we measured Cystatin C (A), occludin (B) and ZO-1 (C) protein levels in REC grown in normal or high glucose and treated with Cystatin C siRNA or scrambled siRNA. Figure 6A shows successful knockdown of cystatin C by the Cystatin C siRNA transfection. Figure 6B–C show that high glucose decreased occludin and ZO-1 levels, which were further reduced by Cystatin C siRNA.

Discussion.

The ischemia/reperfusion (I/R) model has been used as a model of retinal stress in numerous other studies (Abcouwer et al., 2013; Caballero et al., 2007; Liu et al., 2017b; Muthusamy et al., 2014). In the current work, we demonstrated that loss of Cystatin C exacerbated the damage associated with exposure to I/R. Similar findings were observed in RPE cells, where reduced cystatin C levels allowed for increased invasiveness of the cells and enhanced extracellular matrix degradation (Carlsson et al., 2020). Cystatin C is neuroprotective in epilepsy models (Kaur et al., 2010). Cystatin C has been shown to be protective against Alzheimer's disease (Mathews and Levy, 2016), potentially through enhanced amyloid precursor processing (Wang et al., 2016).

In this study, we focused on the role of Cystatin C in inflammation. We found increased inflammatory mediators, HMGB1, IL-1β, and TNFα, in mice lacking Cystatin C. Similar results were obtained in REC grown in high glucose. When these cells were treated with recombinant Cystatin C, inflammatory mediator levels were reduced. Our findings agree with studies showing that cystatin C is involved in multiple steps of immunity (Zi and Xu, 2018). Specifically, mice lacking Cystatin C have a higher incidence of arthritis, with an earlier onset (Backlund et al., 2011). Other studies on monocytes from patients with Icelandic form of hereditary cerebral hemorrhage with amyloidosis showed increased levels of Cystatin C in immune cell types (Warfel et al., 1987). Cystatin C has been shown to regulate levels of various cathepsins, including Cathepsin B and D (Alizadeh et al., 2006). Both Cathepsin B and D are linked to increased inflammatory mediators (Fox et al., 2016; Guicciardi et al., 2000). Thus, our work and the literature strongly support a role for Cystatin C in the regulation of inflammatory pathways. We will explore whether this regulation requires cathepsin B and D in future studies.

In contrast to a vast literature on inflammation and Cystatin C, less has been reported on the role of Cystatin C in regulating cellular permeability. However, in the past 5 years, some

work has shown a role for Cystatin C in decreasing permeability in models of cerebral ischemia. Proteomics studies on mice exposed to a preconditioning model had reduced numbers of infarctions. Cystatin C knockout mice did not have this protection (Fang et al., 2017). Additionally, another group used the ischemia/reperfusion model showed that Cystatin C improved blood-brain-barrier actions, with an increase in occludin (Yang et al., 2020). These findings agree well with our work in the retina showing a protective effect of Cystatin C in the retina against I/R damage.

Our data show that Cystatin C is involved in the regulation of permeability and inflammation in the retina. While the mouse data contrasts the high levels of Cystatin C seen in the later stages of the diabetic retina, this should not detract from the findings for other retinal diseases. We accept that fluorescein angiography is qualitative, but feel that this data, combined with the protein levels and cell culture data, support our conclusions that Cystatin C regulates permeability. Future work will delve more deeply into the mechanisms by which Cystatin C regulates retinal inflammation and permeability, as well as the role of Cystatin-mediated regulation of the cathepsins. We acknowledge that the control mice used for these studies are not an exact match for the cystatin C knockout mice. Mice were age-matched, but there is a possibility that a mismatch was possible. We tried to compare KO to KO with ischemia and control mice to control mice with ischemia. Additionally, retinal images looked similar between both groups of mice, but the different background of the mice confounding some of the data remains a possibility.

Taken together, data strongly suggests that cystatin C is protective against retina stressor through a reduction in inflammatory mediators and improved permeability responses. Future studies need to better explore these actions in animal models of other ocular diseases.

Acknowledgement:

We want to thank Drs. Efrat Levy and Monika Pawlik for the Cystatin C knockout mice. These studies were funded by R01EY0028442 (JJS), R01EY030284, and P30EY04068 Core grant (LDH, PI of Core grant) and an unrestricted grant from Research to Prevent Blindness

References.

