Spleen Tyrosine Kinase Contributes to Müller Glial Expression of Proangiogenic Cytokines in Diabetes

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PURPOSE. Neuroglial dysfunction occurs early in the progression of diabetic retinopathy. In response to diabetes or hypoxia, Müller glia secrete cytokines and growth factors that contribute to disease progression. This study was designed to examine common signaling pathways activated in Müller glia by both type 1 and pre-/type 2 diabetes.

METHODS. RiboTag (Pdgfra-cre;HA-Rpl22) mice were used to compare the impact of streptozotocin (STZ) and a high-fat, high-sucrose (HFHS) diet on ribosome association of mRNAs in Müller glia by RNA sequencing analysis. Human MIO-M1 Müller cells were exposed to either hyperglycemic or hypoxic culture conditions. Genetic manipulation and pharmacologic inhibition were used to interrogate signaling pathways.

RESULTS. Association of mRNAs encoding triggering receptor expressed on myeloid cells 2 (TREM2), DNA-activating protein 12 kDa (DAP12), and colony stimulating factor 1 receptor (CSF1R) with ribosomes isolated from Müller glia was upregulated in both STZ diabetic mice and mice fed an HFHS diet. The TREM2/DAP12 receptor-adaptor complex signals in coordination with CSF1R to activate spleen tyrosine kinase (SYK). SYK activation was enhanced in the retina of diabetic mice and in human MIO-M1 Müller cell cultures exposed to hyperglycemic or hypoxic culture conditions. DAP12 knockout reduced SYK autophosphorylation in Müller cells exposed to hyperglycemic or hypoxic conditions. SYK inhibition or DAP12 knockout suppressed hypoxia-induced expression of the transcription factor hypoxia-inducible factor 1α (HIF1α), as well as expression of vascular endothelial growth factor and angiopoietin-like 4.

CONCLUSIONS. The findings support TREM2/DAP12 receptor-adaptor complex signaling via SYK to promote HIF1α stabilization and increased angiogenic cytokine production by Müller glia.

Keywords: diabetic retinopathy, mRNA translation, glia, hyperglycemia, hypoxia
composed of HIF1α and HIF1β. HIF1 activity is principally regulated through variation in HIF1α protein half-life. Under normoxic conditions, HIF1α is hydroxylated at Pro2/Pro564 by prolyl hydroxylase domain (PHD) enzymes, leading to its ubiquitination and rapid degradation by the proteasome. In response to hypoxia, PHD activity is impaired, resulting in HIF1α accumulation. Cytoplasmic HIF1α enters the nucleus to form a complex with the constitutively expressed HIF1β subunit and bind to the HREs of an array of proangiogenic target genes. HIF1α mRNA expression is observed throughout the human retina but is most highly expressed in Müller glia. Indeed, HIF1α protein localizes to Müller glia in the retina of patients with diabetic eye disease.

The studies here were designed to identify common signaling pathways activated in retinal Müller glia by both type 1 (i.e., streptozotocin [STZ]-induced diabetes) and pre-/type 2 diabetes (i.e., prodiaeticogenic diet). Both of these preclinical murine models develop retinal pathology characterized by gliosis, neurodegeneration, and vascular injury. Sequencing analysis of ribosome-associated mRNAs from retinal Müller glia revealed a common increase in mRNAs encoding triggering receptor expressed on myeloid cells 2 (TREM2), DNAX-activating protein of 12 kDa (DAP12, also known as Tyrobp), and colony stimulating factor 1 receptor (CSF1R). Recent studies support a role for TREM2/DAP12 signaling via SYK to facilitate upregulation of HIF1α and increased cytokine expression by Müller glia.

Materials and Methods

Animals

Pdgfra-cre;HA-Rpl22 (RiboTag) mice were generated as previously described. Male littermates were administered 50 mg/kg STZ versus sodium citrate buffer for 5 consecutive days or fed a high-fat, high-sucrose diet (HFD,ENVIGO TD.88137;Envigo,Indianapolis,IN,USA) versus control chow (ENVIGO TD.08485) to induce diabetes. Diabetic phenotype was assessed with fasting blood glucose levels >250 mg/dL. All procedures were approved by the Penn State College of Medicine Institutional Animal Care and Use Committee and were in accordance with the ARVO statement for the ethical use of animals in ophthalmic and vision research.

