O-GlcNAc Modification of Transcription Factor Sp1 Mediates Hyperglycemia-Induced VEGF-A Upregulation in Retinal Cells

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PURPOSE. Proangiogenic protein VEGF-A contributes significantly to retinal lesions and neovascularization in diabetic retinopathy (DR). In preclinical DR, hyperglycemia can upregulate VEGF-A in retinal cells. The VEGF-A promoter is responsive to the transcription factor specificity protein 1 (Sp1). The O-GlcNAc modification is driven by glucose concentration and has a profound effect on Sp1 activity. This study investigated the effects of hyperglycemia on Sp1-mediated expression of VEGF-A in the retinal endothelium and pigment epithelium.

METHODS. Hyperglycemia-exposed ARPE-19 (human retinal pigment epithelial cells) and TR-1BRB (rat retinal microendothelial cells) were assayed for levels of VEGF-A by qRT-PCR, Western blot, and ELISA. Small molecule inhibitors of O-GlcNAc transferase (OGT) or O-GlcNAcase (OGA) were used to manipulate O-GlcNAc levels. Vascular endothelial growth factor-A protein and transcript were measured in cells depleted of OGT or Sp1 by shRNA. The proximal VEGF-A promoter was analyzed for glucose sensitivity by luciferase assay. Chromatin immunoprecipitation (ChIP) was used to assess Sp1 occupancy on the VEGF-A promoter.

RESULTS. Hyperglycemia increased VEGF-A promoter activity and upregulated VEGF-A transcript and protein. Elevation of O-GlcNAc by OGA inhibitors was sufficient to increase VEGF-A. O-GlcNAc transferase inhibition abrogated glucose-driven VEGF-A. Cellular depletion of OGT or Sp1 by shRNA significantly abrogated glucose-induced changes in VEGF-A. ChIP analysis showed that hyperglycemia significantly increased binding of Sp1 to the VEGF-A promoter.

CONCLUSIONS. Hyperglycemia-driven VEGF-A production is mediated by elevated O-GlcNAc modification of the Sp1 transcription factor. This mechanism may be significant in the pathogenesis of preclinical DR through VEGF-A upregulation.

Keywords: VEGF-A, Sp1, O-GlcNAc, diabetic retinopathy, OGT

Approximately one-third of all diabetic patients have some form of retinopathy,¹ an irreversible, sight-threatening complication associated with diabetes. It has been well established that VEGF-A is intimately involved in the pathogenesis of diabetic retinopathy (DR).² Vascular endothelial growth factor-A potentially stimulates angiogenesis, the complicated process of generating new blood vessels. Vascular endothelial growth factor-A is abundant in the retinal vasculature and the vitreous humor of patients with clinically-significant retinopathy.^{3–5} In DR, VEGF-A directly triggers breakdown, leakiness, and proliferation of blood vessels.⁶ The importance of VEGF-A is highlighted by the number of clinical therapeutics, namely neutralizing antibodies⁷ and decoy receptors⁸ that target this pro-angiogenic protein.

In the earliest stages of the disease (preclinical or subclinical DR), lesions to the retinal microvasculature begin with breakdown of the blood-retina barrier, causing dot-and-blot hemorrhages and localized endothelial proliferation, and finally microaneurysms.⁹ Increased barrier permeability, as well as vessel dilation and proliferation, are well-described effects of

the VEGF-A protein.¹⁰ Elevated VEGF-A has been detected in the eyes of diabetic humans and animals prior to the appearance of discernible vascular lesions,¹¹ supporting the premise that biochemical and molecular changes (including upregulation of VEGF-A) precede damage to the retinal vasculature. Early biochemical changes resulting in VEGF-A elevation are clinically undetectable yet critical in disease pathogenesis. This study is focused on describing a novel mechanism, whereby hyperglycemia can induce VEGF-A production in a preclinical DR model.

Chronic systemic hyperglycemia, the defining feature of diabetes, has been shown to increase VEGF-A protein and transcript in many retinal cell types in the absence of hypoxia,^{12–16} and the severity and progression of DR is tightly associated with glycemic control.¹⁷ Therefore, glucose-dependent transcriptional dysregulation of VEGF-A is a likely mechanism contributing to DR development. Vascular endothelial growth factor-A is transcriptionally modulated by several different transcription factors.¹⁸ Hypoxia-inducible factor 1 (HIF-1) is known to be essential for hypoxia-induced

VEGF-A upregulation. This relationship has been extensively investigated in the eye through studies of retinal development and retinopathy of prematurity.¹⁹ HIF-1 is also heavily implicated in later stages of DR, when vessel occlusion and ischemia are present.^{20,21} These events result in induction of VEGF-A by hypoxia. This hypoxia-driven VEGF-A is distinct from hyperglycemia-driven VEGF-A, which has been shown to occur prior to retinal ischemic insult.¹¹