Abcouwer SF, Lin CM, Shanmugam S, Muthusamy A, Barber AJ, Antonetti DA, 2013. Minocycline prevents retinal inflammation and vascular permeability following ischemia-reperfusion injury. Journal of neuroinflammation 10, 149. [PubMed: 24325836]

Abcouwer SF, Lin CM, Wolpert EB, Shanmugam S, Schaefer EW, Freeman WM, Barber AJ, Antonetti DA, 2010. Effects of ischemic preconditioning and bevacizumab on apoptosis and vascular permeability following retinal ischemia-reperfusion injury. Investigative ophthalmology & visual science 51, 5920–5933. [PubMed: 20554620]

Alizadeh P, Smit-McBride Z, Oltjen SL, Hjelmeland LM, 2006. Regulation of cysteine cathepsin expression by oxidative stress in the retinal pigment epithelium/choroid of the mouse. Experimental eye research 83, 679–687. [PubMed: 16684524]

Backlund A, Holmdahl M, Mattsson R, Hakansson K, Lindstrom V, Nandakumar KS, Grubb A, Holmdahl R, 2011. Cystatin C influences the autoimmune but not inflammatory response to cartilage type II collagen leading to chronic arthritis development. Arthritis Res Ther 13, R54. [PubMed: 21443774]

Butler JM, Sharif U, Ali M, McKibbin M, Thompson JP, Gale R, Yang YC, Inglehearn C, Paraoan L, 2015. A missense variant in CST3 exerts a recessive effect on susceptibility to age-related

macular degeneration resembling its association with Alzheimer's disease. Hum Genet 134, 705–715. [PubMed: 25893795]

Caballero S, Sengupta N, Afzal A, Chang KH, Li Calzi S, Guberski DL, Kern TS, Grant MB, 2007. Ischemic vascular damage can be repaired by healthy, but not diabetic, endothelial progenitor cells. Diabetes 56, 960–967. [PubMed: 17395742]

Carlsson E, Supharattanasitthi W, Jackson M, Paraoan L, 2020. Increased Rate of Retinal Pigment Epithelial Cell Migration and Pro-Angiogenic Potential Ensuing From Reduced Cystatin C Expression. Investigative ophthalmology & visual science 61, 9.

Duan J, Marcellus KA, Qin X, Wang Y, Paudel HK, 2018. Cystatin C promotes tau protein phosphorylation and causes microtubule instability by inhibiting intracellular turnover of GSK3beta in neurons. Mol Cell Neurosci 89, 1–8. [PubMed: 29577984]

Fang Z, Deng J, Wu Z, Dong B, Wang S, Chen X, Nie H, Dong H, Xiong L, 2017. Cystatin C Is a Crucial Endogenous Protective Determinant Against Stroke. Stroke 48, 436–444. [PubMed: 27999137]

Fox C, Cocchiaro P, Oakley F, Howarth R, Callaghan K, Leslie J, Luli S, Wood KM, Genovese F, Sheerin NS, Moles A, 2016. Inhibition of lysosomal protease cathepsin D reduces renal fibrosis in murine chronic kidney disease. Sci Rep 6, 20101. [PubMed: 26831567]

Guicciardi ME, Deussing J, Miyoshi H, Bronk SF, Svingen PA, Peters C, Kaufmann SH, Gores GJ, 2000. Cathepsin B contributes to TNF-alpha-mediated hepatocyte apoptosis by promoting mitochondrial release of cytochrome c. J Clin Invest 106, 1127–1137. [PubMed: 11067865]

He R, Shen J, Zhao J, Zeng H, Li L, Zhao J, Liu F, Jia W, 2013. High cystatin C levels predict severe retinopathy in type 2 diabetes patients. Eur J Epidemiol 28, 775–778. [PubMed: 23918210]

Kaur G, Mohan P, Pawlik M, DeRosa S, Fajiculay J, Che S, Grubb A, Ginsberg SD, Nixon RA, Levy E, 2010. Cystatin C rescues degenerating neurons in a cystatin B-knockout mouse model of progressive myoclonus epilepsy. The American journal of pathology 177, 2256–2267. [PubMed: 20889561]

Kay P, Yang YC, Hiscott P, Gray D, Maminishkis A, Paraoan L, 2014. Age-related changes of cystatin C expression and polarized secretion by retinal pigment epithelium: potential age-related macular degeneration links. Investigative ophthalmology & visual science 55, 926–934. [PubMed: 24458156]

Lee SH, Park SA, Ko SH, Yim HW, Ahn YB, Yoon KH, Cha BY, Son HY, Kwon HS, 2010. Insulin resistance and inflammation may have an additional role in the link between cystatin C and cardiovascular disease in type 2 diabetes mellitus patients. Metabolism 59, 241–246. [PubMed: 19765773]

Levy E, Lopez-Otin C, Ghiso J, Geltner D, Frangione B, 1989. Stroke in Icelandic patients with hereditary amyloid angiopathy is related to a mutation in the cystatin C gene, an inhibitor of cysteine proteases. J Exp Med 169, 1771–1778. [PubMed: 2541223]

Liu L, Jiang Y, Chahine A, Curtiss E, Steinle JJ, 2017a. Epac1 agonist decreased inflammatory proteins in retinal endothelial cells, and loss of Epac1 increased inflammatory proteins in the retinal vasculature of mice. Molecular vision 23, 1–7. [PubMed: 28210097]