Immunofluorescent Microscopy

Immunofluorescent microscopy was performed as previously described. Whole eyes were extracted, placed in 4% paraformaldehyde for 30 minutes, and then washed with PBS. After 48 hours of incubation in 30% sucrose at 4°C, the eyes were placed in optimal cutting temperature compound, flash-frozen, and sectioned. Retinal cryosections (10 μm) were permeablisized with 0.1% Triton-X-100 in PBS, blocked with 10% donkey serum, and incubated in anti–glutamine synthetase (GS) primary antibody followed by Alexa Fluor 488 secondary antibody (Supplementary Table S1). Sections were blocked and incubated in anti-hemagglutinin (HA) primary antibody and Alexa Fluor 647 secondary antibody. Hoechst (1.6 mmol/L) was used for nuclear staining in all sections. Imaging was performed with a Leica SP8 confocal laser microscope (Leica Biosystems,Wetzlar,Germany).

Ribosome-Associated RNA Isolation

Retinas from three mice per group were extracted from RiboTag mice, and HA-tagged ribosomes were immunoprecipitated as previously described. Anti-HA beads (EZview;Sigma,St. Louis,MO,USA) were washed and blocked in 0.5% BSA. Retinas were homogenized in polysome buffer and centrifugated at 10,000 × g at 4°C for 10 minutes. Supernatants were collected and combined with beads at 4°C for 16 hours. The mixture was centrifugated at 8200 × g for 30 seconds, the pellet was collected, and the beads were washed with high-salt buffer. RLT buffer (Qiagen,Hilden,Germany) was added to beads for processing, and RNA was isolated using the RNeasy Micro kit (Qiagen).

Sequencing Analysis

TruSeq Stranded mRNA Library Prep kit (Illumina,San Diego,CA,USA) was used to prepare cDNA libraries as per the manufacturer’s instructions. Briefly, polyA RNA was purified from 200 ng total RNA using oligo (dT) beads. The extracted mRNA fraction was subjected to fragmentation, reverse transcription, end repair, 3′-end adenylation, and adaptor ligation, followed by PCR amplification and SPRI bead purification (Beckman Coulter,Pasadena,CA,USA). Unique dual index sequences (NEXTFLEX Unique Dual Index Barcodes;BioO Scientific,Austin,TX,USA) were incorporated in the adaptors for multiplexed high-throughput sequencing. The final product was assessed for its size distribution and concentration using the BioAnalyzer High Sensitivity DNA Kit (Agilent Technologies,Santa Clara,CA). Libraries were pooled and diluted to 3 nM with 10 mM Tris-HCl, pH 8.5, and then denatured using the Illumina protocol. The denatured libraries were loaded onto an S1 flow cell on an Illumina NovaSeq 6000 (Illumina) and run for 2 × 50 cycles according to the manufacturer’s instructions. De-multiplexed and adapter-trimmed sequencing reads were generated using Illumina bcl2fastq (released version 2.20.0-422), allowing no mismatches in the index read. BBtools (ver. 36.49) was used to trim/filter low-quality sequences using the “qtrim=lr trimq=10 maq=10” option. Reads were then mapped to reference mouse genome mm10 using hisat2 (ver. 2.1.0) with –no-mixed and –no-discordant options. Read counts were calculated using HTseq (ver. 0.11.2) by supplementing Ensembl gene annotation (Mus musculus.GRCm38.78_ERCC92.gtf). Differential analysis was performed using edgeR exactTest, with the biological coefficient of variation manually set to 0.1. Ingenuity Pathway Analysis software was used to perform gene ontology analysis on the significant genes identified by the differential analysis.
Cell Culture

