

TNFAIP3 is anti-inflammatory in the retinal vasculature

Li Liu, Youde Jiang, Jena J. Steinle

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

Purpose: To determine whether tumor necrosis factor alpha-induced protein 3 (TNFAIP3) regulates inflammatory and permeability proteins in the retinal vasculature.

Methods: We used retinal lysates from type 1 diabetic mice and endothelial cell-specific exchange protein for cAMP 1 (Epacl) knockout mice to determine the protein levels of TNFAIP3. We also treated retinal endothelial cells (RECs) in normal (5 mM) and high (25 mM) glucose with an Epacl agonist or with TNFAIP3 siRNA. We performed western blotting for TNFAIP3 and inflammatory and permeability proteins after treatment. TNFAIP3 siRNA was used only in cells grown in high glucose. Immunostaining was performed for localization of ZO-1 and tight junction protein 1.

Results: TNFAIP3 was reduced in the diabetic retinas and the retinas of the Epacl conditional knockout mice. The Epacl agonist increased TNFAIP3 levels in RECs grown in high glucose. Reduction of TNFAIP3 with siRNA led to increased levels of tumor necrosis factor alpha (TNF α) and phosphorylation of nuclear factor kappa beta (NF-kB), while decreasing occludin and zonula occludens 1 (ZO-1) protein levels and inhibitory kappa beta kinase (IkB) phosphorylation. Tumor receptor-associated factor 6 (TRAF6) levels were increased above high glucose levels.

Conclusions: TNFAIP3 serves as an anti-inflammatory factor in the retinal vasculature. Epacl regulates TNFAIP3. TNFAIP3 may offer a new mechanism for regulating inflammation and permeability in the retinal vasculature.

Preproliferative diabetic retinopathy has been associated with increased levels of inflammatory mediators [1,2]. We previously reported increased tumor necrosis factor alpha (TNF α) and interleukin-1-beta (IL-1 β) levels in diabetic retinas and retinal endothelial cells (RECs) grown in high glucose [3,4]. To regulate these inflammatory pathways, other anti-inflammatory pathways must exist to maintain homeostasis in the retina. One such regulator may be tumor necrosis factor alpha-induced protein 3 (TNFAIP3).

TNFAIP3 is an anti-inflammatory protein that plays a role in immune responses and cell death [5]. The impaired function of TNFAIP3 is involved in several autoimmune diseases, including Crohn's, asthma, and chronic obstructive pulmonary disorder [5,6]. Single nucleotide mutations in TNFAIP3 have been reported in type 1 diabetes, lupus, and rheumatoid arthritis [6]. TNFAIP3 encodes a protein (A20) that acts as a ubiquitin-editing enzyme that serves as a negative regulator of nuclear factor kappa beta (NF-kB) [6]. A20 has been shown to block inflammasome actions while also blocking toll-like receptor actions [5]. One potential mechanism by which A20 can regulate inflammation is through TNF receptor-associated protein 6 (TRAF6). Studies have shown that inhibition of A20 increased TRAF6, as well as NF-kB, in early brain injury [7] and in other models of

neuroinflammation [8]. Similarly, A20 has antiosteoclastogenic effects through TRAF6-mediated autophagy in periodontitis [9].

Focusing on endothelial cell actions, TNFAIP3 plays a role in permeability [10]. Endothelial A20 is required for VE-cadherin actions at adherens junctions [10]. Few investigations have focused on TNFAIP3 in the eye. One study showed that TNFAIP3 promoted neovascularization in photoreceptors [11]. Little work has been done on the retinal vasculature. Therefore, we hypothesized that a reduction in TNFAIP3 would increase inflammatory mediator levels in RECs grown in high glucose.

METHODS

Diabetic mice: Eight-week-old male C57BL/6J mice (Jackson Laboratories) were made diabetic with 60 mg/kg injections of streptozotocin dissolved in citrate buffer for up to 5 consecutive days. The control mice received citrate buffer only. Glucose measurements were performed biweekly, with glucose levels >250 mg/dl considered diabetic. Glucose measurements were measured using about 5 μ l blood samples via a hand-held measurement device.