Liu L, Jiang Y, Steinle JJ, 2016. Compound 49b Restores Retinal Thickness and Reduces Degenerate Capillaries in the Rat Retina following Ischemia/Reperfusion. PloS one 11, e0159532. [PubMed: 27439004]

Liu L, Jiang Y, Steinle JJ, 2017b. Inhibition of HMGB1 protects the retina from ischemia-reperfusion, as well as reduces insulin resistance proteins. PloS one 12, e0178236. [PubMed: 28542588]

Liu L, Jiang Y, Steinle JJ, 2017c. Toll-Like Receptor 4 Reduces Occludin and Zonula Occludens 1 to Increase Retinal Permeability Both in vitro and in vivo. J Vasc Res 54, 367–375. [PubMed: 29136627]

Liu L, Jiang Y, Steinle JJ, 2018a. Epac1 protects the retina against ischemia/reperfusion-induced neuronal and vascular damage. PloS one 13, e0204346. [PubMed: 30235337]

Liu L, Jiang Y, Steinle JJ, 2021. Forskolin regulates retinal endothelial cell permeability through TLR4 actions in vitro. Mol Cell Biochem 476, 4487–4492. [PubMed: 34499321]

Liu L, Jiang Y, Steinle JJ, 2022. PKA and Epac1 Reduce Nek7 to Block the NLRP3 Inflammasome Proteins in the Retinal Vasculature. Investigative ophthalmology & visual science 63, 14.

Liu L, Patel P, Steinle JJ, 2018b. PKA regulates HMGB1 through activation of IGFBP-3 and SIRT1 in human retinal endothelial cells cultured in high glucose. Inflamm Res 67, 1013–1019. [PubMed: 30328477]

Liu Y, Jiao Y, He Y, Ding X, Su Q, Zhao Y, Jiang J, 2019. Expression levels of cathepsin L and cystatin C in a hyperglycemic environment were associated with aortic aneurysm development in a mouse model. J Int Med Res 47, 2499–2506. [PubMed: 31096818]

Mathews PM, Levy E, 2016. Cystatin C in aging and in Alzheimer's disease. Ageing Res Rev 32, 38–50. [PubMed: 27333827]

Mi W, Pawlik M, Sastre M, Jung SS, Radvinsky DS, Klein AM, Sommer J, Schmidt SD, Nixon RA, Mathews PM, Levy E, 2007. Cystatin C inhibits amyloid-beta deposition in Alzheimer's disease mouse models. Nat Genet 39, 1440–1442. [PubMed: 18026100]

Murakami T, Felinski EA, Antonetti DA, 2009. Occludin phosphorylation and ubiquitination regulate tight junction trafficking and vascular endothelial growth factor-induced permeability. The Journal of biological chemistry 284, 21036–21046. [PubMed: 19478092]

Muthusamy A, Lin CM, Shanmugam S, Lindner HM, Abcouwer SF, Antonetti DA, 2014. Ischemia-reperfusion injury induces occludin phosphorylation/ubiquitination and retinal vascular permeability in a VEGFR-2-dependent manner. J Cereb Blood Flow Metab 34, 522–531. [PubMed: 24398936]

Paraoan L, Grierson I, Maden BE, 2000. Analysis of expressed sequence tags of retinal pigment epithelium: cystatin C is an abundant transcript. Int J Biochem Cell Biol 32, 417–426. [PubMed: 10762067]

Paraoan L, Hiscott P, Gosden C, Grierson I, 2010. Cystatin C in macular and neuronal degenerations: implications for mechanism(s) of age-related macular degeneration. Vision Res 50, 737–742. [PubMed: 19917302]

Paraoan L, Ratnayaka A, Spiller DG, Hiscott P, White MR, Grierson I, 2004. Unexpected intracellular localization of the AMD-associated cystatin C variant. Traffic 5, 884–895. [PubMed: 15479453]

Paraoan L, Sharif U, Carlsson E, Supharattanasitthi W, Mahmud NM, Kamalden TA, Hiscott P, Jackson M, Grierson I, 2020. Secretory proteostasis of the retinal pigmented epithelium: Impairment links to age-related macular degeneration. Progress in retinal and eye research 79, 100859. [PubMed: 32278708]

Pirttila TJ, Lukasiuk K, Hakansson K, Grubb A, Abrahamson M, Pitkanen A, 2005. Cystatin C modulates neurodegeneration and neurogenesis following status epilepticus in mouse. Neurobiol Dis 20, 241–253. [PubMed: 16242633]

Seidel A, Liu L, Jiang Y, Steinle JJ, 2021. Loss of TLR4 in endothelial cells but not Muller cells protects the diabetic retina. Experimental eye research 206, 108557. [PubMed: 33789141]

Shimizu A, Horikoshi S, Rinnno H, Kobata M, Saito K, Tomino Y, 2003. Serum cystatin C may predict the early prognostic stages of patients with type 2 diabetic nephropathy. J Clin Lab Anal 17, 164–167. [PubMed: 12938144]