Human MIO-M1 Müller cells were obtained from the UCL Institute of Ophthalmology (London, UK) and cultured at 37°C and 5% CO2 in Dulbecco's modified eagle medium (DMEM) containing 1 g/L glucose with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin-streptomycin (P/S). Culture medium was supplemented with 25 mM D-glucose (BioChemika, Steinheim, Germany) to model hyperglycemic conditions. For hypoxic conditions, cells were cultured in a hypoxia chamber (Hypoxia Chamber Cat# 27320; STEMCELL Technologies, Vancouver, Canada) containing mixed gas (1% O2, 94% N2, and 5% CO2). In some studies, culture medium was supplemented with CoCl2 (150 μM), deferoxamine mesylate (DFO, 100 μM), or carbobenzoxy-Leu-Leu-leucinal (MG132, 20 μM). ER-2739 maleate (30 μM) (Tocris Bioscience, Bristol, United Kingdom) or fostamatinib disodium (R788, 10 μM) was added to culture medium 15 minutes prior to other manipulations. HEK293 cells were cultured in high-glucose (4.5 g/L) DMEM, 10% FBS, and 1% P/S. HEK293 cells were transfected with plasmids encoding empty vector, HIF1α wild-type, or HIF1α P402A/P564A using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA). HA-HIF1α-pcDNA3 and HA-HIF1α-pcDNA3 were a gift from William Kaelin (Addgene plasmids #18949 & 18955). pLKO-shDAP12 plasmid for lentiviral short hairpin RNA (shRNA) knockdown was obtained from the Penn State College of Medicine shRNA Library Core. pLKO-1-TRC control shRNA plasmid was a gift from David Root (Addgene plasmid #10879). HEK293FT cells were used to generate lentivirus containing either the control shRNA or an shRNA targeting DAP12 (5′-CCGGGCTCTCCTGCTGCTGTAAGCTCGAG ACTTACGCCAGCAAGGAGTTTTTG-3′). Lentivirus was applied to MIO-M1 cells to obtain cell lines with stable shRNA expression as previously described.19

Western Blotting

Cells were lysed, combined with Laemmli sample buffer, and boiled at 100°C for 5 minutes. Then, 4% to 20% Criterion gels (Bio-Rad, Hercules, CA, USA) were used for SDS-PAGE. Proteins were transferred to nitrocellulose (Thermo Scientific) or polyvinylidene fluoride (PVDF) membrane (Bio-Rad), which was blocked with 5% milk in TBST (50 mM Tris, pH 7.6; 0.9% NaCl; and 0.1% Tween-20) and exposed to the appropriate primary and secondary antibody (Supplementary Table S1). Protein bands were visualized with enhanced chemiluminescence Clarity Reagent (Bio-Rad) using a ProteinSimple FluorChem E.

Quantitative RT-PCR

Cells were collected with TRIzol for homogenization, and RNA was extracted following the organic-extraction protocol (Invitrogen, Carlsbad, CA, USA). Whole murine retinas were homogenized in TRIzol, combined with chloroform, and centrifuged to separate the organic and aqueous phases. The aqueous phase was collected and RNA was isolated using an RNaseasy micro kit (Qiagen). A High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) was used to generate cDNA. Quantitative RT-PCR was conducted using a QuantiTect SYBR Green PCR Kit (Qiagen). Primer sequences are listed in Supplementary Table S2. Results were normalized to GAPDH using the 2-ΔΔCT calculations.

Data Analysis and Presentation

Statistical analyses were performed using GraphPad Prism software (GraphPad Software, La Jolla, CA, USA) with P < 0.05 defined as statistically significant. Data were analyzed overall with either Student’s t-test or one-way or two-way analysis of variance. Trend test and pairwise comparisons were conducted with the Tukey test for multiple comparisons. Figures were assembled with Adobe Illustrator (Adobe, San Jose, CA, USA). Graphics were created with BioRender.com.