Epacl endothelial cell-specific knockout mice: Epacl floxed mice (B6;129S2-Rapgef3^{tm1Geno/J} mice) and B6 FVB-Tg (cdh5-cre)7Mlia/J Cre mice were purchased from Jackson Laboratories (Bar Harbor, ME). Epacl floxed mice were bred with cdh5-Cre mice to generate a line of conditional knockout mice with Epacl eradicated from vascular endothelial cells [12].

Correspondence to: Jena J. Steinle, Department of Ophthalmology, Visual, and Anatomical Sciences, 9314 Scott Hall, Detroit, MI 48202. Phone (313) 577-9731; FAX: (313) 577-3125 email: jsteinle@med.wayne.edu

Retinal lysates from Epacl floxed and Epacl Cre-Lox mice were collected at 2 months of age. All animal procedures met the Association for Research in Vision and Ophthalmology requirements, were approved by the Institutional Animal Care and Use Committee of Wayne State University, and conformed to NIH guidelines.

Western blotting: Western blotting was performed as previously described [12,13]. Briefly, cell lysates or whole retina lysates were separated onto precast tris-glycine gels (Invitrogen, Carlsbad, CA) and then blotted onto nitrocellulose membrane. After blocking in TBST, membranes were treated with Epacl, TNFAIP3, TRAF6, TNF α , phosphorylated and total NF-kB, phosphorylated and total I κ B, occludin, ZO-1 (Abcam, Cambridge, MA), or beta actin (Santa Cruz Biotechnology, Santa Cruz, CA) primary antibodies. Secondary species-appropriate antibodies labeled with horseradish peroxidase were applied after the primary antibodies. Chemiluminescence (Thermo Scientific, Pittsburgh, PA) was used to visualize antigen-antibody complexes. Images were acquired on an Azure C500 (Azure Biosystems, Dublin, CA), and optical densities were measured using Image Studio Lite software (Lincoln, NE).

Primary retinal endothelial cells: Primary human RECs were purchased from Cell System Corporation (CSC, Kirkland, WA). The cells were grown in normal glucose medium (Cell Systems) with the addition of microvascular growth supplements (Invitrogen), 10 μ g/ml gentamycin, and 0.25 μ g/ml amphotericin B. Once confluent, some cells were transferred to high (25 mM) glucose medium (Cell Systems) for 3 days. Only primary cells within passage 6 were used. Cells were quiesced by incubating in a high or normal glucose medium without growth factors for 24 h and used to perform the experiments unless otherwise indicated.

Some cells were treated with an Epacl agonist (8-CPT-2'-O-Me-cAMP, 10 μ M, 24 h), as we have done previously [14]. Additional cells in high glucose were transfected with TNFAIP3 siRNA (Qiagen, Germantown, MD) or scrambled siRNA (Qiagen).

Immunostaining: REC were grown in normal and high glucose medium for 1 week before they were placed in multi-chamber slides. Once cells reached confluence following TNFAIP3 siRNA treatment, the medium was removed, and 4% paraformaldehyde was added for 20 min. After washing in PBS (1X; 120 mM NaCl, 20 mM KCl, 10 mM NaPO₄, 5 mM KPO₄, pH 7.4), the slides were blocked with 5% bovine serum albumin (BSA) for 1 h at room temperature to block nonspecific staining. The slides were then incubated with rabbit antitight junction protein 1 (1:200, Novus) or mouse anti-ZO-1 (1:200, Invitrogen) at 4 °C overnight. After rising in

0.1% Triton/PBS, slides were incubated with a goat anti-rabbit secondary antibody conjugated to Alexa Fluor 594 (1:500, Life Technologies, Carlsbad, CA) or donkey anti-mouse conjugated to Alexa Fluor 488 (1:500, Life Technologies) for 2 h at room temperature. The slides were then rinsed in PBS and counterstained with 4',6-diamidino-2-phenylindole (DAPI). Finally, the slides were mounted with FluorSave Reagent Sigma (St. Louis, MO) and examined on a Leica Confocal (Wetzlar, Germany) microscope.

