506 Diabetic Retinopathy: Neurodegeneration and pathology associated with the neurovascular unit.
Thursday, May 11, 2017 8:30 AM–10:15 AM
Exhibit/Poster Hall Poster Session
Program #/Board # Range: 5189–5205/B0076–B0092
Organizing Section: Retinal Cell Biology

Program Number: 5189 Poster Board Number: B0076
Presentation Time: 8:30 AM–10:15 AM
Diabetes Reduces Phosphorylation in Human Retina of αA-crystallin on T148, a Site Regulating its Protective Function in Neurons and Glia
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Purpose: Intrinsic protective mechanisms including those involving αA-crystallin play an important role in retinal neuroprotection. We previously reported that rodent models of diabetes presented with high levels of αA-crystallin and reduced phosphorylation on residue 148. This study tested the hypothesis that phosphorylation of αA-crystallin on T148 is negatively regulated by diabetes in human retina and that this phosphorylation site affects αA-crystallin protective function.

Methods: Ocular tissues from 8 non-diabetic, 10 diabetic without retinopathy and 10 diabetic with retinopathy donors were used in this study. One eye was rapidly fixed in formaldehyde to assess regional changes by histology and immunohistochemistry. The contralateral eye was rapidly dissected to isolate and analyze the expression, phosphorylation and subcellular localization of αA-crystallin, Bax and other targets in the different regions of the retina using gene expression, biochemical, and proteomic-based methods.

Results: αA-crystallin expression was significantly increased in peripheral and central retinal regions of donors with diabetes but without signs of retinopathy (44 and 84% increase respectively; p<0.03). While similar increases were detected in donors with diabetic retinopathy, we also found by mass spectrometry that phosphorylation of αA-crystallin on T148 was reduced by 83% in these donors (n=3; p=0.08). Immunolocalization analysis demonstrated expression of αA-crystallin in both neurons and glia shown by a strong colocalization with GFAP and glutamine synthetase (Müller glia), and Neurofilament-H (ganglion cells). Overexpression of the αA-crystallin 148D phosphomimetic mutant protects neurons (differentiated R28) and glia (MIOM1) from serum deprivation while the non-phosphorylatable 148A mutant does not (p<0.01).

Conclusions: Our results strongly support our hypothesis that phosphorylation on T148 controls the protective function of αA-crystallin. They also show that while αA-crystallin is induced in both diabetic donors with and without retinopathy, its phosphorylation is highly reduced in those with retinopathy, consistent with its necessity for the protective function of αA-crystallin. The impact of this change is currently being investigated, including through RNA-seq examination of human donor tissues with and without retinopathy.

Commercial Relationships: Patrice E. Fort, Anne Ruebsam, None; Kevin Schey, None; Yang Shan, None
Support: Fight for Sight, Eversight, DiaComp, NIH EY020895 and P30 EY007003

Program Number: 5190 Poster Board Number: B0077
Presentation Time: 8:30 AM–10:15 AM
Effect of hypoxia on autophagy in R28 cells under low and high glucose conditions
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Purpose: Retinal ischemia/reperfusion (I/R) injury occurs in various ocular diseases, such as diabetic retinopathy, and is a common cause of visual impairment and blindness. There has been increasing interest in autophagy as a potential therapeutic target for ocular diseases but the role of autophagy in retinal I/R injury remains controversial. Our previous in vivo research showed that retinal I/R injury (middle cerebral artery occlusion stroke model) resulted in autophagy upregulation in the inner retinæ of Akita (type 1 diabetic) and wild-type mouse. In this study, we used an in vitro model to further investigate the role of autophagy in retinal cells exposed to hypoxic conditions under different glucose levels.

Methods: The R28 retinal precursor cell line (R28) was chosen for its rat retinal origin and heterogenous nature, which reflects the diversity of cell types found in the retina. R28 cells were cultured in low glucose DMEM medium with 10% fetal bovine serum and pCPT-cyclic AMP to induce cell differentiation into a more neuronal-like phenotype. To mimic a hyperglycemic state, cells were incubated with additional glucose. Hypoxia was chemically induced in both low and high glucose-treated cells using cobalt (II) chloride (CoCl2) for 24 hours. Cell viability was determined using the MTS assay. Autophagy was assessed through the detection of microtubule-associated light chain protein LC3 (an autophagosome marker) by Western blot.

Results: Cells subjected to CoCl2-induced hypoxia had lower cell viability as compared with untreated cells (control group). Hypoxia resulted in significant autophagic upregulation in both low glucose and high glucose-treated cells. LC3 expression was higher in high glucose-treated cells as compared with low glucose-treated cells after hypoxia induction.