Uruska A, Araszkiewicz A, Zozulinska-Ziolkiewicz D, Wegner M, Grzelka A, Wierusz-Wysocka B, 2014. Does serum cystatin C level reflect insulin resistance in patients with type 1 diabetes? Clin Biochem 47, 1235–1238. [PubMed: 24956263]

Veenstra A, Liu H, Lee CA, Du Y, Tang J, Kern TS, 2015. Diabetic Retinopathy: Retina-Specific Methods for Maintenance of Diabetic Rodents and Evaluation of Vascular Histopathology and Molecular Abnormalities. Curr Protoc Mouse Biol 5, 247–270. [PubMed: 26331759]

Vigil L, Lopez M, Condes E, Varela M, Lorence D, Garcia-Carretero R, Ruiz J, 2009. Cystatin C is associated with the metabolic syndrome and other cardiovascular risk factors in a hypertensive population. J Am Soc Hypertens 3, 201–209. [PubMed: 20409960]

Wang XF, Liu DX, Liang Y, Xing LL, Zhao WH, Qin XX, Shang DS, Li B, Fang WG, Cao L, Zhao WD, Chen YH, 2016. Cystatin C Shifts APP Processing from Amyloid-beta Production towards Non-Amyloidgenic Pathway in Brain Endothelial Cells. PloS one 11, e0161093. [PubMed: 27532339]

Warfel AH, Zucker-Franklin D, Frangione B, Ghiso J, 1987. Constitutive secretion of cystatin C (gamma-trace) by monocytes and macrophages and its downregulation after stimulation. J Exp Med 166, 1912–1917. [PubMed: 3119764]

Wong CW, Teo BW, Lamoureux E, Ikram MK, Wang JJ, Tai ES, Sethi S, Wong TY, Sabanayagam C, 2015. Serum Cystatin C, Markers of Chronic Kidney Disease, and Retinopathy in Persons with Diabetes. Journal of diabetes research 2015, 404280. [PubMed: 26576434]

Yang B, Xu J, Chang L, Miao Z, Heang D, Pu Y, Zhou X, Zhang L, Xie H, 2020. Cystatin C improves blood-brain barrier integrity after ischemic brain injury in mice. J Neurochem 153, 413–425. [PubMed: 31603990]

Zavrsnik J, Butinar M, Prebanda MT, Krajnc A, Vidmar R, Fonovic M, Grubb A, Turk V, Turk B, Vasiljeva O, 2017. Cystatin C deficiency suppresses tumor growth in a breast cancer model through decreased proliferation of tumor cells. Oncotarget 8, 73793–73809. [PubMed: 29088746]

Zhang Q, Guy K, Pagadala J, Jiang Y, Walker RJ, Liu L, Soderland C, Kern TS, Ferry R Jr., He H, Yates CR, Miller DD, Steinle JJ, 2012. Compound 49b Prevents Diabetes-Induced Apoptosis through Increased IGFBP-3 Levels. Investigative ophthalmology & visual science 53, 3004–3013. [PubMed: 22467575]

Zi M, Xu Y, 2018. Involvement of cystatin C in immunity and apoptosis. Immunol Lett 196, 80–90. [PubMed: 29355583]

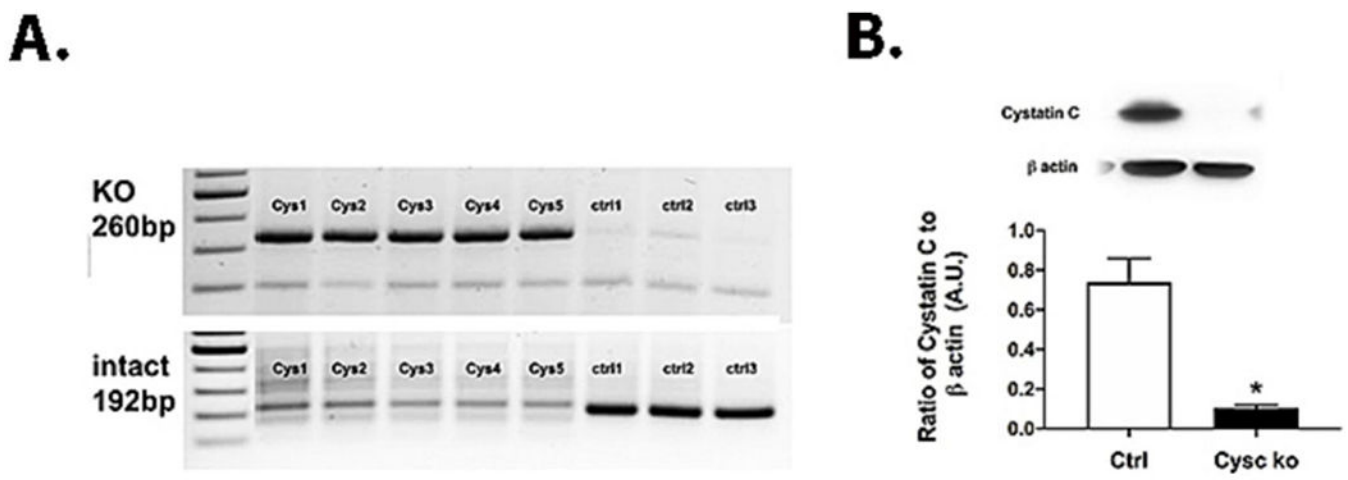

Figure 1.