Statistical analysis: One-way ANOVA with Tukey's post-hoc test or unpaired *t* tests were used for statistical analyses. All statistical analyses were performed using Prism software (GraphPad, San Diego, CA). Data are presented as mean \pm standard error of the mean (SEM). A *p* value of less than 0.05 was considered statistically significant.

RESULTS

TNFAIP3 is reduced in the retinas of diabetic mice and cdh5Cre-Epacl mice: We measured TNFAIP3 levels in the retinas from streptozotocin-treated diabetic mice and mice with Epacl eliminated in endothelial cells. Figure 1A shows that diabetes led to significantly reduced TNFAIP3 levels. Similarly, Figure 1B shows that the loss of Epacl in endothelial cells led to decreased TNFAIP3 levels.

Epacl increases TNFAIP3 in RECs grown in high glucose: As the retinal lysates from cdh5Cre-Epacl mice had decreased TNFAIP3 levels, RECs were grown in normal and high glucose and treated with an Epacl agonist. Figure 2 shows that high glucose decreased TNFAIP3 levels, which were increased by the Epacl agonist.

ZO-1 and occludin levels are decreased when TNFAIP3 siRNA is used in RECs grown in high glucose: ZO-1 and occludin levels are decreased in diabetic retinas [15,16]. As we saw that TNFAIP3 was decreased in the diabetic retinas, we wanted to investigate whether TNFAIP3 regulated these permeability-associated proteins. Figure 3 shows that high glucose had decreased ZO-1 (Figure 3B) and occludin (Figure 3C) levels. These levels were further reduced when TNFAIP3 siRNA was used. Figure 3A is a control to show the successful knockdown of TNFAIP3 by siRNA transfection. To support the western blotting data, we performed immunostaining for tight junction protein 1 (Figure 3D) and ZO-1 (Figure 3E). Occludin antibodies did not work well for immunostaining on cells.

TNFAIP3 siRNA leads to increased inflammatory mediators: We previously showed increased inflammatory mediators in diabetic retinas and the retinas of cdh5Cre-Epacl mice [17]. As TNFAIP3 should work as an anti-inflammatory agent, we

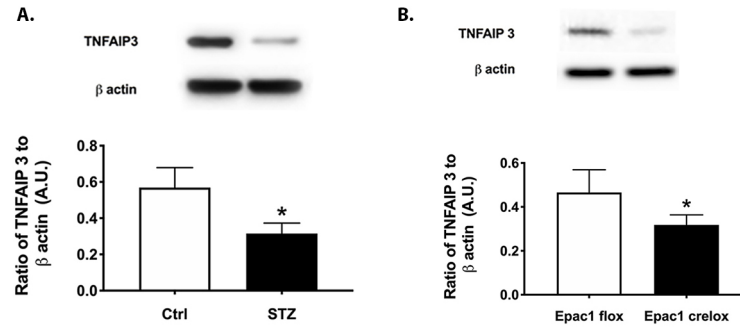


Figure 1. TNFAIP3 is reduced in retinal lysates from diabetic retinas and *cdh5Cre-Epac1* mice. Whole retinal lysates were collected from control and streptozotocin-treated (A) and *Epac1* floxed and *cdh5Cre-Epac1* mice (B) and processed for western blotting for TNFAIP3. * $p < 0.05$ versus ctrl or *Epac1* floxed. Data are mean \pm standard error of the mean (SEM). $n = 5$.

treated RECs in high glucose with TNFAIP3 siRNA. Figure 4 shows that high glucose culturing conditions significantly increased the TNF α (Figure 4A) and phosphorylated NF- κ B levels (Figure 4B). These levels were further increased when TNFAIP3 siRNA was transfected into the cells. The changes in NF- κ B were likely regulated by I κ B, as I κ B phosphorylation was reduced after TNFAIP3 siRNA was transfected into cells grown in high glucose (Figure 4C).