RESULTS

Examination of mRNA Translation in Retinal Müller Glia

To specifically assess ribosome-associated mRNAs in retinal Müller glia, a previously developed Ribotag mouse that expresses an epitope-tagged variant of the ribosomal protein Rpl22 under the control of Cre recombinase was used.20 In Cre-positive cells, wild-type exon 4 of Rpl22 was replaced with an HA-tagged exon 4 (Fig. 1A). Rpl22HA expression was directed to Müller glia under the control of Pdgfra-cre recombinase.21 Specificity of Rpl22HA expression in Müller glia of Rpl22HA; Pdgfra-cre mice17 was assessed by examining retinal cross sections. Consistent with the prior analysis,17 Rpl22HA was observed in radially oriented processes that spanned the entire neuroretina and in branching end feet that surrounded ganglion cells (Fig. 1B). Rpl22HA strongly colocalized with the Müller glia marker GS. Rpl22HA forms a functional 60S ribosomal subunit that allowed translationally active mRNAs from Pdgfra-cre expressing Müller glia to be isolated from whole retina tissue homogenate by affinity purification (Fig. 1C). To evaluate the impact of type 1 diabetes on Müller glial mRNA translation, mice were administered STZ. After 6 weeks of STZ-induced diabetes, mice exhibited increased fasting blood glucose concentrations (Fig. 1D). To evaluate the impact of type 2 diabetes, mice were fed an HFS diet. After 6 weeks of the prodiabetogenic diet, fasting blood glucose concentrations were elevated (Fig. 1E) and weight gain increased (Fig. 1F), resulting in greater body weight (Fig. 1G). RNA sequencing was performed on mRNAs associated with Rpl22HA ribosomes in the retina of STZ diabetic versus nondiabetic control mice (Fig. 1H, Supplementary Table S3). Most transcripts observed in the analysis of STZ versus vehicle control mice (Fig. 1H, Supplementary Table S3) did not exhibit a change in abundance (R2 = 0.999). A similar sequencing analysis was also performed on Rpl22HA-associated mRNAs from the retina of mice fed either an HFHS diet or control chow (R2 = 0.999, Fig. 1I, Supplementary Table S4). Whereas the abundance of Rpl22HA ribosome-associated mRNAs was highly similar in nondiabetic control versus Chow-fed mice (R2 = 0.999, Fig. 1I), increased variation was observed in the abundance of Rpl22HA ribosome-associated mRNAs from the retina of STZ diabetic versus HFHS diet-fed mice (R2 = 0.994, Fig. 1K). The analysis supports variation in ribosome-association of mRNAs within each preclinical model of diabetes and substantial variation between the two experimental models.

Upregulation of Müller Glial Trem2/DAP12 Signaling in Diabetes

STZ diabetes enhanced the abundance of 28 mRNAs in the Rpl22HA ribosome immunoprecipitate and reduced the
FIGURE 1. Ribosome-associated mRNAs isolated from retinal Müller glia of diabetic mice were examined by sequencing analysis. (A) Müller glia-specific expression of HA-tagged Rpl22 protein was achieved by Cre-Lox recombination, resulting in deletion of the wild-type (WT) exon 4 of Rpl22 and replacement with an HA-tagged Rpl22 exon in the presence of Pdgfra-cre. (B) Whole eyes were isolated, fixed, and sectioned longitudinally. Localization of HA-Rpl22 (red) and the Müller glia marker GS (green) was evaluated by immunofluorescence. Nuclei were visualized with Hoechst (blue). (C) Ribosomes from Müller glia of Rpl22HA-expressing homozygous mouse retinas were isolated by immunoprecipitation. Cell-specific expression of Rpl22HA allows translationally active mRNAs from Müller glia to be isolated from whole retina tissue homogenate by affinity purification, as all cre-negative cells express wild-type Rpl22 that lacks an affinity tag. (D) Diabetes was induced in Rpl22HA mice by administration of STZ. Control mice received a vehicle (Veh). Fasting blood glucose concentrations were measured after 6 weeks. (E–G) Mice were fed either an HFHS diet or control chow for 6 weeks. Fasting blood glucose concentrations were measured (E). Weight gain (F) and final body weight (G) were determined. (H) Retinas were analyzed after 6 weeks of STZ diabetes. Rpl22HA ribosome-associated mRNAs were evaluated by RNA sequencing. Variation in Rpl22HA ribosome-associated mRNA abundances in mice receiving STZ or Veh was visualized. (I) Retinas were analyzed 6 weeks after initiation of HFHS or chow diet. Changes in Rpl22HA ribosome-associated mRNAs in mice receiving the HFHS diet or chow were compared. (J) Rpl22HA ribosome-associated mRNA sequencing analysis for control mice receiving either Veh or chow diet was compared. (K) Rpl22HA ribosome-associated mRNAs from Müller glia of mice receiving either STZ or HFHS diet were compared. Values are means ± SD. *P < 0.05 versus Veh or chow. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; RPE, retinal pigment epithelium; TPM, transcript per million.