The transcription factor specificity protein 1 (Sp1) is known to regulate basal VEGF-A expression.^{18,22} Not surprisingly, it is also linked to excessive VEGF-A production in cardiovascular disease, and pancreatic, gastric, prostate, and colon cancer.^{23–25} Sp1 is a member of the Krüppel-like factor family, and has a DNA-binding domain containing three C₂H₂-type zinc-finger motifs. These zinc-fingers bind the GC-rich consensus sequence GGCGGG.²⁶ The proximal promoter of human VEGF-A contains four Sp1 binding sites: –238/–233, –94/–89, –84/–79, and –57/–52.¹⁸ Sp1 activity is tightly regulated in many ways, including by posttranslational modifications (PTM). Sp1 PTMs include phosphorylation, acetylation, ubiquitination, glycosylation, and more.²⁶ These PTMs control Sp1 activity, stability, degradation, signaling, and localization.^{27–31} Sp1 is particularly highly glycosylated with the glucose-derived sugar moiety O-linked β ,N-acetylglucosamine (O-GlcNAc).^{27,29} The O-GlcNAc modification is a regulatory and reversible signal (analogous to phosphorylation) that cycles rapidly in response to cell cycle and glucose concentration. Glycolysis directly fuels the hexosamine biosynthetic pathway (HSP), which generates a donor molecule that is O-linked by the enzyme O-GlcNAc transferase (OGT) to serine and threonine residues on target proteins.³² The consequences of this modification for VEGF-A regulation remain unclear. O-GlcNAc transferase is the only enzyme that catalyzes this modification, and O-GlcNAcase (OGA) is the only enzyme that removes it, allowing O-GlcNAc to be tightly controlled and relatively easily studied.^{33,34} Elevation in ambient glucose (as in diabetes) increases flux through the HSP, promoting overall protein O-GlcNAcylation.³⁵ This phenotype is seen in vitro, in the tissues of diabetic animals, and in vivo in the endothelial cells and kidneys of type II diabetic patients.^{36–40}

O-GlcNAcylation of Sp1 has been shown to increase its stability, localization to the nucleus and transcriptional activity.^{30,41–43} Many groups have demonstrated the enhanced transcriptional activity of Sp1 under diabetic conditions, and it has been previously implicated in diabetic complications.^{30,42–46} In this study, we use an in vitro model of preclinical DR to show that hyperglycemia upregulates VEGF-A through increased O-GlcNAcylation and transcriptional activity of Sp1.

MATERIALS AND METHODS

Cells

Human retinal pigment epithelial cells (ARPE-19; ATCC, Manassas, VA, USA)⁴⁷ and rat retinal microendothelial cells (TR-iBRB; kind gift from Peter Kador at the University of Nebraska Medical Center, Omaha, NE, USA)⁴⁸ were cultured in Dulbecco's modified Eagle's medium (DMEM; Lonza, Basel, Switzerland) containing 1 g/L glucose, 10% FBS (Gemini, West Sacramento, CA, USA), 110 mg/mL sodium pyruvate (Sigma-Aldrich Corp., St. Louis, MO, USA), 0.2 mg/mL penicillin, 60 μ g/mL streptomycin, 4 mM L-glutamine, 50 nM insulin, and 15 ng/mL endothelial cell growth factor (TR-iBRB only; Sigma-Aldrich). TR-iBRB were grown on plates coated with rat tail tendon collagen type I (Sigma-Aldrich). Cells were incubated at 37°C in a humidified atmosphere of 5% CO₂. The HEK293T viral packaging cell line was grown in DMEM containing 10%

heat-inactivated FBS, 2 mM L-glutamine, 110 mg/mL sodium pyruvate, and Pen-Strep at 37°C in a humidified atmosphere of 5% CO₂.

All transduced cells were selected for approximately 3 to 4 days after transduction using 10 mg/mL puromycin, 20 mg/mL neomycin (G418), or 5 mg/mL blasticidin. Cells harboring the tet-inducible shRNAs were clonally selected; clones showing greatest protein depletion were used. To induce tet-on shRNA expression, 10 μ g/mL of doxycycline (Sigma-Aldrich) was used. Cells were exposed to doxycycline for 72 hours before experimentation. Doxycycline was replaced every 48 hours for the duration of the experiment.

Drugs and Treatments

Cells were treated with O-GlcNAcase inhibitors Thiamet-G (Sigma-Aldrich), or NButGT⁴⁹ (a generous gift from David Vocadlo, Simon Fraser University, Burnaby, BC, Canada). Thiamet-G was dissolved in DMSO and NButGT in PBS. O-GlcNAc transferase inhibitor Ac5sGlcNAc⁵⁰ (a gift from David J. Vocadlo, PhD) was dissolved in dimethyl sulfoxide.

Enzyme-Linked Immunosorbent Assay (ELISA)

Cells were treated for the desired amount of time, and 1 mL of medium was collected and stored at –80°C until use. Medium was brought to room temperature for ELISA assay. The manufacturer's protocol for the human VEGF Quantikine ELISA kit (R&D Systems, Minneapolis, MN, USA) was followed. Samples were visualized using a TECAN plate reader (TECAN, Männedorf, Switzerland) at the recommended wavelength. Samples were assayed in duplicate and measured in duplicate; raw values were averaged. Experiments were combined and normalized to the 5 mM glucose control to show fold changes in extracellular VEGF-A.