Conclusions: CoCl2-induced hypoxia induces autophagy activation in retinal cells. Both our in vivo and in vitro results suggest that elevated glucose levels may further exacerbate retina I/R injury-induced autophagy. Autophagy modulation may represent a potential therapeutic strategy for retinal I/R injury related diseases.

Commercial Relationships: Larissa H. Tang, None; Frederic K. Fung, None; Amy C. Lo, None
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Program Number: 5191 Poster Board Number: B0078
Presentation Time: 8:30 AM–10:15 AM
Conocral nerve fiber morphology and neurodegeneration of the retina in diabetic rats
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Purpose: Diabetes-induced degeneration of the inner retina occurs in both animals and humans. Recent clinical evidence suggests that corneal nerve fibers may also degenerate in diabetes. The aim of this study was to compare retinal cell layer thickness, using spectral domain optical coherence tomography (SD-OCT), with corneal nerve density, in streptozotocin (STZ)-diabetic rats.

Methods: Long-Evans rats were made diabetic by STZ injection (100 mg/kg, i.v., n=6) and compared to age-matched controls (n=6). Retinal morphology was measured by SD-OCT (Envisu)
2210. Biopitgen) 9 weeks later. Rats were sacrificed after 10 weeks of diabetes and corneas were dissected, labeled for β-tubulin and flat-mounted for confocal microscopy (Leica SP8). Images from 5 random 116.25 µm² regions were obtained. Corneal nerve fiber density (number/mm²) and corneal nerve fiber length (mm/mm²) were measured by image analysis using Neuron J. Statistical comparisons were made by two-tailed t-test with p<0.05 considered significant (Pryam, Graphpad). Degeneration was also confirmed by cell death ELISA on one retina from each rat.

**Results:** There was significantly more cell death in STZ-diabetic rat retinas compared to controls (p<0.05). SD-OCT data revealed that the inner plexiform and inner nuclear layers were significantly thinner in the STZ-diabetic rats compared to controls (p<0.05 and p<0.01 respectively), while the outer nuclear and photoreceptor layers were significantly thicker (p<0.001 and p<0.05 respectively). Morphological analysis of the corneal nerve fibers revealed no significant differences between STZ-diabetic and control groups.

**Conclusions:** Cell death and reduction of the inner plexiform and nuclear layers indicate neurodegeneration with a potential loss of synaptic connectivity, as established previously. Thickening of the outer nuclear and photoreceptor layer is most likely due to fluid accumulation caused by increased permeability of the blood retinal barrier, or possibly by increased resistance to fluid clearance through the pigment epithelium. This study found no significant difference in the corneal nerve fiber morphology, suggesting that this degeneration cannot be detected in rats after 10 weeks of diabetes. Together our data suggest that inner retinal degeneration and outer retinal swelling occur in parallel during the first 2-3 months of STZ-diabetes but is not accompanied by changes in corneal neuron morphology.

**Commercial Relationships:** Sean D. Kim, None; Oliver Schmachtenberg, None

**Program Number:** 5192 Poster Board Number: B0079

**Presentation Time:** 8:30 AM–10:15 AM

**Simulating diabetic retinopathy in organotypic retinal explant cultures: Comparison of diabetic conditions on early vs. late post-natal retina**

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**Purpose:** Diabetic retinopathy (DR) is one of the leading causes of vision impairment worldwide. Unfortunately, DR research is hindered by a lack of disease models that faithfully reproduce the retinal phenotype of diabetes, in particular for type 2 diabetes. Previously, we have shown that a number of aspects of DR can be faithfully reproduced in early (P5) post-natal organotypic retinal explant cultures (Valdès et al., ALTEX 33:459-464, 2016). However, since the retina is not fully developed at this stage, it remains unclear whether the deleterious effects of diabetes-like conditions on the early postnatal retina are representative of mature retinal pathophysiology.

**Methods:** Mouse retinas were explanted at P14 (around eye opening) and treated from P16 to different with different experimental conditions (i.e. no-insulin, high-glucose, no-insulin + high-glucose, 2-deoxyglucose; 2-DG) to simulate type 1 and 2 diabetic conditions. The treatment effects were assessed on histological preparations using photoreceptor row counts, TUNEL assay for cell death detection, and immunostaining for cone arrestin.