Validation of the Cystatin C KO mice. Panel A is genotyping, Panel B is immunostaining and Panel C is Western blot data confirming the lack of Cystatin C in the knockout mice compared to control. In Panel A, Ctrl 1-3 are samples from C57BL/6 mice and Cst 1-5 are samples from Cystatin C KO mice. The ladder is 1kB. 270bp is the expected size of knockout cystatin levels, while 192bp is the expected size for normal cystatin C. Panel B is Western blotting for Cystatin C in whole retinal lysates. *P<0.05 vs. C57BL/6.

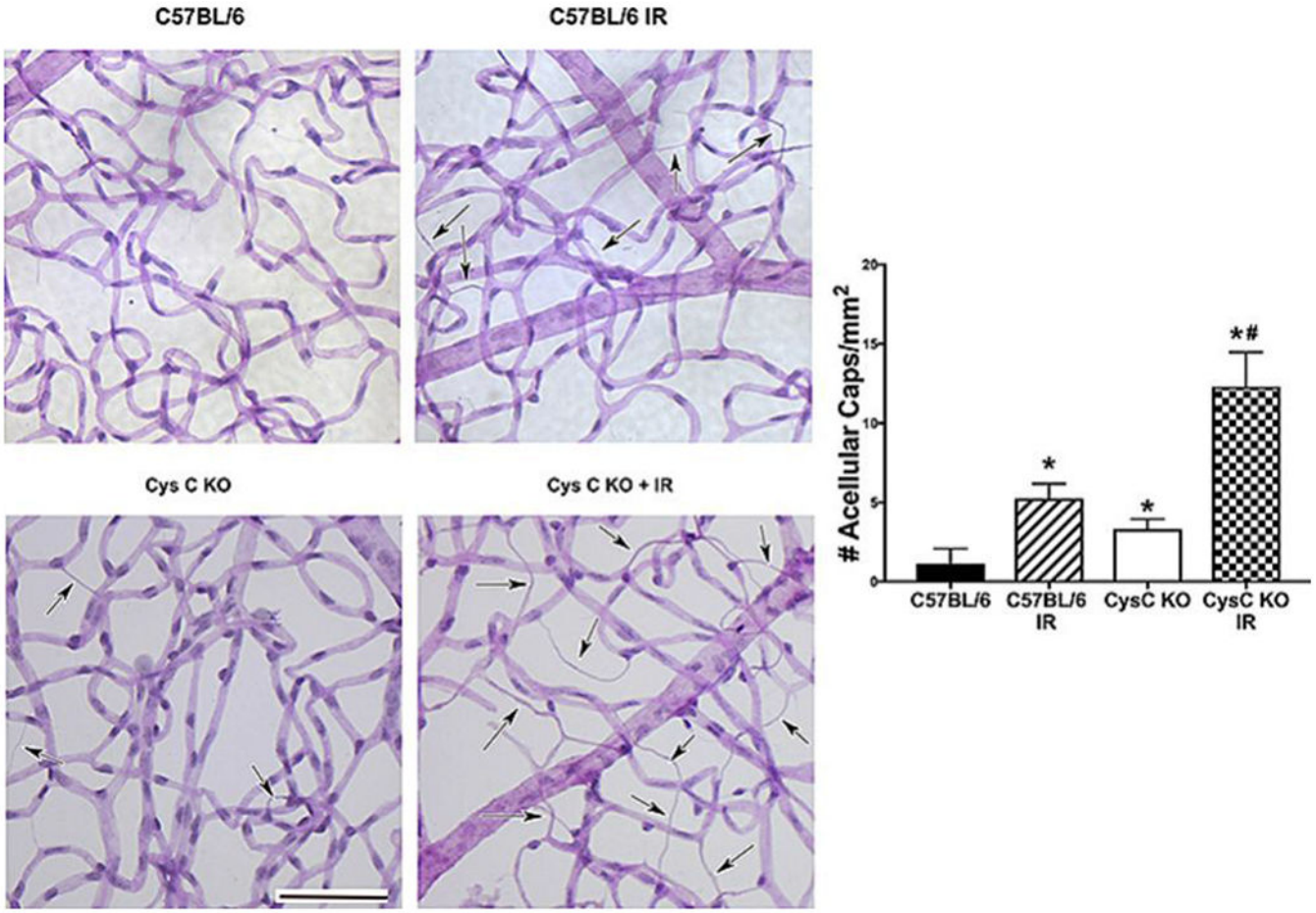

Figure 2.