TRAF6 levels are regulated by TNFAIP3 in RECs: One key pathway that TNFAIP3 works through is the inhibition of TRAF6 [6]. To determine whether this occurs in the retina, RECs were grown in high glucose and treated with TNFAIP3 siRNA. Figure 5 shows that high glucose increased TRAF6 levels in RECs. TNFAIP3 siRNA further increased TRAF6 levels, demonstrating that TNFAIP3 regulates TRAF6 in the retinal vasculature.

DISCUSSION

Inflammation is a key factor in the complications of diabetes. There is strong literature linking inflammatory cytokines to islet damage in the pancreas [18,19]. Much less is known about the eye, particularly the retina. We found that diabetes decreased TNFAIP3 levels in the retina, which matches the literature from other organs affected by diabetes [20,21]. Additionally, we found that TNFAIP3 regulated inflammatory mediators [19,22]. We also observed that reduced TNFAIP3 led to decreased occludin and ZO-1 levels in the retina, two proteins involved in retinal permeability [15]. These findings well match work on intestinal permeability [23]. Other studies have shown that TNFAIP3 was key to VE-cadherin signaling in the lung, where TNFAIP3 regulated permeability [10].

Although little has been explored on TNFAIP3 actions in the retinal vasculature, there is some literature in other eye diseases that support the present findings. Research on retinal vasculitis showed that TNFAIP3 was a key gene involved

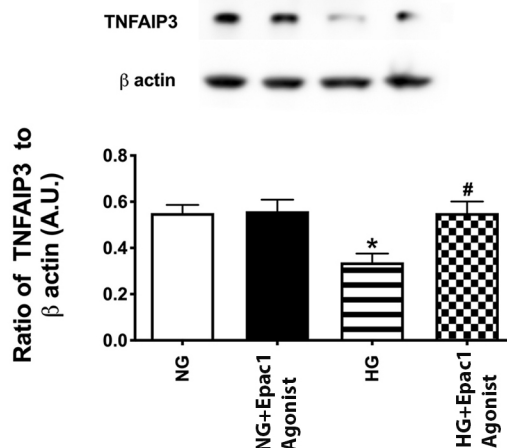


Figure 2. *Epac1* regulates TNFAIP3. Retinal endothelial cells (RECs) grown in normal glucose (NG) or high glucose (HG) were treated with an *Epac1* agonist. Western blotting was performed for TNFAIP3. * $p < 0.05$ versus NG, # $p < 0.05$ versus HG. Data are mean \pm standard error of the mean (SEM). $n = 5$.