abundance of 48 mRNAs, as compared to nondiabetic control mice (Fig. 2A). Rpl22HA ribosome association was increased for 58 mRNAs in the retina of mice fed an HFHS as compared to control chow, and 61 mRNAs were decreased (Fig. 2B). To assess the potential impact of the observed changes, functional relationships between mRNAs that exhibited altered ribosome association were investigated. The top canonical pathways identified by Ingenuity Pathway Analysis as being altered by STZ diabetes included Interferon Signaling and the GP6 Signaling Pathway (Supplementary Fig. S1). The top scoring pathways identified as being altered by the HFHS diet included Regulation of Epithelial-Mesenchymal Transition Pathway and IL-4 Signaling (Supplementary Fig. S2).

Surprisingly, there was limited overlap in the mRNAs that were altered in both mouse models, as only 18 mRNAs exhibited codirectional changes (Figs. 2C, 2D). Nineteen mRNAs were upregulated with the HFHS diet but downregulated with STZ diabetes. The opposite case, in which an mRNA was upregulated with STZ diabetes but downregulated by the HFHS diet, was not observed. Notably, mRNAs encoding all three subunits of the complement protein C1q were upregulated in both models (Fig. 2D), and codirectional changes were observed for several mRNAs encoding crystallins. Additionally, mRNAs encoding DAP12, TREM2, and CSF1R all exhibited enhanced association with Rpl22HA ribosomes in both STZ diabetic mice and mice fed an HFHS diet. TREM2 and CSF1R are cell receptors that cross-talk to drive glial activation (Fig. 2E). DAP12 binds the intracellular domain of TREM2, and CSF1R cross-talks with TREM2/DAP12 by promoting Src-dependent phosphorylation of DAP12.22 In contrast with the sequencing
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FIGURE 2. Ribosome association of mRNAs encoding proteins in the TREM2/DAP12 signaling pathway was increased in Müller glia of diabetic mice. Diabetes was induced in Rpl22HA mice by administration of STZ. Control mice received a vehicle (Veh). Retinas were analyzed after 6 weeks of diabetes. Ribosomes from Müller glia of Rpl22HA-expressing homozygous mice were isolated from retina homogenates by immunoprecipitation (IP) and examined by RNA sequencing. Mice were fed either an HFHS diet or control chow. Retinas were analyzed 6 weeks after initiation of diet. (A, B) Volcano plot of sequencing analysis for STZ diabetic versus Veh control mice (A) and HFHS diet versus chow (B) with mRNAs exhibiting increased (green) or decreased (red) Rpl22HA association shown. (C) Overlap in mRNAs exhibiting changes in Rpl22HA association in mice receiving STZ versus Veh or HFHS diet versus chow was visualized by Venn diagram. (D) Heatmap (left) illustrates mRNAs exhibiting changes in ribosome association in both models. Expanded view of heatmap (right) depicts only the mRNAs exhibiting codirectional changes in the two models, with log fold change (FC) value shown in white. (E) Cartoon of TREM2/DAP12 signaling to activate Syk kinase is shown. (F) Results from the sequencing analysis for DAP12 (Tyrobp), TREM2, and CSF1R. (G) DAP12, TREM2, and CSF1R mRNA abundance was examined in whole retinal lysates by quantitative PCR (qPCR). (H) Ribosome-associated mRNAs from Müller glia were isolated by immunoprecipitation. DAP12, TREM2, and CSF1R mRNA abundance was examined in retinal immunoprecipitate by qPCR. Values are means ± SD. *P < 0.05 versus Veh or chow.

analysis of Rpl22HA ribosome-associated mRNAs (Fig. 2F), DAP12, TREM2, and CSF1R mRNA abundance in homogenates from whole retina was not altered with either STZ diabetes or the HFHS diet (Fig. 2G). In support of the sequencing data, PCR analysis confirmed increased DAP12 and TREM2 association with Rpl22HA ribosomes from the retina of STZ diabetic mice or mice fed an HFHS diet (Fig. 2H). Increased CSF1R association with Rpl22HA ribosomes was also observed in mice fed an HFHS diet, and there was a trend toward upregulation in STZ diabetic mice (P = 0.0937).