**Results:** In the no-insulin, high-glucose, and no-insulin + high-glucose treatment paradigms the photoreceptor row counts, numbers of TUNEL, and cone arrestin-positive cells did not change significantly when compared to control. However, the 2-DG treatment significantly increased the numbers of dying TUNEL-positive cells in the outer nuclear layer (ONL; control: 1.22% ± 0.3 STD; 2-DG: 13.76% ± 0.8, p<0.001) and reduced the number of arrestin-positive cones per 100 µM of retinal circumference (control: 4.7 ± 0.7; 2-DG: 0.4 ± 0.3, p<0.001). Yet, the overall photoreceptor row count was not significantly altered (control: 8.0 ± 0.6; 2-DG: 8.1 ± 0.4).

**Conclusions:** Compared to the early post-natal retina, older retinas responded less strongly to simulated diabetic conditions, yet, cone photoreceptors were highly vulnerable to type 1 diabetes-like conditions caused by 2-DG treatment. Taken together, we have further validated and extended an in vitro model of DR which may prove useful for studies into the etiopathology of DR and for DR-related drug screening.

**Commercial Relationships:** Francois Paquet-Durand, None; Stavros Vagionitis, None; Ksenija Martinovic, None; Joaquin Valdes, None; Dragana Trifunovic, None; Maria Miranda, None; Oliver Schmachtenberg, None

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**Program Number:** 5193 Poster Board Number: B0080

**Presentation Time:** 8:30 AM–10:15 AM

**Upregulation of activated Müller cell marker independent of local ischemia in diabetic retinopathy**


**Purpose:** Microvascular abnormalities and non-perfusion underlie deterioration of the retina and progressive loss of vision in diabetic retinopathy (DR). However, ischemia is usually not panretinal, but rather affects clearly localised regions. How well non-perfused and perfused retinal regions correlate with neural changes in DR within the same eye has not been well studied. Here, we present a case study describing the spatial relationship between vascular degeneration and neuronal/glial changes in a post-mortem retina with DR.

**Methods:** Post-mortem donor tissue was obtained through the Moorfields Eye Bank. The donor age ranged between 39 and 80 years (mean age 58 years). A total of 6 eyes from patients diagnosed with diabetes were histologically assessed for evidence of vessel damage by staining retinal blood vessels with UEA agglutinin in retinal wholemounts. One eye was found with apparent proliferative DR (evidence of laser scars) and regions of severe vascular dropout. This specimen was further studied and compared to a healthy eye and a diabetic case with no vascular pathology. The specimen was paraffin embedded and serially sectioned. The distribution of blood vessels within sections was then used to establish the exact position within the wholemount. Further sections were immunostained with a panel of antibodies for neural, glial and vascular proteins (Crystallin alpha A, Collagen IV, CRALBP, GFAP, Glutamine Synthetase, MAP2, Opsin, Rhodopsin, Vimentin).

**Results:** As expected, a localised region with loss of blood vessels showed severe hypotrophy due to a complete loss of inner retinal cells. However, photoreceptors were also reduced in this region. Interestingly, Crystallin alpha A was markedly upregulated compared
to controls throughout the retina, independent of localised vascular loss.

**Conclusions:** In this case of DR, our detailed histopathological analysis identified evidence of reactive Müller cells (Crystallin alpha A upregulation) throughout the retina, demonstrating that neural changes can occur in the diabetic retina independent of blood vessels loss. Further DR specimens will have to be studied to confirm this and to establish effects on neurons in more detail. Understanding early pathological changes in DR, will provide insight into disease mechanisms and may also reveal therapeutic targets.

**Commercial Relationships:** Marina V. Yasvoina; Almas Dawood; None; Michael B. Powner; None; Marcus Fruttiger, None

**Program Number:** 5194 Poster Board Number: B0081

**Presentation Time:** 8:30 AM–10:15 AM

**Newly Designed Culture System to Study Effects of Hyperglycemia on the Cross-Talk between Müller and Retinal Endothelial Cells**

Susanne Mohr; Brandon Coughlin. Department of Physiology, Michigan State University, East Lansing, MI.

**Purpose:** Considering the increasing numbers of diabetic patients there is a dire need to find new therapeutic strategies to effectively treat or even better cure diabetic retinopathy. However, therapies developed using rodent models rarely translate into successful treatments for patients with diabetic retinopathy. Therefore, the goal of this study was to establish a physiologically relevant flow based culture system using human retinal cells that will allow to identify effects of hyperglycemia on human retinal cell cross-talk and to test potential new therapies.