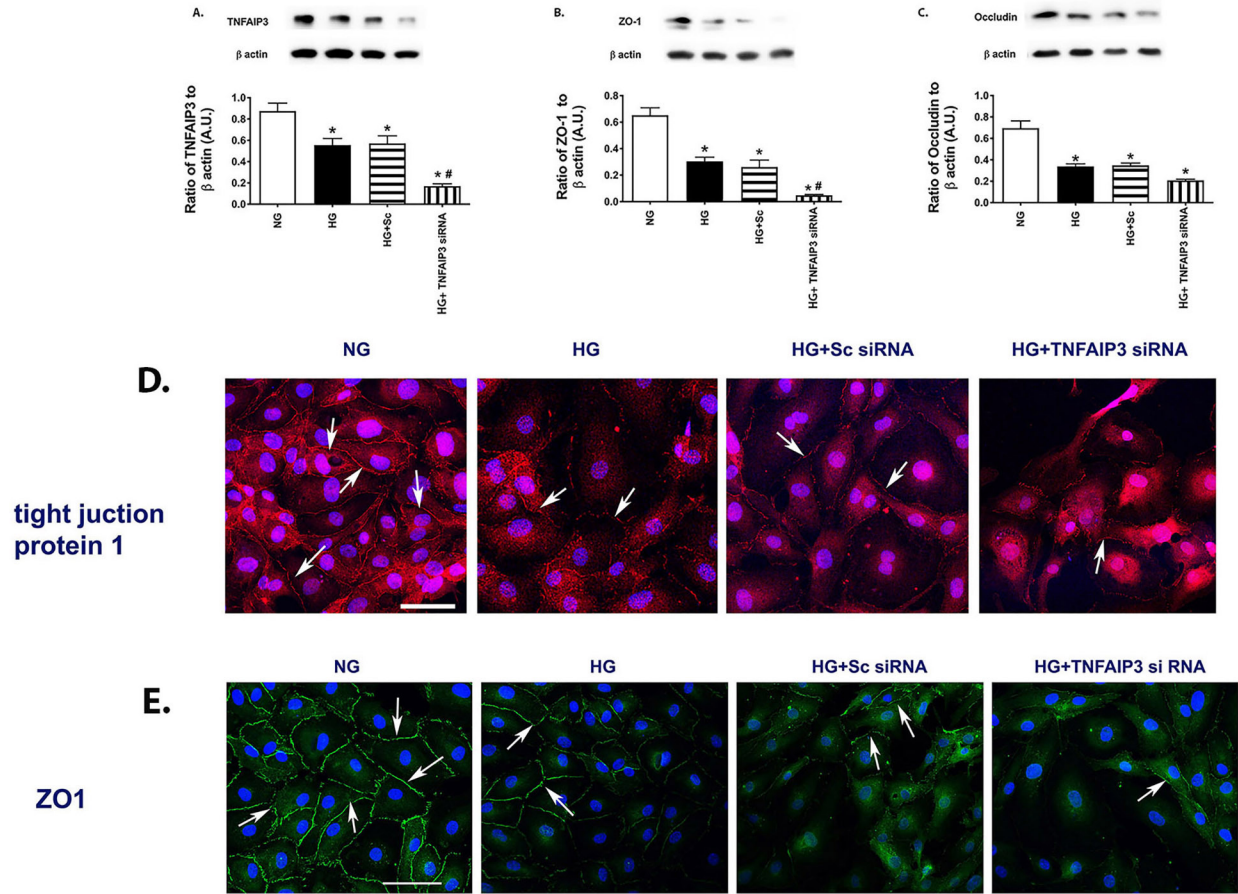


Figure 3. TNFAIP3 siRNA decreased ZO-1 and occludin levels. Retinal endothelial cells (RECs) grown in normal glucose (NG) or high glucose (HG). Some cells in HG were treated with scrambled siRNA or TNFAIP3 siRNA. Western blotting was performed for TNFAIP3 (A), ZO-1 (B), and occludin (C). Panels D and E show immunostaining for tight junction protein 1 (D) and ZO-1 (E). * $p < 0.05$ versus NG, # $p < 0.05$ versus HG. Data are mean \pm standard error of the mean (SEM) $n = 5$.