Constructs

pLKO-shRNA vector for Sp1 knockdown was purchased from Sigma-Aldrich. The pLKO-shRNA vector for OGT knockdown was generously provided by Mauricio Reginato (Drexel University College of Medicine, Philadelphia, PA, USA). A nontargeting (NT) sequence in the same pLKO backbone was used as a control. Tetracycline-inducible pLKO vectors were constructed as previously described.⁵¹ Constructs containing the VEGF-A promoter spanning –2018/+50, –66/+50, and –52/+50 bp were kind gifts from Stephen Safe (Texas A&M University College of Veterinary Medicine, College Station, TX, USA).⁵² The –2018/+50 bp promoter was excised from its original backbone using SalI and BsaWI and cloned into a lentivirally packageable promoterless luciferase construct (Addgene #19166) originally generated by Eric Campeau (University of Massachusetts Medical School, Worcester, MA, USA). This VEGF-A promoter fragment was ligated into the luciferase vector multiple cloning site at XhoI and AgeI. The two smaller promoter fragments, –66/+50 bp (containing an Sp1 binding site) and –52/+50 bp (excluding Sp1 binding sites) were excised using KpnI and NheI. These fragments were ligated into a modified pUC18 construct, excised with AgeI and XhoI, and ligated into the promoterless luciferase vector via the same restriction endonuclease sites. All three of these constructs were sequence-verified prior to use. All vectors were packaged as described.

Lentivirus Production

HEK293T cells were transfected with 10 μ g of plasmid using GenDrill transfection reagent (BamaGen, Gaithersburg, MD,

FIGURE 1. Hyperglycemia increases pan-cellular O-GlcNAc and VEGF-A in ARPE-19 and TR-iBRB cells. ARPE-19 (A, C) and TR-iBRB (B, D) cells were exposed to high glucose (25 mM) or normal glucose (5 mM) as a control. Protein lysates and total RNA samples were collected at the indicated timepoints. (A, B) Western blotting was performed using antibodies specific for VEGF-A, O-GlcNAc modification, or nucleolin, a housekeeping gene. *Graphs* show densitometry analysis of Western blots for VEGF-A normalized to nucleolin loading control. *Error bars* show ± 1 SEM. (C, D) Quantitative RT-PCR was used to analyze samples with verified primers for VEGF-A; 18s rRNA was used as a reference gene. Data were processed by the $\Delta\Delta C_t$ method, and 25 mM glucose values were normalized to corresponding 5 mM glucose values, *error bars* indicate ± 1 SEM. $n = 3$ independent experiments for all.

USA) following manufacturer's instructions, along with viral packaging vectors pCMV-VSV-G, pRSV-Rev, and pMDLg/pRRE,⁵³ which were kindly provided by Mauricio Reginato. Virus was collected 48 hours after transfection, filtered, and stored at -80°C until further use.

Chromatin Immunoprecipitation

The protocol described by Beishline et al.⁵⁴ was followed closely. Briefly, ARPE-19 cells were grown to confluence on 15-cm dishes and exposed to 5 or 25mM glucose for 72 hours. Cells were fixed, and collected in PBS. Samples were lysed in chromatin immunoprecipitation (ChIP) buffer (50 mM Tris [pH7.5], 5 mM EDTA, 150 mM NaCl, 0.5% NP-40, 1% Triton X-100, and protease and phosphatase inhibitors) and sonicated to shear DNA. Protein was normalized after bicinchoninic acid (BCA) assay, and 20% of the sample was reserved for DNA isolation for input. The remaining sample was used for overnight immunoprecipitation with beads pre-conjugated with Sp1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or isotype control. Samples were then washed 5 $\times$ with ChIP buffer and purified for DNA with Chelex-100 resin. Fold induction of binding was calculated using the $\Delta\Delta C_t$ method, in which untreated and treated IP sample values are normalized to the difference in input DNA. Threshold cycle (C_T) values for control immunoprecipitations and dihydrofolate reductase control primers were used to set gates for background amplification.

FIGURE 2. Elevation of pan-cellular O-GlcNAc is sufficient to upregulate VEGF-A. ARPE-19 and TR-iBRB cells cultured in 5 mM glucose were exposed to O-GlcNAcase inhibitor 150 μM NBuGT (A) for 72 hours. (B, C) ARPE-19 were exposed to 50 μM Thiamet-G for the indicated amount of time. Protein lysates were collected and processed. (A–C) Western blotting was performed for VEGF-A, O-GlcNAc modification, or nucleolin. (C) Densitometry of the Western blots for Thiamet-G-treated ARPE-19 cells. *Error bars* show ± 1 SEM. $n = 3$ independent experiments.

qRT-PCR

RNA was isolated from cells with Qiagen RNeasy Kit (QIAGEN, Hilden, Germany). Quantitative PCR was performed with iTaq Universal SYBR Green Supermix (Bio Rad, Hercules, CA, USA) using the CFX 96-real time PCR detection system (Bio Rad). Fold changes were calculated from raw data using a modified $\Delta\Delta C_T$ method.⁵⁵ Relative fold changes were produced by normalizing experimental fold change to the fold change of the respective control. The following primer sequences were used in this study:

1. VEGF-A (rat)⁵⁶ F: tgcacccacgacagaaggga R: tcaccgcctt ggcttgcatcat
2. β -Actin (rat)⁵⁷ F: gtccaccttcacgacatg R: ctcaagtaacagt ccgcctag
3. VEGF-A (human)⁵⁸ F: gctactgccatccaatcgag R: ctctcctatgt gctggcctt
4. 18s rRNA (human)⁵⁹ F: gtaaccgggtgaacccatt R: ccataccaa tcgtagtagcg
5. VEGF-A promoter (human)⁶⁰ F: ggctgagcttcccttca R: gat cctcccgcctaccag

Antibodies and Western Blotting

Protein lysates were collected in Laemmli buffer (12.5 mM Tris [pH 6.8], 20% glycerol, 4% [wt/vol] SDS), and protein concentration was assessed by BCA assay. For a standard SDS-PAGE, 5 to 12 μg of protein was used. Blots were transferred to