**Methods:** The cell culture system consisting of a pump and a cartridge fits in a regular incubator (37°C/5% CO₂). The cartridge consists of a network of hollow fiber artificial capillaries surrounded by an extracellular compartment (ECS). The fibers (0.5 μm pores) allow for exchange of proteins between inside of the capillary (LS) and the outside (ECS). Human retinal Müller (hMC) and endothelial cells (HREC) were isolated from donors with no history of diabetes and inflammatory diseases. HREC cells (10ml of 3x10⁵ cells/ml HREC suspension) were injected into the LS. hMCS (3x10⁵ cells) from the same donor were added to the ECS. The cartridges were allowed to settle in for 4 weeks. Formation of capillary layers was confirmed using electron microscopy (EM). Effects of hyperglycemia and VEGF on permeability were assessed using trypan blue and expressed as mean area under the curve (AUC) ± SDEV.

**Results:** Under normal (5mmol/l) glucose condition, HREC formed a monolayer within the capillary fiber as determined by EM. HREC also formed a tight barrier. Following VEGF (2ng/ml) injection into the LS, permeability significantly increased (9.47AUC compared to 2.5AUC). Interestingly, hyperglycemia (25mmol/l) itself had no effect on permeability (2.3±0.3AUC vs 2.46±0.4AUC). For co-culture, attachment of hMCS on outside of the fiber was confirmed by EM. Hyperglycemia caused significant and irreversible barrier breakdown at 4-6 months (2.13AUC vs 4.87AUC) but not an acute breakdown.

**Conclusions:** We have established a flow based culture system that seems to mimic physiological relevant cross-talk and microenvironment between human retinal Müller and endothelial cells. This system can be used to identify and test new therapies in order to develop more efficient treatments for diabetic patients.

**Commercial Relationships:** Susanne Mohr, None; Brandon Coughlin, None

**Support:** NH grant EY-017206 and EY-007739

**Program Number:** 5195 Poster Board Number: B0082

**Presentation Time:** 8:30 AM–10:15 AM

**Diabetes induced neurodegeneration in the retina and the brain of mice are associated and independent of microvasculopathy**

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**Purpose:** Diabetes causes structural changes in the brain, primarily in the cortex, thalamus and caudate and putamen, and these have been ascribed to microvascular changes and ischemia. As we have recently discovered that in the retina, neuroretinal degeneration precedes any form of microvascular damage, we sought to determine whether this retinal neurodegeneration, associated with DM is associated with neural loss in the brain. Performing this study on Lp-PLA₂ knockout and control mice allowed us to determine an effect of Lp-PLA₂ inhibition on DM-induced neurodegeneration in the retina and brain, as well as an effect on microvascularopathy in the retina.

**Methods:** Male Lp-PLA₂⁻/⁻ (n=30) and Lp-PLA₂⁺/⁺ (n=30) mice at 12 weeks of age received streptozotocin to induce hyperglycemia. At baseline, 12 and 24 weeks after DM was confirmed, mice underwent OCT imaging, MRI and immunohistologic analysis. Whole mount retinas were used for quantification of pericyte density, acellular capillaries, and ganglion density. NFL/GCL thickness were automatically segmented using our Iowa Reference Algorithms. T2 MRI scans (Varian 4.7T) were automatically segmented using our fully automated and validated brain segmentation algorithm to quantify total brain, hippocampus and caudate and putamen volume. Statistical analysis was performed in R and GraphPad prism 7.

**Results:** DM Lp-PLA₂⁻/⁻ (n=11) had significantly greater pericyte density (*p=0.012), and decrease in acellular capillaries (*p=0.035) compared to DM Lp-PLA₂⁺/⁺ control (n=9) at 24 weeks but not at 12 weeks after induction of DM. There was no difference in the number of total cells or ganglion cells at week 12 and 24 post-induction of DM. NFL/GCL complex and brian volume were not significantly different between Lp-PLA₂⁻/⁻ and Lp-PLA₂⁺/⁺ mice at baseline, weeks 12 and 24. Correlation coefficient (r) was significant between retinal NFL/GCL thickness and total brain volume at 0.32 (95% CI, 0.06 – 0.54), also for specific brain structures: hippocampus with r = 0.37 (95% CI, 0.11 – 0.58) and caudate and putamen with r=0.36 (95% CI, 0.10 – 0.57).

**Conclusions:** Lp-PLA₂⁻/⁻ may be protected against DM-induced retinal vascular loss. No protective effect on retinal neurodegeneration or cerebral neurodegeneration was found in DM Lp-PLA₂⁺/⁺ mice. There may be an association between diabetes induced retinal neurodegeneration and cerebral neurodegeneration.