SYK Activation was Enhanced in the Retina of Diabetic Mice

To further explore TREM2/DAP12 signaling, we investigated SYK activation by assessing autophosphorylation of the kinase. SYK autophosphorylation within its activation loop at Tyr525/Tyr526 is required for SYK downstream signaling.23 SYK phosphorylation was enhanced in retinal lysates from STZ diabetic mice as compared to nondiabetic controls (Fig. 3A). Enhanced SYK phosphorylation was also observed in mice fed an HFHS diet as compared to a chow diet (Fig. 3B). Together, the data provide direct evidence of enhanced SYK activation in the retina of diabetic mice.

FIGURE 3. SYK autophosphorylation was enhanced in the retina of diabetic mice. (A) Phosphorylation of SYK at Tyr525/Tyr526 was evaluated in retinal lysates from mice administered either STZ or vehicle (Veh) by Western blotting. Representative blots are shown. Protein molecular mass in kDa is indicated at right of blots. (B) SYK phosphorylation was evaluated in retinal lysates from mice fed either an HFHS diet or control chow. Values are means ± SD. *P < 0.05 versus Veh or chow.
DAP12 Contributes to Enhanced SYK Activation in Müller Glia

To investigate SYK activation in human Müller glia, SYK phosphorylation was examined in immortalized MIO-M1 Müller cell cultures. Phosphorylated SYK was increased in cells exposed to either hyperglycemic (Fig. 4A) or hypoxic culture conditions (Figs. 4B, 4C). To evaluate a role for TREM2/DAP12 signaling in SYK activation, DAP12 was knocked down by stable expression of an shRNA (Fig. 4D). DAP12 knockdown prevented an increase in phosphorylated SYK in cells exposed to either hyperglycemic (Fig. 4E) or hypoxic culture conditions (Fig. 4G). Upregulation of phosphorylated SYK was also observed upon exposure to the hypoxia-mimetic CoCl₂ (Fig. 4G), in coordination with the gliosis marker GFAP (Supplementary Fig. S3B). DAP12 knockdown also prevented an increase in phosphorylated SYK with CoCl₂ exposure (Fig. 4G).

SYK Inhibition Suppressed HIF1α Expression Under Hypoxic Conditions

To investigate the consequence of hypoxia-induced TREM2/DAP12 signaling via SYK, HIF1α expression was examined in Müller cell cultures exposed to hypoxic conditions. Hypoxia promoted HIF1α expression in MIO-M1 cells (Supplementary Fig. S3C), and the effect was blunted by DAP12 knockdown (Fig. 5A, Supplementary Fig. S3D). Hypoxia prevents proteasomal degradation of HIF1α by suppressing PHD activity, which is modeled by CoCl₂- and DFO-induced PHD inhibition. HIF1α expression was increased in MIO-M1 cells exposed to CoCl₂, and DAP12 knockdown attenuated the effect (Fig. 5B). In support of a role for SYK signaling in promoting HIF1α expression, SYK inhibition with ER-27391 prevented the increase in HIF1α expression with either hypoxia (Fig. 5C) or CoCl₂ (Fig. 5D). SYK inhibition also prevented an increase in HIF1α expression in cells exposed to the iron chelator.