Ischemia/reperfusion in the Cystatin C mice exacerbates retinal damage. Data is from control mice, mice exposed to ischemia/reperfusion (I/R), and cystatin C knockout mice exposed to nothing or I/R. Data show vascular changes (degenerate capillaries). *P<0.05 vs. C57BL/6, #P<0.05 vs. I/R. N=5.

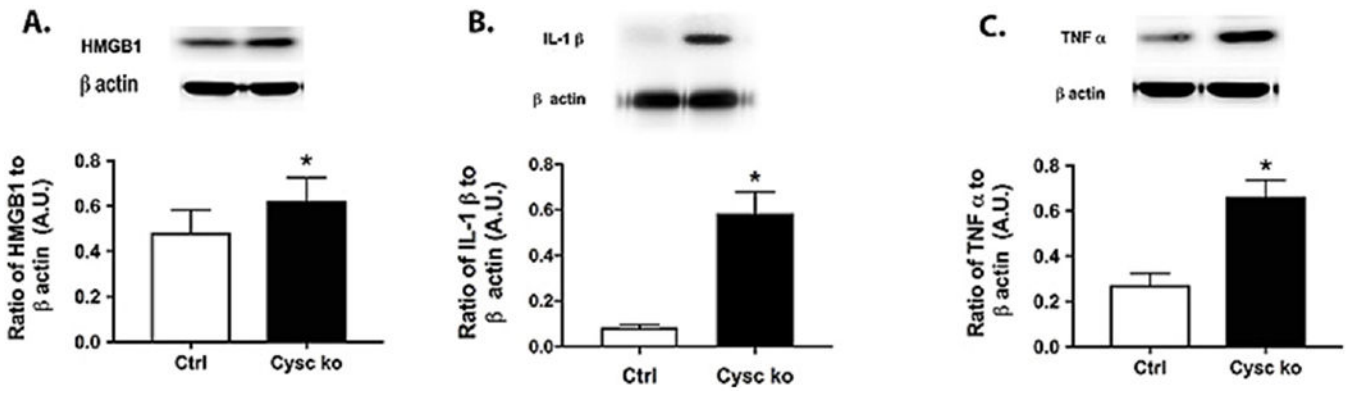

Figure 3.

Cystatin C regulates inflammatory mediators in mouse retina. Western blot data from whole retinal lysates from Cystatin C knockout (Cys C KO) and C57BL/6 (Ctrl) mice for HMGB1 (A), IL-1B (B) and TNFα (C). *P<0.05 vs. C57BL/6. N=5. Data are mean ± SEM.