**Commercial Relationships:** Chunhua Jiao, None; Michael D. Abramoff, University of Iowa (P), IDX LLC (I), IDX LLC (C); Kyungmoo Lee, None; Ipek Oguz, None; Peter Adamson, GlaxoSmithKline Pharmaceutical company (E); Adam Hedberg-Buenz, None; Michael G. Anderson, None; Elliott Sohn, None

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Dysfunction in Diabetic Retinopathy

**JINGMING LI** 2, GUO CHEN2, FENGJUN ZHANG2, GUOULONG DING2, QIUPING LIU2. 1Ophthalmology, First Affiliated Hospital of Xi’an Jiaotong University, Xian, China; 2Ophthalmology, Affiliated Eye Hospital of Nanchang University, Nanchang, China.

**Purpose:** Oxidative stress through excessive generation of reactive oxygen species (ROS) is involved vascular leakage and degeneration in diabetic retinopathy (DR). Paraaxonase 2 (Pon2) plays a critical role in anti-oxidative defense. However, nothing is known about its role in retinas. The aim of this study is to elucidate Pon2’s function in regulating of retinal oxidative stress and vascular damage during DR.

**Methods:** A mouse model of diabetes was set up by intraperitoneal injection of streptozotocin. Retinal expression of Pon2 were determined by immunofluorescence. Induction of Pon2 in mouse retinas was achieved by intravitreal injection of AAV-PON2 or by feeding with Niacin. Retinal ROS generation, expression of vascular endothelial growth factor (VEGF), leukostasis, vascular leakage and formation of acellular capillaries were determined in cellrox staining, western-blot analysis, Con A-lectin perfusion and trypsin digestion, respectively.

**Results:** Retinal expression of Pon2 were increased in retinas of diabetic mice, which was mainly expressed by Muller cells. Retinal ROS generation and VEGF expression were significantly increased in diabetic mice, together with enhanced leukocytes adhesion to retinal vasculature, exacerbated vascular leakage and prominent acellular capillaries. Uprogregation of AAV-PON2 markedly inhibited diabetes-induced retinal ROS generation and VEGF expression. In parallel, retinal leukostasis, vascular leakage and amount of acellular capillaries were dramatically reduced in diabetic mice with overexpression of Pon2.

**Conclusions:** Taken together, our data indicate that Pon2 plays a pivotal role in anti-oxidative defense in DR. Induction of Pon2 could be a potential therapeutic strategy of diabetic microvascular complications.

**Commercial Relationships:** JINGMING LI, None; GUO CHEN, None; FENGJUN ZHANG, None; GUOULONG DING, None; QIUPING LIU, None

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**Program Number:** 5196 Poster Board Number: B0083

**Presentation Time:** 8:30 AM–10:15 AM

**Muller Cell-derived Paraoxonase 2 Reverses Vascular Dysfunction in Diabetic Retinopathy**

**Yohi Tomita1, 3, Yukihiro Miwa1, 3, Maki Miyachi1, 3, Ayako Ishida1, 3, Hiromitsu Kunimi2, 3, Yusaku Katada1, 3, Kazuo Tsubota1, 3, Toshihide Kurihara2, 3. 1Laboratory of Photobiology, Keio University School of Medicine, Tokyo, Japan; 2Ophthalmology, Keio University School of Medicine, Tokyo, Japan.

**Purpose:** Spermine Oxidase: a novel mediator of diabetes-induced retinal neurodegeneration

**Program Number:** 5198 Poster Board Number: B0085

**Presentation Time:** 8:30 AM–10:15 AM

**Spermine Oxidase: a novel mediator of diabetes-induced retinal neurodegeneration**

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**Purpose:** Deregulation of the polyamine metabolic pathway has been implicated in various neurodegenerative diseases. Studies from our laboratory have demonstrated that polyamine oxidation is involved in neuronal and vascular damage in retina (Narayanan et al 2014; Patel et al 2016). The impact of polyamine oxidation and its contribution to retinal neuronal damage induced by diabetes is not yet studied. The current study was undertaken to investigate the role of spermine oxidase (SOMO, a polyamine oxidase) in mediating neuronal damage and dysfunction in diabetic retina.