FIGURE 3. O-GlcNAc transferase activity is necessary for glucose-driven VEGF-A production. (A) ARPE-19 cells were exposed to 5 or 25 mM glucose and 50 μ M Ac5sGlcNAc or vehicle for the indicated amount of time. Protein lysates were collected and processed by Western blotting. *n* = at least 2 independent experiments. *Graph* shows densitometry analysis of Western blots. Error bars show ± 1 SEM. *n* = at least 2 independent experiments. (B) ARPE-19 cells were cultured in 5 mM glucose, 25 mM glucose and DMSO, or 25 mM glucose and OGT inhibitor Ac5sGlcNAc (50 μ M) for 72 hours. Protein lysates were collected and analyzed by Western blotting. (C) Medium was collected from ARPE-19 cells treated for 72 hours with the indicated treatments (vehicle or Ac5sGlcNAc at 50 μ M). Medium was assayed for VEGF-A by ELISA. Samples were normalized to the 5 mM glucose control. Fold change of 1 indicates 2365.5 pg/mL of extracellular VEGF-A. Error bars show ± 1 SEM. *n* = 2 independent experiments. (D, G) ARPE-19 cells or TR-iBRB expressing nontargeting shRNA or shRNA against OGT were exposed to 5 mM or 25 mM glucose for the indicated amount of time. Knockdown was confirmed by Western blot prior to experiment (Supplementary Fig. S3). Protein lysates were collected and processed by Western blotting. *Graphs* show densitometry analysis of Western blots. Error bars show experimental values normalized to control. Error bars

show ± 1 SEM. (D) $n =$ at least 3 independent experiments. (G) $n = 2$ independent experiments. (E) ARPE-19 cells expressing shNT or shOGT were treated for 72 hours with the indicated treatments. Medium was collected from and assayed for VEGF-A by ELISA. Samples were normalized to the 5 mM glucose control. Fold change of 1 indicates 3379 pg/mL of extracellular VEGF-A. Error bars show ± 1 SEM. $n = 3$ independent experiments. (F) ARPE-19 cells expressing shNT or shOGT were exposed to 5 or 25 mM glucose for indicated timepoints. Samples were processed for qRT-PCR; VEGF-A was the target gene, 18s rRNA was used as a reference. $n = 2$ independent experiments.

polyvinylidene fluoride (PVDF) membrane at 60 V for 2 hours, blocked in 5% BSA in tris-buffered saline with Tween-20 (TBST), and probed with primary antibody overnight at 4°C. Primary antibodies were VEGF-A (Rockland, Gilbertsville, PA, USA; Abcam, Cambridge, United Kingdom), O-GlcNAc (Novus, Littleton, CO, USA), Sp1 (pAb581),⁶¹ OGT (Sigma), and Nucelolin (Santa Cruz Biotechnologies, Dallas, TX, USA). Immunodetection was performed using LI-COR infrared imaging (LI-COR, Lincoln, NE, USA) or horse-radish peroxidase, via film. Western blot bands were quantified using ImageJ software (<http://imagej.nih.gov/ij/>; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA). Each band was measured twice and raw values were averaged. All bands were normalized to their respective loading controls and normalized to the untreated control sample. Fold change values from independent experiments were combined to produce graphs shown in figures.

Immunoprecipitation

The immunoprecipitation protocol was adapted from a previously described method.⁵⁴ ARPE-19 cells were treated with 5 or 25 mM glucose for 72 hours before being washed twice with PBS on ice and collected in 500- μ L cold TGN buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 1% Tween 20, 0.3% NP-40, and protease and phosphatase inhibitors). Cells were disrupted 5 $\times$ with a tuberculin syringe and then sonicated twice at 40% duty for 10 seconds on ice. Protein was quantified

and 1.8 to 2.5 mg of protein lysate was used for each IP. A total of 10% of the lysate was saved for input. Lysates were immunoprecipitated with pre-conjugated Sp1 beads (Santa Cruz Biotechnologies, Dallas, TX) that had been pre-blocked for 1 hour with 1 mg/mL BSA and incubated overnight at 4°C. Beads were washed twice with TGN buffer and twice with Tris-buffered saline (TBS). Protein was eluted with Laemmli buffer and assessed by Western blotting as described.

Statistical Analysis

Statistical significance was determined using two-tailed Student's *t*-test. *P* values are indicated by nonsignificant ($P > 0.05$), * ($P < 0.05$), ** ($P < 0.01$), or *** ($P < 0.001$). Data without an explicit indication of statistical significance should be considered to have a *P* value greater than 0.05.

RESULTS

Hyperglycemia Increases Pan-Cellular O-GlcNAcylation and VEGF-A in ARPE-19 and TR-iBRB Cells