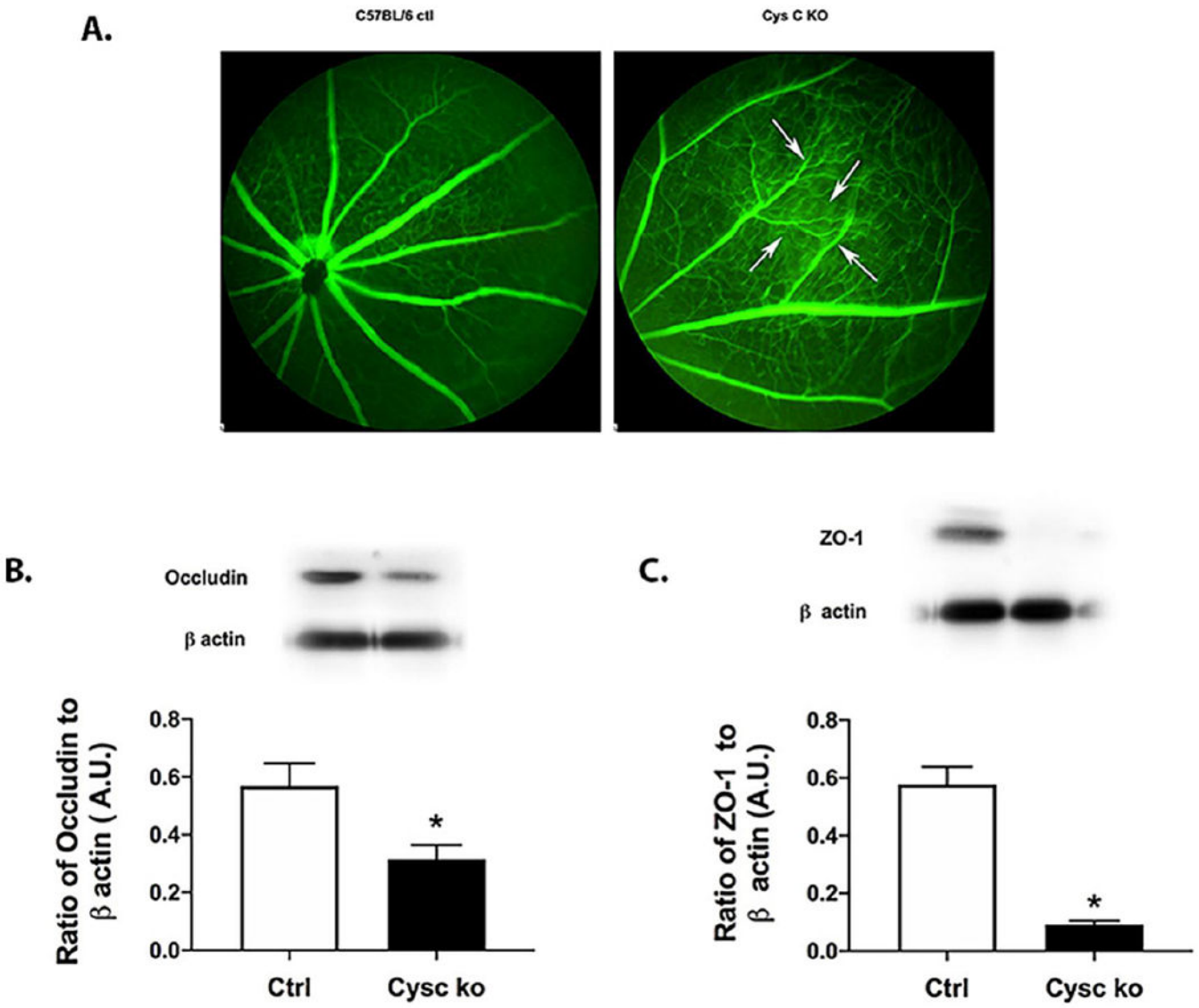

Figure 4.

Cystatin C regulates permeability in the mouse retina. Panel A shows fluorescein angiography in Cystatin C knockout (Cys C KO) vs. C57BL/6 (Ctrl) mice. Panels B-C show Western blot data from whole retinal lysates for occludin (B) and ZO-1 (C). *P<0.05 vs. C57BL/6. N=7 for fluorescein angiography, N=5 for western blot samples. Data are mean ± SEM.

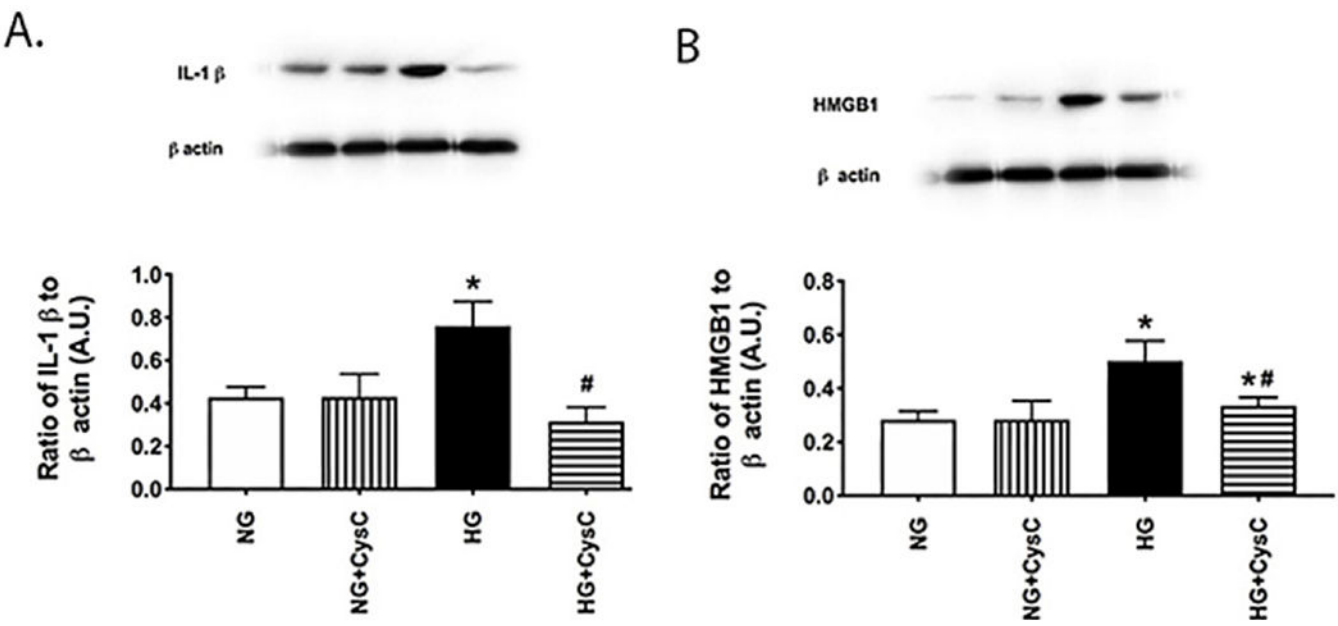

Figure 5.