Figure 4. Phosphorylated SYK was enhanced in Müller cells by hyperglycemic or hypoxic conditions. (A) MIO-M1 Müller cells were exposed to medium containing 30 mM glucose (high glucose, HG) for up to 8 hours. SYK phosphorylation at Tyr525/Tyr526 was evaluated by Western blotting. Representative blots are shown. Protein molecular mass in kDa is indicated at right of blots. Phosphorylated SYK was quantified after 8 hours of exposure to HG. (B, C) SYK phosphorylation was evaluated in MIO-M1 cells exposed to hypoxic conditions for 4 (B) or 24 (C) hours. (D) DAP12 expression was knocked down in MIO-M1 cells by stable expression of an shRNA (shDAP12). (E) MIO-M1 cells were exposed to HG for 24 hours and SYK phosphorylation was quantified. (F) MIO-M1 cells were exposed to 1% oxygen for 4 hours and SYK phosphorylation was quantified. (G) MIO-M1 cells were exposed to medium supplemented with CoCl₂ for up to 4 hours. SYK phosphorylation was quantified after 0 or 4 hours of exposure to CoCl₂ (G). Values are means ± SD. *P < 0.05 versus time 0; #P < 0.05 versus without shDAP12.
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SYK Inhibition Prevented Hypoxia-Induced Proangiogenic Cytokine Expression

VEGFA and angiopoietin-like 4 (ANGPTL4) are increased in the eyes of patients with diabetic macular edema and act synergistically to drive retinal permeability. In MIO-M1 cells exposed to hypoxic conditions, mRNA abundance of VEGFA (Fig. 6A) and ANGPTL4 (Fig. 6B) was increased. DAP12 knockdown attenuated hypoxia-induced expression of both VEGFA and ANGPTL4 mRNAs. A similar suppressive effect was observed with SYK inhibition, as hypoxia-induced expression of VEGFA and ANGPTL4 was absent in cells treated with ER-27391 (Figs. 6C, 6D). CoCl₂ also promoted VEGFA and ANGPTL4 mRNA expression in MIO-M1 cells, and DAP12 knockdown reduced the effect (Supplementary Figs. S3F, S3G). Similarly, SYK inhibition with either ER-27391 (Figs. 6E, 6F) or R778 (Figs. 6G, 6H) attenuated CoCl₂-induced VEGFA and ANGPTL4 mRNA expression. In addition to secreting proangiogenic factors, Müller glia have also been implicated in the production of proinflammatory cytokines that contribute to DR progression. To determine if SYK inhibition had a similar effect on inflammatory cytokine expression, TNFα, IL-1β, and IL6 expression were evaluated in MIO-M1 cells exposed to CoCl₂ (Supplementary Figs. S4A–C). Consistent with the suppressive effect on VEGFA and ANGPTL4, SYK inhibition reduced the expression of TNFα, IL-1β, and IL6 in cells exposed to CoCl₂ (Supplementary Figs. S4D–F).
FIGURE 6. SYK contributed to pro-angiogenic cytokine expression in Müller cells. (A, B) DAP12 expression was knocked down in MIO-M1 Müller cells by stable expression of shRNA (shDAP12) or a control shRNA (shCTRL). Cells were exposed to 1% oxygen for 4 hours as indicated. (C–F) MIO-M1 cells were treated with the SYK inhibitor ER-27391 or a vehicle (Veh) control and subsequently exposed to 1% oxygen (C, D) or CoCl2 (E, F) for 4 hours. (G, H) MIO-M1 cells were treated with the SYK inhibitor fostamatinib (R788) or a vehicle (Veh) control and subsequently exposed to CoCl2 for 4 hours. Expression of mRNAs encoding VEGFA (A, C, E, G) and ANGPTL4 (B, D, F, H) was evaluated by RT-PCR. Values are means ± SD. *P < 0.05 versus no hypoxia or CoCl2. #P < 0.05 versus shCTRL or vehicle.

DISCUSSION

Retinal Müller glia provide critical homeostatic and trophic support to maintain both activity of retinal neurons and integrity of the blood–retinal barrier. Müller cells recycle neurotransmitters to prevent excitotoxicity, regulate metabolite levels, reabsorb fluid to prevent edema, and even contribute to the visual cycle. In response to diabetes, Müller glia are known to produce a range of angiogenic and inflammatory cytokines that contribute to development of DR. The present study used Pdgfra-cre RiboTag mice to compare the impact of STZ-induced diabetes and HFHS diet on ribosome association of mRNAs in Müller glia. Association of mRNAs encoding TREM2, DAP12, and CSF1R with ribosomes isolated from Müller glia was upregulated in both models. Overall, the findings support a model wherein the TREM2/DAP12 receptor-adaptor complex signals via SYK to promote HIF1α stabilization and increased proangiogenic cytokine production by Müller glia (Supplementary Fig. S5).