**Methods:** Eight weeks old C57BL6 male mice were made diabetic by streptozotocin injection (STZ, 65 mg/kg, pH 4.5, i.p.) and compared to age-matched controls (CNT). Following the onset of diabetes animals were treated with either vehicle or SMO inhibitor, MDL 72527 (20 mg/kg of body weight, in saline, i.p., 3 times/week). Retinal function was analyzed by electrotoretinography. Retinal cryostat sections were used for immunofluorescence studies and fresh frozen retinas for Western blot analysis.
Hypoxic Condition

Results: The current data indicate that hypoxia induces spermine oxidation and increases H₂O₂ generation in Müller glial cells under hypoxic condition.

Commercial Relationships: Di Wu, None; Kousuke Noda, None; Miyuki Murata, None; Ye Liu, None; Atsuhiro Kanda, None; Susumu Ishida, None

Program Number: 5200 Poster Board Number: B0087
Presentation Time: 8:30 AM–10:15 AM
Alpha-1-Anti-Trypsin increased Na⁺/K⁺-ATPase expression in an in vitro Müller cells diabetic retinopathy model
MARÍA CONSTANZA POTILINSKI, Gustavo Ortiz, Juan E. Gallo
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Purpose: The ophthalmic therapy for diabetic retinopathy is focused on severe stages of the disease. Previous results obtained in our group show that Alpha-1-Anti-Trypsin (A1AT) acts like an anti-inflammatory agent that could play a role on diabetic retinopathy treatment. It is important to evaluate A1AT impact on cellular components that are essential to retina function like Na⁺/K⁺-ATPase (NKA). This protein is responsible of Na⁺ and K⁺ gradients in cells and it is involved in synaptic activity and action potentials in this tissue. It is known that NKA activity and expression is diminished in diabetic retinopathy. A1AT may stimulate NKA expression through different cellular mechanisms. For this reason we aimed at evaluating NKA with A1AT treatment in an in vitro diabetic retinopathy cell model.

Methods: Eight mouse retinas were obtained from freshly enucleated eyes incubated with collagenase I and Trypsin. Retinas were desegregated and incubated with DMEM for 5 days to allow the enrichment of Müller cells population. Müller cells obtained were incubated 24h with DMEM 30mM glucose (Control), DMEM 30mM glucose + 4.5mg/ml A1AT (Control + A1AT), DMEM 100mM glucose (Diabetic), DMEM 100mM glucose + 4.5mg/ml A1AT (Diabetic + A1AT). Cells were harvested with RIPA buffer for Western Blot Assay or Fixed for Immunohistochemistry. Western blot and Immunohistochemistry were performed with using specific primary antibodies for total α-Na⁺/K⁺-ATPase (H-300, Santa Cruz Biotechnology, California, USA).

Results: Alpha subunit of Na⁺/K⁺-ATPase expression was increased in A1AT treated cells.

Conclusions: Results support the hypothesis that A1AT promotes Na⁺/K⁺-ATPase expression. This is a novel aspect about NKA expression modulation. Although molecular mechanisms involved remained unknown, A1AT might play a new role in diabetic retinopathy treatment.

Commercial Relationships: MARÍA CONSTANZA POTILINSKI, None; Gustavo Ortiz, None; Juan E. Gallo, None

Program Number: 5201 Poster Board Number: B0088
Presentation Time: 8:30 AM–10:15 AM
Alpha-1-antitrypsin ameliorates features of oxidative stress and structural retinal damage in diabetic mice
Gustavo Ortiz, MARÍA CONSTANZA POTILINSKI, Juan P. Salica, Juan E. Gallo
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Purpose: Oxidative stress has been implicated in the etiology of several diseases and in aging, including the pathogenesis of diabetic retinopathy (DR). Indeed nitric oxide (NO) was found higher in patients with proliferative DR. It is known that Muller cells (MC) are responsible for the secretion of several molecules that are involved in the regulation of retinal homeostasis and play key role in the interaction between endothelium and neural retina. The aim of our work was to evaluate the levels of 2,7-dichlorodihydrofluorescein (DCFC)
on supernatant of MC and human microvascular endothelial cells (Hmvec-1) exposed to hyperglycemia. Besides, we evaluated the retinal thickness and the loss of ganglion cells (GC) in c57BL6J mice with 8 weeks of diabetes treated with hAAT.