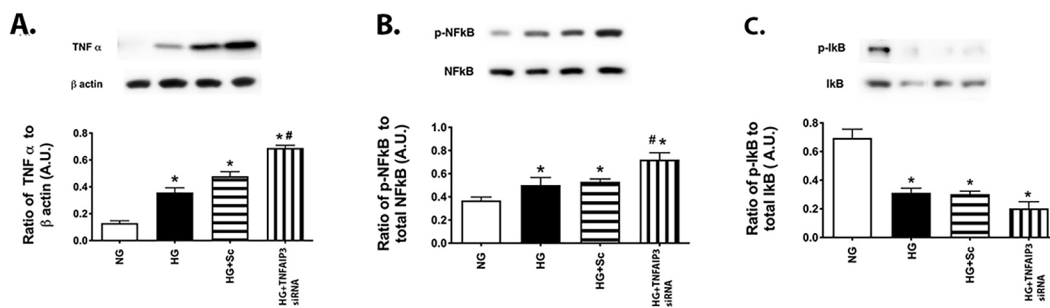


Figure 4. TNFAIP3 siRNA increased inflammatory mediators. Retinal endothelial cells (RECs) grown in normal glucose (NG) or high glucose (HG). Some cells in HG were treated with scrambled siRNA or TNFAIP3 siRNA. Western blotting was performed for TNF α (A) and phosphorylated NF-kB to total NF-kB (B), and phosphorylated to total IkB (C). * $p < 0.05$ versus NG, # $p < 0.05$ versus HG. Data are mean \pm standard error of the mean (SEM) $n = 5$.

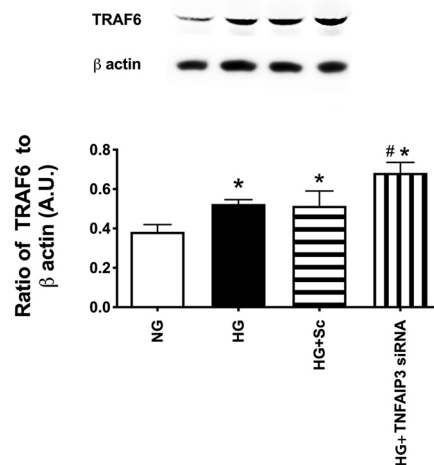


Figure 5. TNFAIP3 regulates TRAF6. Retinal endothelial cells (RECs) grown in normal glucose (NG) or high glucose (HG). Some cells in HG were treated with scrambled siRNA or TNFAIP3 siRNA. Western blotting was performed for TRAF6. * $p < 0.05$ versus NG, # $p < 0.05$ versus HG. Data are mean \pm standard error of the mean (SEM). $n = 5$.

[24]. Additionally, a study on uveitis showed that A20 was key to inflammation, with a focus on CD4 T+ cells and RPE cells [25]. Thus, the present findings of reduced TNFAIP3 levels in the retina leading to increased inflammation agree with the literature in other ocular tissues. Although few studies directly investigated TNFAIP3 in retinal inflammation, there is ample literature on inflammation in the retina due to diabetes [1,2,26], suggesting that diabetes leads to increased inflammation and permeability. There is no literature on the role of Epacl in regulating TNFAIP3; however, we previously showed that Epacl reduced retinal inflammation in diabetes [17]. These findings suggest that TNFAIP3 may offer another mechanism by which Epacl works to reduce retinal inflammation [12,27].

We acknowledge that these studies were performed primarily in primary retinal endothelial cells, with some support from whole retinal lysates from diabetic and Epacl conditional knockout mice. The finding that the Epacl agonist did not alter TNFAIP3 levels in normal glucose is counter to the findings in the mice. Perhaps this is genetic versus pharmacological differences, but we may explore this in the future. We would also have preferred to perform a permeability assay in vitro. Due to the use of TNFAIP3 siRNA transfection, it was difficult to obtain 100% confluence, which was required for a successful permeability assay in vitro. We also attempted to stain cells with three different occludin antibodies to no avail. Therefore, we used a different junctional protein to further demonstrate the effects of TNFAIP3 on permeability proteins. To address this and the limited options available as pharmacological tools for probing TNFAIP3 actions, we plan to develop conditional knockout mice to explore TNFAIP3 in the retinal vasculature alone and in the setting of diabetes.

In conclusion, the data demonstrated that diabetes reduced TNFAIP3 levels in the retina. Epacl regulated TNFAIP3 in retinal endothelial cells. High glucose culturing conditions in RECs increased inflammatory mediators, while decreasing occludin and ZO-1 protein levels. The use of TNFAIP3 siRNA further increased inflammatory mediators and reduced permeability proteins, suggesting that increasing TNFAIP3 levels may reduce retinal inflammation.