Cystatin C decreased inflammatory mediators in retinal endothelial cells. Retinal endothelial cells (REC) grown in normal or high glucose were treated with recombinant Cystatin C. The data show that IL-1β (A) and HMGB1 (B) were decreased compared to high glucose alone. *P<0.05 vs. normal glucose, #P<0.05 vs. high glucose. N=5. Data are mean ± SEM.

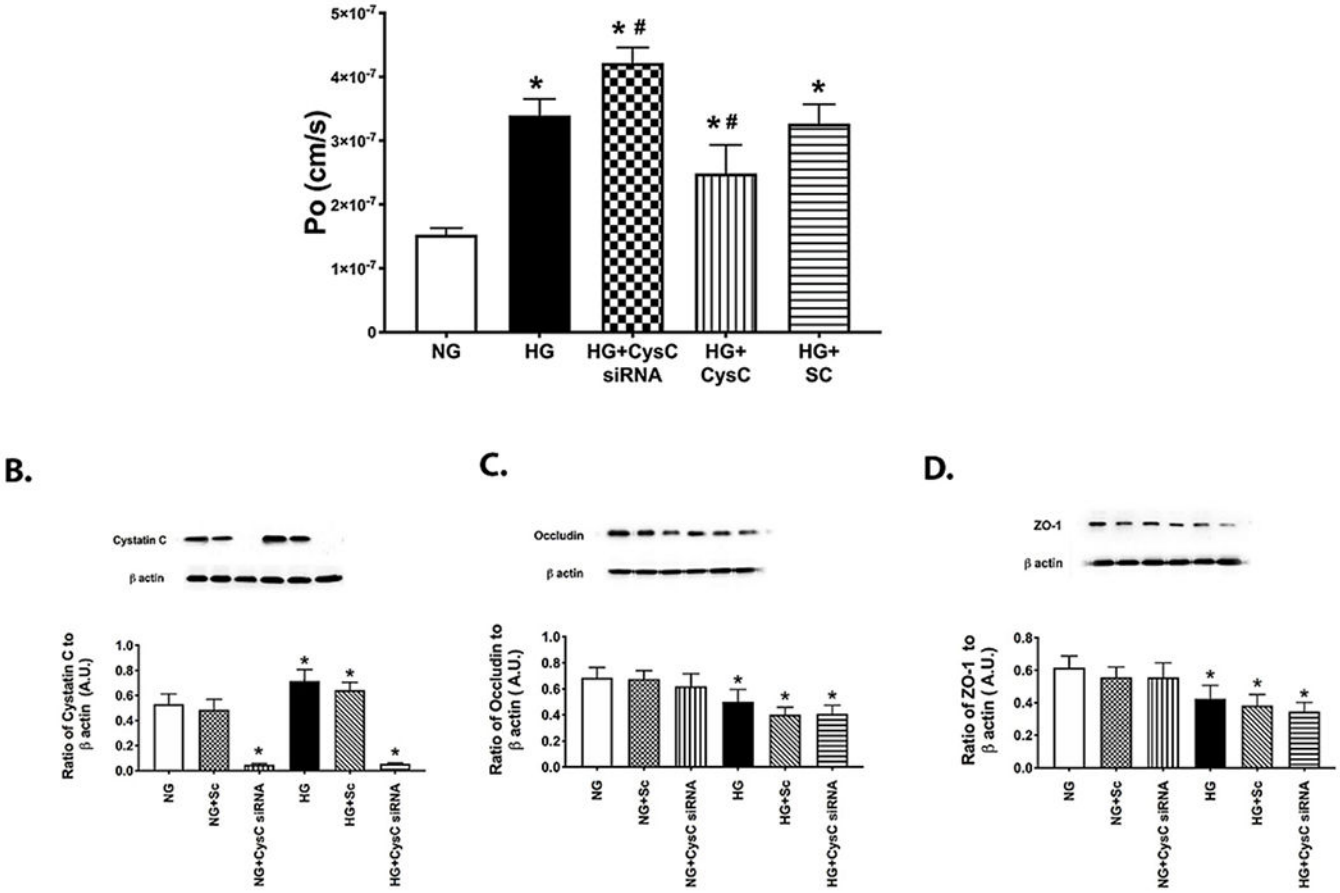

Figure 6.

Loss of Cystatin C decreased permeability, as well as ZO-1 and occludin protein levels. Retinal endothelial cells (REC) were grown in normal or high glucose and treated with Cystatin C siRNA, recombinant cystatin C, or scrambled siRNA. Panel A is a permeability assay for cells treated with either cystatin C siRNA, recombinant Cystatin C or scrambled siRNA. Panel B-D shows protein levels in cells treated with cystatin C siRNA. Panel B is a control to show successful knockdown of Cystatin C with siRNA. Panel C (occludin) and Panel D (ZO-1) show that protein levels are decreased compared to high glucose alone. *P<0.05 vs. normal glucose, #P<0.05 vs. high glucose. N=5. Data are mean ± SEM.