It has been convincingly demonstrated by many investigators that hyperglycemia increases VEGF-A protein in several retinal cell types.^{12–16} Separately, it has been shown that hyperglycemia elevates protein modification by O-GlcNAc.^{36,62} We observed both results in monolayers of ARPE-19 and TR-iBRB. Cells were exposed to 5 mM (control) glucose or 25 mM (high) glucose for 24, 48, and 72 hours. To mimic the serum glucose level of an uncontrolled diabetic, 25 mM glucose was chosen. Mannitol is frequently used as an osmotic control for D-glucose; we saw no changes in VEGF-A in response to treatment with 20 mM mannitol with 5 mM glucose (Supplementary Fig. S1). As expected, 25 mM glucose treatment increased total protein O-GlcNAcylation (Fig. 1). Samples from the same experiment showed a small, but statistically significant, and highly reproducible increase in VEGF-A protein in both cell lines (Figs. 1A, 1B). We next sought to determine whether VEGF-A transcript was also elevated by hyperglycemia. RNA was collected from both cell lines under identical conditions, and qRT-PCR analysis revealed an elevation in VEGF-A transcript (Figs. 1C, 1D). Increases in mean normalized VEGF-A transcript were statistically significant at 72 hours for both cell lines. The intracellular levels of VEGF-A also correspond in magnitude to those published in other reports.^{15,16,63} Since VEGF-A is a secreted protein, we have performed ELISA using conditioned medium from ARPE-19 cells, and determined that VEGF-A secretion is increased by 50% in response to 25 mM glucose, as discussed in the next section.

Increased O-GlcNAc Modification is Sufficient to Elevate VEGF-A

To explore the connection between protein O-GlcNAcylation and VEGF-A, we used small-molecule inhibitors of the OGA enzyme. O-GlcNAcase inhibitors NButGT and Thiamet-G prevent the removal of O-GlcNAc modification from proteins, effectively increasing O-GlcNAc levels without hyperglycemic

FIGURE 4. Hyperglycemia activates the proximal VEGF-A promoter through transcription factor Sp1. ARPE-19 cells were transduced with lentiviruses containing three different successive truncations of the human VEGF-A promoter driving the luciferase gene. Selected cells were then cultured in 5 or 25 mM glucose for 72 hours. (A–C) Protein lysates were collected and BCA assayed; normalized protein samples were analyzed for luminescence. Bars show raw luminescence values from high glucose samples normalized to their respective low glucose controls, ± 1 SEM. For (A) $n = 5$, (B) $n = 12$, and (C) $n = 6$ independent experiments. (D) Chromatin immunoprecipitation for Sp1 was done after 72 hours of 5 or 25 mM glucose treatment in ARPE-19 cells. –199/+3 of the proximal VEGF-A promoter was analyzed by verified primers using qRT-PCR, the DHFR promoter was used as reference gene. Triplicate raw values were processed by the $\Delta\Delta C_t$ method. Error bars show ± 1 SEM; $n = 3$ independent experiments.

FIGURE 5. Sp1 is required for VEGF-A production driven by glucose and O-GlcNAc. (A, B) ARPE-19 cells were depleted of Sp1 by tetracycline-inducible shRNA expression, control cells expressed a NT shRNA. Sp1 knockdown was verified prior to experiments. Sp1 knockdown cells were exposed to 5 or 25 mM glucose for the indicated amount of time and processed for Western blotting or qRT-PCR. Densitometry was performed for Western blots. Error bars show experimental values normalized to control values; error bars indicate ± 1 SEM. (C) ARPE-19 cells expressing shNT or shSp1 were treated for 72 hours with the indicated treatments. Medium was collected and assayed for VEGF-A by ELISA. Samples were

normalized to the 5 mM glucose control. Fold change of 1 indicates 3379 pg/mL of extracellular VEGF-A. *Error bars* show ± 1 SEM. $n = 2$ independent experiments. (D) Nontargeting or Sp1 knockdown ARPE-19 cells were exposed to the O-GlcNAcase inhibitor Thiamet-G (50 μ M) or vehicle for the indicated amount of time; protein lysates were collected for Western blotting. (E) Immunoprecipitation of Sp1 was performed using ARPE-19 cells exposed to 5 or 25 mM glucose for 72 hours. Nonspecific isotype and species-matched IgG was used as a control. Samples were analyzed by Western blot using antibodies against Sp1 or the O-GlcNAc modification. $n = 3$ independent experiments for (A, B). $n = 2$ independent experiments for (C, D).

treatment. Cells exposed to either of these inhibitors (NButGT at 72 hours, or Thiamet-G at 24, 48, and 72 hours) exhibit a concomitant increase in O-GlcNAc and VEGF-A protein (Figs. 2A–C). Figure 2B is a positive control for the Thiamet-G as an OGA inhibitor. Thiamet-G-treated ARPE-19 cells showed a statistically significant increase in VEGF-A production at 72 hours post treatment. A similar trend was observed in TR-IBRB cells (Supplementary Fig. S2).