**Methods:** MC were obtained from retinas of c57BL6J mice. Isolated cells were used in immunofluorescence for CRALBP, GFAP and Vimentin markers. Cells were grown in standard conditions and were exposed to different concentration of hAAT for 16hs. Intracellular oxidants were detected by flow cytometry after incubating with 5 µm 2,7-dichlorodihydrofluorescein (DCFH) 45 min. Mice c57BL6J of 8 weeks old were injected with 2 doses of 100mg/kg of streptozotocin spaced 48 hours. Blood glucose test was performed and levels of 200 mg/dl or higher were considered diabetic (DBT). Animals were injected weekly, with a single dose of 60mg/kg of hAAT. Cells of the ganglion cell layer (GCL) were quantified by counting cells in the middle part of the retina and thickness measurements of the entire retina were taken.

**Results:** The oxidation of DCFH originates DCFC, a fluorescent compound in presence of H2O2 and other ROS, such as HO and ROO. DCFH fluorescence decreased by ROS generation in MC exposed to high glucose is notably decreased by hAAT. GC count was significantly lower in untreated DBT animals while DBT animals treated with hAAT had results close to control values. In turn the retinal thickness was also decreased in DBT animals treated but without treatment. Furthermore, a decrease in mRNA levels of iNOS was found in DBT mice treated with hAAT as well as an increased in mRNA levels of Arg1.

**Conclusions:** A better understanding on how the oxidative stress induced by chronic hyperglycemia occurs in DR is necessary. The use of hAAT as an anti-oxidative stress molecule in early DR could be a promising approach for this potentially blinding disease.

**Commercial Relationships:** Gustavo Ortiz, None; MARIA CONSTANZA POTILINSKI, None; Juan P. Salica, None; Juan E. Gallo, None

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**Program Number:** 5202 Poster Board Number: B0089  
**Presentation Time:** 8:30 AM–10:15 AM

High glucose induces mitochondrial dysfunction and mitophagy in retinal Müller cells: Role of TXNIP  
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**Purpose:** Thioredoxin-interacting protein (TXNIP) is involved in oxidative stress and apoptosis in diabetic retinopathy (DR). However, the role of TXNIP in the removal of damaged mitochondria (MT) via mitophagy, a process of macroautophagy, remains unexplored. Here, we investigate the associated cellular and molecular mechanisms underlying mitophagy in retinal cells under sustained hyperglycemia.

**Methods:** We maintained a rat Müller cell line (rMC1) under high glucose (25 mM, HG) or low glucose (5.5 mM, LG) condition for 5 days. MitoSox and JC1 assays were used to detect mitochondrial ROS and membrane depolarization, respectively. QPCR and western blotting measured mRNA and protein levels while confocal microscopy (immunofluorescence analysis) determined protein co-localization in mitochondria. Unpaired two-tailed t-test or one-way ANOVA and Bonferroni post-hoc test determine differences among means/−/sem in multiple sets of experiments. A p-value of <0.05 was considered to be statistically significant.

**Results:** HG upregulates TXNIP in the cytosol as well as in mitochondria. Moreover, mitochondrial ROS stress and membrane depolarization occur under prolonged hyperglycemia leading to fragmentation. These damaged MT are targeted to lysosome for mitophagic degradation as is evident by co-localization of mitochondrial protein COXIV with autophagosome marker LC3BII and the lysosomal membrane protein LAMP2A. In addition, under HG there is accumulation of dynamin-related fission protein Drp1 and E3 ubiquitin ligase Parkin in damaged MT suggesting their roles in mitochondrial fragmentation and ubiquitination, respectively, which is absent in LG. Subsequently, ubiquitin receptors, optineurin (OPTN) and p62/sequestrome 1, bind to the damaged MT and target them to LC3BII autophagosomes, which then fuse with lysosomes via LAMP2A for degradation. Conversely, TXNIP knockout via CRISPR/Cas9 and TXNIP grNA prevents the HG-induced mitochondrial damage and mitophagy in mMC1.

**Conclusions:** TXNIP plays an important role in mitophagy induction in Müller glia under diabetic conditions. We proposed that TXNIP is a potential target for preventing ocular complications of diabetes.

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**Program Number:** 5203 Poster Board Number: B0090  
**Presentation Time:** 8:30 AM–10:15 AM

TLR4 plays an important role in regulating ZO-1 and occludin levels, as well as retinal damage following ischemia-reperfusion injury  
Li Liu1, Youde Jiang1, Elizabeth Currier1, Jena J. Steinle1, 2.
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**Purpose:** Recent work has suggested that diabetic retinopathy has a strong inflammatory component. Our group reported that a novel b-adrenergic receptor agonist, Compound 49b, reduced toll-like receptor 4 (TLR4) signaling in the diabetic retina and in retinal endothelial cells. In this study, we wanted to investigate whether TLR4 levels regulate endothelial cell permeability, including zonula occluden 1 (ZO-1) and occludin levels, as well as, if TLR4 plays an
important role in ischemia-reperfusion (I/R)-induced retinal vascular damage and neuronal loss.