ACKNOWLEDGMENTS

These studies were funded by RO1EY028442 (JJS) and P30EY04068 (LDH) and a unrestricted grant from Research to Prevent Blindness.

REFERENCES

1. Tang J, Kern TS. Inflammation in diabetic retinopathy. *Prog Retin Eye Res* 2011; 30:343-58. [PMID: 21635964].
2. Aveleira CA, Lin CM, Abcouwer SF, Ambrosio AF, Antonetti DA. TNF-alpha signals through PKCzeta/NF-kappaB to alter the tight junction complex and increase retinal endothelial cell permeability. *Diabetes* 2010; 59:2872-82. [PMID: 20693346].
3. 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. Compound 49b Prevents Diabetes-Induced Apoptosis through Increased IGFBP-3 Levels. *Invest Ophthalmol Vis Sci* 2012; 53:3004-13. [PMID: 22467575].
4. Zhang Q, Soderland D, Steinle JJ. TNFalpha inhibits IGFBP-3 through activation of p38alpha and casein kinase 2 in human retinal endothelial cells. *PLoS One* 2014; 9:e103578. [PMID: 25073020].

5. Wu Y, He X, Huang N, Yu J, Shao B. A20: a master regulator of arthritis. *Arthritis Res Ther* 2020; 22:220-[PMID: 32958016].
6. Momtazi G, Lambrecht BN, Naranjo JR, Schock BC. Regulators of A20 (TNFAIP3): new drug-able targets in inflammation. *Am J Physiol Lung Cell Mol Physiol* 2019; 316:L456-69. [PMID: 30543305].
7. Deng HJ, Deji Q, Zhaba W, Liu JQ, Gao SQ, Han YL, Zhou ML, Wang CX. A20 Establishes Negative Feedback With TRAF6/NF-kappaB and Attenuates Early Brain Injury After Experimental Subarachnoid Hemorrhage. *Front Immunol* 2021; 12:623256-[PMID: 34381441].
8. Li Z, Chu S, He W, Zhang Z, Liu J, Cui L, Yan X, Li D, Chen N. A20 as a novel target for the anti-neuroinflammatory effect of chrysin via inhibition of NF-kappaB signaling pathway. *Brain Behav Immun* 2019; 79:228-35. [PMID: 30738841].
9. Yan K, Wu C, Ye Y, Li L, Wang X, He W, Ren S, Xu Y. A20 inhibits osteoclastogenesis via TRAF6-dependent autophagy in human periodontal ligament cells under hypoxia. *Cell Prolif* 2020; 53:e12778-[PMID: 32027437].
10. Soni D, Wang DM, Regmi SC, Mittal M, Vogel SM, Schluter D, Tirupathi C. Deubiquitinase function of A20 maintains and repairs endothelial barrier after lung vascular injury. *Cell Death Dis* 2018; 4:60-[PMID: 29796309].
11. Sun Y, Lin Z, Liu CH, Gong Y, Liegl R, Fredrick TW, Meng SS, Burnim SB, Wang Z, Akula JD, Pu WT, Chen J, Smith LEH. Inflammatory signals from photoreceptor modulate pathological retinal angiogenesis via c-Fos. *J Exp Med* 2017; 214:1753-67. [PMID: 28465464].
12. Liu L, Jiang Y, Chahine A, Curtiss E, Steinle JJ. Epacl agonist decreased inflammatory proteins in retinal endothelial cells, and loss of Epacl increased inflammatory proteins in the retinal vasculature of mice. *Mol Vis* 2017; 23:1-7. [PMID: 28210097].
13. Jiang Y, Liu L, Steinle JJ. Epacl deacetylates HMGB1 through increased IGFBP-3 and SIRT1 levels in the retinal vasculature. *Mol Vis* 2018; 24:727-32. [PMID: 30581279].
14. Jiang Y, Liu L, Curtiss E, Steinle JJ. Epacl Blocks NLRP3 Inflammasome to Reduce IL-1beta in Retinal Endothelial Cells and Mouse Retinal Vasculature. *Mediators Inflamm* 2017; 2017:2860956-[PMID: 28348460].