O-GlcNAc Modification by OGT is Critical for Hyperglycemic Induction of VEGF-A

To assess the importance of O-GlcNAc modification of proteins in the pro-angiogenic effect of hyperglycemia, we used the small molecule inhibitor Ac5sGlcNAc to inhibit the OGT enzyme. In the presence of hyperglycemia in ARPE-19, 50 μ M Ac5sGlcNAc effectively reduced protein O-GlcNAcylation (Fig. 3B). ARPE-19 conditioned medium was assessed by ELISA for extracellular VEGF-A at 72 hours post treatment. Results showed a statistically significant increase in VEGF-A in response to 25 mM glucose. This induction was completely abrogated by the OGT inhibitor Ac5sGlcNAc (Fig. 3C). The Ac5sGlcNAc inhibitor also prevented intracellular glucose-induced VEGF-A expression in ARPE-19 cells at 72 hours. (Fig. 3A). To further test the role of OGT, ARPE-19 cells were depleted of the OGT enzyme by shRNA. An NT shRNA sequence was expressed as a control. Western blot verification and densitometry analysis showed a 93% OGT knockdown (Supplementary Fig. S3). O-GlcNAc transferase-depleted ARPE-19 cells do not show hyperglycemia-induced upregulation of VEGF-A protein after a 72 hour exposure, similar to the effect of OGT inhibition (Fig. 3D). Both OGT knockdown and OGT inhibition by Ac5sGlcNAc showed statistically significant reductions in VEGF-A at 72 hours post 25-mM glucose treatment. A similar trend was observed for OGT-depleted TR-IBRB cells (45% knockdown efficiency; Supplementary Fig. S3, Fig. 3G). Transcript data in OGT-depleted ARPE-19 cells are statistically significant at the 72-hour timepoint (Fig. 3F). Medium collected from shNT or shOGT expressing ARPE-19 cells showed a significant abrogation of extracellular VEGF-A after 72 hours (Fig. 3E). Notably, OGT inhibition and knockdown do not fully abrogate all VEGF-A expression; remaining low levels of VEGF-A transcript and protein are highly reproducible. By contrast, control cells, including those expressing the NT shRNA, were able to upregulate VEGF-A in response to 25 mM glucose (Figs. 3A, D–G). With these two forms of OGT interference, we show that O-GlcNAcylation of proteins is necessary in RPE and retinal endothelial cells for upregulation of VEGF-A by hyperglycemia.

Hyperglycemia Influences VEGF-A Promoter Activity Through the Transcription Factor Sp1

The VEGF-A promoter was analyzed for glucose-responsive activity by luciferase assay in the ARPE-19 cell line, which was chosen because we wished to study the human VEGF-A promoter specifically in human cells. Cells were transduced with a lentivirus construct containing the luciferase gene driven by $-2018/+50$ bp of the proximal VEGF-A promoter (Fig. 4A), or a truncated version of the promoter ($-66/+50$ bp)

that includes one of the three clustered Sp1 binding sites (Fig. 4B). A minimal promoter, $-52/+50$ bp (excluding Sp1 sites) was used as an experimental control (Fig. 4C). Cells harboring these integrated constructs were clonally selected. The resultant cell lines were exposed to 5 or 25 mM glucose for up to 72 hours. Assay results show a small but statistically significant and highly reproducible increase in luminescence with the $-2018/+50$ and $-66/+50$ bp VEGF-A promoter constructs. The minimal control promoter showed no change in luciferase expression in response to hyperglycemia. These data indicate that the promoter retained its glucose-sensitivity when Sp1 sites were included, and lost its glucose-sensitivity when Sp1 sites were excluded. Data are presented as the normalized mean of multiple experiments, performed in duplicate and measured in triplicate. Notably, the minimal promoter not only lost its glucose sensitivity, but also had an approximately 100-fold lower luminescent signal compared to the $-2018/+50$ bp proximal promoter. Luciferase production from the Sp1 site-containing truncation was approximately one-half that of the $-2018/+50$ bp promoter region (data not shown). This can be attributed to the elimination of binding sites for various activators (including 2 Sp1 sites) that were included in the $-2018/+50$ bp proximal promoter region. Although the Sp1 site-truncation had diminished luciferase activity, it retained its sensitivity to glucose, which was the critical parameter of interest. The minimal promoter not only lost its ability to respond to hyperglycemia, but also had very significantly decreased basal activity.

To further explore the possibility that Sp1 was the responsible factor in this phenotype, we used ChIP to analyze Sp1 occupancy of the proximal VEGF-A promoter (Fig. 4D). ARPE-19 cells exposed to 25 mM glucose for 72 hours showed a 64% increase in Sp1 binding to the proximal VEGF-A promoter ($-199/+3$ bp). Taken together, the promoter analysis and ChIP results demonstrate that the Sp1 sites in the proximal VEGF-A promoter are necessary for glucose sensitivity of the promoter, and that occupancy of the Sp1 sites in the VEGF-A promoter is increased by hyperglycemia.

Sp1 Mediates Glucose and O-GlcNAc-Driven VEGF-A Production in RPE Cells

To address the role of Sp1 in the O-GlcNAc-mediated induction of VEGF-A, we used ARPE-19 cells depleted of Sp1 by shRNA. Control cells expressed a NT shRNA sequence. Since Sp1 is a critical transcription factor for cell viability, the shRNA against Sp1 was designed to be tetracycline-inducible. Transduced and selected cells were treated with doxycycline for 72 hours to allow for full knockdown of the factor prior to experimentation. Sp1 knockdown was verified by Western blot prior to experiments; densitometry analysis showed an 83% knockdown of Sp1 in ARPE-19 cells (Supplementary Fig. S3B). Cells depleted of Sp1 were exposed to 25 mM glucose for up to 72 hours, and Western blotting and qRT-PCR showed no increase in VEGF-A protein or mRNA. In contrast, cells expressing the NT shRNA showed upregulation of VEGF-A protein and mRNA in response to hyperglycemia, as previously observed (Fig. 5A). Changes in VEGF-A protein and transcript were statistically significant at the 72-hour timepoint (Figs. 5A, 5B). Extracellular VEGF-A, as measured by ELISA, showed that cellular depletion

FIGURE 6. Proposed model of Sp1-mediated glucose-driven VEGF-A production. Hyperglycemia increases ambient intracellular glucose, thereby increasing flux and output of the hexosamine biosynthesis pathway. The O-GlcNAc transferase enzyme catalyzes the addition of the donor molecule UDP-GlcNAc to target ser/thr residues on proteins. Transcription factor Sp1 is particularly highly O-glycosylated, increasing its stability, nuclear localization, and transcriptional activity. Hyperglycosylated Sp1 binds the proximal VEGF-A promoter and upregulates VEGF-A expression.