**Methods:** TLR4 floxed mice (B6 Cg-Tlr4tm1.lkap/J) and B6, FVB-Tg (ed85-crcr) Mlia/J Cre mice purchased from Jackson lab were cross bred to produce endothelial cell specific TLR4 conditional knockout mice. TLR4 overexpressing mice were acquired from Dr. Fukuchi. TLR4 knockout and overexpression mice were selected by genotyping and kept for 3 months prior the experiments. Some mice from each group were used for Western blotting for TLR4, ZO-1, and occludin. Additionally, additional knockout and overexpressing mice were subjected to ischemia-reperfusion (I/R) followed by neuronal analyses at 2 days and vascular analyses at 10 days. Mice in all groups were assessed for changes in permeability using fluorescein angiography.

**Results:** Protein levels of ZO-1 and occludin were decreased in the TLR4 floxed mice compared to TLR4 Cre-Lox. TLR4 overexpressing mice had much lower ZO-1 and occludin levels than their control littermates. Ten days after I/R, TLR4 overexpressing mice had significantly more degenerate capillaries than their littermates. TLR4 Cre-Lox had fewer degenerate capillaries than floxed mates. At 24 hour after I/R, TLR4 overexpressing and TLR4 floxed mice had increased leakage when compared to the control littermates and TLR4 Cre-Lox, respectively.

**Conclusions:** The data demonstrate that TLR4 plays an important role in regulation of the tight junction permeability, as well as vascular and neuronal damage in the retina. TLR4 overexpression enhanced leakage following I/R, which was associated with more degenerate capillaries and neuronal loss. Taken together, the data suggest that inhibition of TLR4 may be protective to the damaged retina.

**Commercial Relationships:** Li Liu, None; Youde Jiang, None; Elizabeth Curtis, None; Jena J. Steine, None

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Circadian Arrhythmia Alters Retinal Kir4.1 Expression

**Ashay D. Bhatwadekar, Osama Mufti, Qianyi Luo. Ophthalmology, Eugene and Marilyn Glick Eye Institute, Indianapolis, IN.**

**Purpose:** The Müller cell, a major glia of the retina maintains K+ balance through inwardly rectifying Kir4.1 channels. Studies in our laboratory suggest that the Kir4.1 channels exhibit a diurnal rhythm. Clock-controlled genes regulate about 10% of the transcriptome; therefore, improper regulation of circadian rhythms likely contributes to disorders that include obesity, metabolic syndrome, and type 2 diabetes (T2D). However, it remains unknown whether circadian rhythm disruption (CRD) affects retinal Kir4.1 expression, therefore the purpose of this investigation is to determine the influence of CRD on Kir4.1 regulation and Müller cell dysfunction.

**Methods:** The T2D mice (db/db) and control mice (db/m) were maintained under regular light-dark (LD) conditions or exposed to a constant dark to induce the CRD in circadian cabinets. The locomotor activity was evaluated using the Clock-Lab software. After two weeks, the plasma levels of insulin and leptin were evaluated. The retinal expression of clock genes Per2, Bmal1 and Kir4.1 was determined.

**Results:** The db/m mice in the LD group exhibited a locomotor activity consistent with the nocturnal nature of animals. However, the movements of the diabetic mice were sporadic. The CRD altered the normal locomotor behavior of both db/m and db/db mice with a profound decrease in the mean onset of activity (p<0.05). In db/m mice, CRD lead to hyperinsulinemia and hyperleptinemia with a decrease in the levels of retinal clock genes, Per2 (1.8-fold p<0.05) and Bmal1 (1.7-fold; p<0.05). The db/db mice demonstrated a greater reduction of clock genes. The Kir4.1 expression exhibited a diurnal rhythm in the retina of db/m mice, however, the diabetic mice were devoid of this rhythm. CRD resulted in disruption of diurnal rhythm in both db/m and db/db animals.

**Conclusions:** Our studies demonstrate that CRD leads to a decrease in Kir4.1 expression, which may result in Müller cell dysfunction. Thus, circadian rhythm regulation is critical for protection from Müller cell dysfunction in diabetes.

**Commercial Relationships:** Ashay D. Bhatwadekar, Indiana University (E); Osama Mufti, None; Qianyi Luo, Indiana University (E)

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