Prior investigations focused on Müller glial gene expression and more recent retina-wide single-cell sequencing have used techniques such as microarray and RNA sequencing to identify “steady-state” differences in mRNA abundance. However, mRNA abundances are a relatively poor correlate for protein expression, and some studies suggest that variation in mRNA accounts for only ~40% of the global variation in protein abundance. Translational control facilitates the selective recruitment of ribosomes to specific mRNAs to provide a rapid and reversible change in expression of specific proteins. Notably, mRNAs sensitive to translational control are most enriched in the gene categories of angiogenesis, synaptic transmission, and cell adhesion, which are all key processes disrupted in the retina by diabetes. To investigate gene expression changes in Müller glia that were associated with early diabetes, sequencing analysis was performed on ribosome-associated mRNAs from the retina of RiboTag mice. Whereas there was no change in the abundance of mRNAs encoding TREM2, DAP12, and CSF1R in whole retina, association of these mRNAs with Müller glial ribosomes was enhanced by STZ diabetes or HFHS diet.

RiboTag mice express a fully functional HA-tagged ribosome in cells with Cre recombinase activity, allowing cell-specific isolation of translationally active mRNAs from heterologous tissues such as the retina. We previously demonstrated that RNA isolated from the retina of Pdgfra-cre;RiboTag mice was enriched for Müller cell markers. An important limitation of this analysis is that in addition to Müller glia, other retinal glia may also exhibit recombinase activity. In particular, PDGFRa has been previously reported in astrocytes of the developing retina. However, Pdgfra-cre recombinase-dependent nuclear lacZ expression in adult murine retina is exclusively localized to the inner nuclear layer, whereas the cell bodies of astrocytes are more commonly found in the retinal nerve fiber layer. Consistent with Müller cell specificity in adult murine retina, Rpl22HA expression was observed in radially oriented processes that strongly colocalized with the Müller glia-specific marker GS.

TREM2 and DAP12 form a receptor-adaptor complex that is associated with neurodegenerative diseases, including Alzheimer and Parkinson diseases. TREM2/DAP12...
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HIF1α expression. While less well explored than proteasomal degradation, chaperone-mediated autophagy (CMA) is also a major regulator of HIF1α expression. CMA mediates lysosomal degradation of specific KFERQ-like motif-bearing proteins in response to changes in the cellular environment. Thus, one possibility is that SYK acts to prevent HIF1α degradation via CMA.

HIF1α protein expression is enhanced in the retina of STZ diabetic mice, and HIF1α deletion specifically in Müller glia is sufficient to prevent the increase. The observation supports that diabetes specifically promotes HIF1α expression in Müller cells and is consistent with localization of HIF1α protein to Müller glia in the retina of diabetic patients. Importantly, HIF1α deletion in Müller cells attenuates retinal vascular leakage and prevents increased VEGF production with diabetes. Evidence also supports that Müller glia-derived VEGF is a key contributor to retinal vascular pathology in preclinical diabetes models. Together, the prior studies support a key role for HIF1-dependent VEGF expression in the development of the vascular pathology that defines DR. Antibodies that block VEGF signaling have dramatically improved treatment outcomes in patients with DR. However, VEGF is a relatively poor correlate with the extent of retinal edema, implying that it likely acts in coordination with multiple other growth factors and cytokines to drive pathologic neovascularization. Indeed, Müller glia also upregulate production of ANGPTL4 to promote retinal vascular permeability. The findings of this study support a role for diabetes-induced upregulation of TREM2/DAP12 signaling in Müller glia that acts via SYK to promote HIF1α stabilization and increased production of VEGF and ANGPTL4. Thus, local retinal SYK inhibition may represent a therapeutic step forward by not only suppressing VEGF mRNA transcription but also targeting the production of other critical factors that influence vascular permeability in both type 1 and type 2 diabetes.

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