15. Murakami T, Felinski EA, Antonetti DA. Occludin phosphorylation and ubiquitination regulate tight junction trafficking and vascular endothelial growth factor-induced permeability. *J Biol Chem* 2009; 284:21036-46. [PMID: 19478092].
16. Erickson KK, Sundstrom JM, Antonetti DA. Vascular permeability in ocular disease and the role of tight junctions. *Angiogenesis* 2007; 10:103-17. [PMID: 17340211].
17. Liu L, Jiang Y, Steinle JJ. Epacl and Glycyrrhizin Both Inhibit HMGB1 Levels to Reduce Diabetes-Induced Neuronal and Vascular Damage in the Mouse Retina. *J Clin Med* 2019; 8:772-[PMID: 31159195].
18. Zammit NW, Grey ST. Emerging roles for A20 in islet biology and pathology. *Adv Exp Med Biol* 2014; 809:141-62. [PMID: 25302370].
19. Grey ST, Arvelo MB, Hasenkamp W, Bach FH, Ferran C. A20 inhibits cytokine-induced apoptosis and nuclear factor kappaB-dependent gene activation in islets. *J Exp Med* 1999; 190:1135-46. [PMID: 10523611].
20. Cheng L, Zhang D, Jiang Y, Deng W, Wu Q, Jiang X, Chen B. Decreased A20 mRNA and protein expression in peripheral blood mononuclear cells in patients with type 2 diabetes and latent autoimmune diabetes in adults. *Diabetes Res Clin Pract* 2014; 106:611-6. [PMID: 25451900].
21. Yu LY, Lin B, Zhang ZL, Guo LH. Direct transfer of A20 gene into pancreas protected mice from streptozotocin-induced diabetes. *Acta Pharmacol Sin* 2004; 25:721-6. [PMID: 15169622].
22. Gao W, Du J, Chi Y, Zhu R, Gao X, Yang L. Minocycline prevents the inflammatory response after retinal detachment, where microglia phenotypes being regulated through A20. *Exp Eye Res* 2021; 203:108403-[PMID: 33326811].
23. Chen D, Ma L, Hu T, Liu J, Chen B, Yang P, Liu Z. A20 Restores Impaired Intestinal Permeability and Inhibits Th2 Response in Mice with Colitis. *Dig Dis Sci* 2020; 65:1340-7. [PMID: 31584137].
24. Rosenbaum JT, Sibley CH, Lin P. Retinal vasculitis. *Curr Opin Rheumatol* 2016; 28:228-35. [PMID: 26945335].
25. Hu J, Yi S, Wang C, Zhang Y, Tang J, Huang X, Yang L, Yang J, Li H. A20 Inhibits Intraocular Inflammation in Mice by Regulating the Function of CD4+T Cells and RPE Cells. *Front Immunol* 2020; 11:603939-[PMID: 33613524].
26. Abcouwer SF, Lin CM, Shanmugam S, Muthusamy A, Barber AJ, Antonetti DA. Minocycline prevents retinal inflammation and vascular permeability following ischemia-reperfusion injury. *J Neuroinflammation* 2013; 10:149-[PMID: 24325836].
27. Jiang Y, Steinle JJ. Epacl Requires AMPK Phosphorylation to Regulate HMGB1 in the Retinal Vasculature. *Invest Ophthalmol Vis Sci* 2020; 61:33-[PMID: 32940662].

Articles are provided courtesy of Emory University and the Zhongshan Ophthalmic Center, Sun Yat-sen University, P.R. China. The print version of this article was created on 30 June 2022. This reflects all typographical corrections and errata to the article through that date. Details of any changes may be found in the online version of the article.