of Sp1 prevented glucose-sensitive VEGF-A production. However, Sp1 knockdown did not reduce VEGF-A secretion (Fig. 5C). This result is consistent with the abnormally high level of VEGF-A seen in the 5 mM Sp1 KD glucose control in Figure 5A. Nevertheless, both importantly show glucose insensitivity without Sp1. Knockdown of Sp1 also prevented the increase in VEGF-A induced by the OGA inhibitor Thiamet-G (Fig. 5D). Sp1 was immunoprecipitated from ARPE-19 cells after 72 hours of 5 or 25 mM glucose treatment. Western blotting shows that the Sp1 protein remains highly O-GlcNAcylated up to 72 hours after 25 mM glucose administration (Fig. 5E). Together with the luciferase and ChIP data, these results consistently implicate Sp1 as an O-GlcNAc-sensitive transcription factor critical in mediating hyperglycemic induction of VEGF-A in ARPE-19 cells.

DISCUSSION

It has been known for some time that elevation in ambient glucose concentration can upregulate VEGF-A in retinal cells. The data presented in this paper reveal a new mechanism, whereby hyperglycemia induces VEGF-A expression in retinal cells through an increase in Sp1 interaction with the VEGF-A promoter. We also demonstrate that pan-cellular elevation in the O-GlcNAc posttranslational modification plays a significant role in modulating this process, presumably via the highly O-GlcNAcylated transcription factor Sp1. We have included a schematic model of the proposed mechanism based upon the data presented in this report (Fig. 6). It is unclear exactly how O-GlcNAcylation modifies the activity and localization of Sp1. While this phenomenon has been described before,^{27,41,43,46} it has not been explored in relation to VEGF-A upregulation and overproduction in DR.

Retinal endothelial and pigmented epithelial cells, like many other cell types, tolerate Sp1 KD, likely as a result of a level of transcriptional redundancy with other members of the Sp family. The observed slight increase in basal VEGF-A protein in RPE cells depleted of Sp1 (Fig. 5C) was not anticipated. It may be the result of a general effect of Sp1 KD on the cells, such as stress induction. Important to this study however, the Sp1 KD cells do not upregulate VEGF-A in response to hyperglycemia.

Many different retinal cell types have been shown to upregulate VEGF-A in response to hyperglycemia. Although we chose to study the RPE and endothelial cells, we have also considered the participation of pericytes and Müller cells. Primary cells from all four of these retinal lineages have demonstrated glucose-driven VEGF-A production.^{12–15} Müller cells in particular have been extensively studied for their contribution to retinal VEGF-A in DR.^{11,21} It is very likely that RPE, endothelial, pericytes, and Müller cells are all important to preclinical VEGF-A production, as all of them have been implicated in DR pathogenesis. It would be interesting to investigate whether the OGT-mediated mechanism for VEGF-A upregulation holds true in these remaining retinal cell types and in vivo.

It is clear that Sp1 is a target for signal transduction through its many posttranslational modifications, which affect its transactivation and DNA binding activity.^{27,31,64} While some proteins are glycosylated and phosphorylated on the same residues, the relationship between phosphorylation and O-glycosylation is more complicated than competition for identical residues or simple steric hindrance. Their exact relationship in regard to Sp1 is yet to be elucidated, and is further obscured by the fact that Sp1 has 164 Ser and Thr residues and is hyperglycosylated on up to eight residues.²⁹ There are conflicting reports regarding the effect of O-GlcNAc on Sp1 localization and function. Tissue-specificity and variability in Sp1's phosphorylation/O-glycosylation status can very likely account for these differences. Sp1 is a ubiquitous transcription factor responsible for expression of housekeeping genes, where it clearly plays a role in recruiting the general transcription factors.⁶⁵ Sp1 is also capable of responding to various extra- and intracellular signals to modulate expression of many genes, including those involved in proliferation, cell cycle, DNA repair, and apoptosis.^{31,66,67} Therefore, dysregulation of Sp1 activity and function, as in the diabetic state, is potentially very problematic for the cell.

O-glycosylation of Sp1 is reported to have different outcomes depending upon where on Sp1 it occurs. N-terminal modification of Sp1 has been demonstrated to reduce its transactivation activity by preventing protein-protein interactions with critical transcriptional mediators, such as TFIID.⁶⁸ Conversely, in mesangial cells of the glomerulus, Goldberg et al.⁴⁶ showed that Sp1 activity was increased by OGA inhibition, while dominant negative OGT, RNAi against OGT, or OGA overexpression decreased the transcriptional activity of Sp1. These data correspond strongly with our findings in retinal cells. A few additional groups have identified Sp1 as having increased transcriptional activity under diabetic conditions. These include studies by Geraldès et al.³⁰ in a mouse model of DR, and by Du et al.⁴³ in an in vitro model of diabetes. In these reports, and others, hyperglycemia drove Sp1 to transcriptionally upregulate Shp-1 and PAI-1.^{42,46} Transforming growth factor- α and - β are also regulated by Sp1 and upregulated by hyperglycemia and glucosamine; however, Sp1 has not been causally linked to these changes.^{69–71} All of these proteins are involved in the pathogenesis of diabetic complications. Du et al.⁴³ used bovine endothelial cells to demonstrate that O-GlcNAc modification of Sp1 was the specific cause of its increased transcriptional activity, which is consistent with our findings. In relation to Geraldès et al.,³⁰ we used qRT-PCR to analyze Shp-1 transcript levels in our model; an increase in Shp-1 transcript would be consistent with a global increase in Sp1 transcriptional activity. In agreement with these reported results, we also found upregulation of Shp-1 transcript in our model (unpublished data). Many other genes that are regulated by Sp1 are linked to the pathogenesis of diabetic complications, including VEGFR-2/KDR and IGF-1 receptor, both of which are increased in the diabetic human eye.^{72–74} Other

relevant gene targets include leptin, insulin receptor, and the glucagon receptor.^{75,76} Leptin is of particular interest, as Sp1 has been shown to regulate its expression in response to insulin signaling, HSP flux, and increased O-GlcNAc modification.^{44,77} This may be interesting to assess in the future as the role of Sp1 is further characterized in the diabetic state.

Hyperglycosylation also affects the stability of Sp1. O-GlcNAcylated Sp1 has been shown to resist degradation by the proteasome.²⁸ Increased half-life of glycosylated Sp1 elevates the likelihood of interaction with its targets. It is unknown whether O-GlcNAcylation has any effect on the homo-oligomerization of Sp1, which is important for its trans-activity.⁷⁸ Sp1 is also transcriptionally self-regulated; it is unclear how O-glycosylation affects Sp1 expression in light of its resistance to degradation, and the effect of O-GlcNAc on its function. Interestingly, Yoshida-Hata et al.⁴⁵ found that Sp1 is transcriptionally upregulated and accumulates in epiretinal membranes of patients with DR within the membrane; Sp1 was colocalized with VEGF-A.

Sp1 activation by O-GlcNAcylation may also be related to the structure of the Sp1 consensus binding site. Many promoters, including the proximal VEGF-A promoter are purported to contain a G-quadruplex,⁷⁹ a three-dimensional formation generated from a GC-rich sequence that spontaneously produces secondary DNA structure. It is estimated that the majority (approximately 87%) of Sp1 binding sites overlap with G-quadruplexes. Sp1 binds these G-quadruplexes with high affinity via its zinc-finger motifs.⁸⁰ Interestingly, many of the residues on which Sp1 is O-GlcNAcylated fall within the conserved zinc-finger DNA binding regions. It is possible that hyperglycosylation of these closely apposed residues may influence the DNA-binding affinity of Sp1. Studies are currently in progress to understand the interaction between hyperglycosylated Sp1 and G-quadruplexes, as well as to systematically determine which residues on Sp1 are critical to this phenomenon.

O-GlcNAcylation and OGT have been implicated in the pathogenesis of many diseases including diabetes, Alzheimer's disease, various cancers, and AIDS.^{81–83} O-GlcNAc has also been explored in relation to cardiovascular protection, stress, neurodegeneration, and embryogenesis. This modification has emerged as a highly influential and intricately regulated nucleocytoplasmic signal.^{35,84} On a subcellular level, O-GlcNAc is involved in nearly every cellular process, making its regulation vital to proper cellular function.⁸⁵ In spite of its importance, and the growing interest in its study, there are no nanomolar range inhibitors of OGT currently available; however, several groups are dedicated to the search for an effective inhibitor, as OGT seems to be a promising therapeutic target for many diseases.^{86,87} In this investigation, inhibition of OGT with available small-molecule inhibitors or by RNAi prevented glucose-driven VEGF-A production without completely abrogating all VEGF-A expression. This is a critical detail, because low levels of VEGF-A are required for maintenance of the mature neuroretina.^{88,89} In fact, full blockage of VEGF-A, as with therapeutic antibodies against VEGF-A, can be detrimental.⁹⁰ The premise of using OGT as a therapeutic target for DR is obviously not centered solely on Sp1. There are hundreds of proteins known to be O-GlcNAcylated; the majority of these are transcription factors. Inhibition of OGT in the setting of high-glucose concentration would shield these influential proteins from dysregulation by inappropriate O-glycosylation. A thorough analysis of the effect of O-GlcNAcylation on additional transcription factors including p53, STAT5, PDX-1, NF- κ B, FOXO1, LXR, and c-Myc could increase the rationale for OGT inhibition as a therapy. Other significant regulatory proteins and enzymes such as AKT, PFK1, and synapsin I^{91–93} are also impacted by O-glycosylation. Modification to these

proteins can be stimulatory or inhibitory. O-GlcNAc is a dynamic modification driven by glucose flux, tight glycemic control and insulin therapy are primary ways to manage oscillating glucose concentrations, and therefore also output of the hexosamine biosynthetic pathway.

We have presented data to show that VEGF-A produced in preclinical DR could be attributed to Sp1 O-GlcNAcylation by an unknown mechanism. Vascular endothelial growth factor-A at this early stage is likely responsible for the vascular lesions seen in nonproliferative DR, namely microaneurysms from hyperproliferative endothelial cells, increased vessel permeability, and loss of blood-retina barrier integrity. In other words, this phenomenon may be one of the complex factors and cascades that initiate the process of vessel occlusion and breakdown, hypoxia, inflammation, and subsequent vessel proliferation. Vascular endothelial growth factor-A protein has been shown to be elevated in the eyes of diabetic patients without discernible signs of DR; OGT may be an attractive target to slow the onset of early disease by preventing the abnormal cellular signaling that occurs in a high-glucose environment.

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