High Glucose-induced Retinal Pericyte Apoptosis Depends on Association of GAPDH and Siah1*

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Diabetic retinopathy (DR) is a leading cause of blindness worldwide, and its prevalence is growing. Current therapies for DR address only the later stages of the disease, are invasive, and have limited effectiveness. Retinal pericyte death is an early pathologic feature of DR. Although it has been observed in diabetic patients and in animal models of DR, the cause of pericyte death remains unknown. A novel pro-apoptotic pathway initiated by the interaction between glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and the E3 ubiquitin ligase, seven in absentia homolog 1 (Siah1), was recently identified in ocular tissues. In this article we examined the involvement of the GAPDH/Siah1 interaction in human retinal pericyte (hrp) apoptosis. HRP were cultured in 5 mM normal glucose, 25 mM L- or D-glucose for 48 h (osmotic control and high glucose treatments, respectively). Siah1 siRNA was used to down-regulate Siah1 expression. TAT-FLAG GAPDH and/or Siah1-directed peptides were used to block GAPDH and Siah1 interaction. Co-immunoprecipitation assays were conducted to analyze the effect of high glucose on the association of GAPDH and Siah1. Apoptosis was measured by Annexin V staining and caspase-3 enzymatic activity assay. High glucose increased Siah1 total protein levels, induced the association between GAPDH and Siah1, and led to GAPDH nuclear translocation. Our findings demonstrate that dissociation of the GAPDH/Siah1 pro-apoptotic complex can block high glucose-induced pericyte apoptosis, widely considered a hallmark feature of DR. Thus, the work presented in this article can provide a foundation to identify novel targets for early treatment of DR.

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2 The abbreviations used are: DR, diabetic retinopathy; Siah1, seven in absentia homolog 1; hrp, human retinal pericytes; EC, endothelial cell; Z, benzoyloxy carbonyl.
Role of GAPDH/Siah1 Signaling in Pericyte Apoptosis

Although evidence from diabetic patients and animals suggests a pathogenic role for pericyte apoptosis, the cellular and molecular mechanisms contributing to the onset and progression of DR are not fully understood (5, 13). Pericytes are perivascular cells intimately associated with retinal capillaries; they promote blood-retinal barrier integrity, endothelial cell (EC) maintenance, and survival (14, 15). These beneficial trophic functions of pericytes cease upon their death, and this is hypothesized to lead to EC death and ultimately vasoregression (16–19).

Although glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is historically known for its role in glycolysis, other biologic functions have been ascribed to it, including regulatory functions related to cell death (20–22). A pro-apoptotic complex comprised of GAPDH and Siah1 has been identified and characterized in neuronal and non-neuronal cell types (23–26). Siah1 is an E3-ubiquitin-ligase involved in ubiquitination and proteasome-mediated degradation of proteins. This E3-ligase contains a N-terminal RING finger domain, responsible for proteolysis, and a substrate-binding domain (27). Nitric oxide (NO) has been shown to induce translocation of GAPDH from the cytosol to the nucleus; however, GAPDH lacks a nuclear location signal, and experiments have shown that this translocation event depends on an obligatory association with nuclear location signal-containing Siah1 (24, 27–29). This association stabilizes the typically short-lived Siah1, reducing its high turnover rate and increasing the ubiquitination and degradation of nuclear proteins (23, 30–32). Once in the nucleus, Siah1 degrades target proteins, such as N-COR, resulting in cellular instability and ultimately cell death (24, 33, 34). It has also been shown that nuclear GAPDH can cause cell death by acetylating CBP/300, which in turn causes activation of downstream targets including the pro-apoptotic p53 (35).

Because there is abundant evidence suggesting a role for retinal pericyte death in the pathogenesis of diabetic retinopathy, it is desirable to elucidate pro-apoptotic pathways in these cells under diabetes-relevant conditions. If these pathways can be identified and characterized, rational therapeutic strategies can be developed to block retinal pericyte death and retinal vasoregression. These strategies may offer distinct advantages over those that are currently being used for clinical treatment of DR. Therefore, in this study we investigated the GAPDH/Siah1 pathway in high glucose-treated human retinal pericytes, to determine whether it demonstrates pro-apoptotic activity in these cells. Thus, the work presented in this article can provide the necessary initial steps to developing therapeutics designed at the early stages of DR.

Experimental Procedures

**HRP Treatment**—Primary cultures of human retinal pericytes (hRP) (Cell Systems, Kirkland, WA) were seeded into tissue culture flask-coated with attachment factor (Cell Signaling, Danvers, MA). HRP were grown and cultured in Dulbecco’s modified Eagle’s medium normal glucose (5.5 mm DMEM 1X, Life Technologies) supplemented with 10% FBS, and cell growth supplements, including antibiotics (Lonza, Basel, Switzerland). All cultures were incubated at 37 °C, 5% CO₂ and 95% relative humidity (20.9% oxygen). Passages 5 to 7 were used for all experiments. HRP identity was confirmed by immunoreactivity of neuron glial 2 (EMD Millipore, Temecula, CA). At 80% confluence hRP were treated with 10% FBS medium containing normal D-glucose (5.5 mm), high D-glucose (25 mm Sigma), or L-glucose (25 mm Acros Organics, Geel, Belgium), which served as an osmotic control. For TAT-FLAG peptide treatment, 1 μM control peptide, 1 μM GAPDH peptide, and/or 1 μM Siah1 peptide was added to Hanks’ balanced salt solution (Life Technologies). GAPDH peptide competitively blocks the GAPDH binding site on Siah1 and the Siah1 peptide competitively blocks the Siah1 binding site on GAPDH. Peptide solution was incubated at 37 °C for 30 min before being added to each well. Cells were incubated with each peptide solution for 2 h before experimental treatments were added. In cases where peptides were used in combination, each original concentration was used for each peptide. Specific sequence identification of each TAT peptide can be found in Table 1. The N-terminal of each TAT-peptide is acetylated and the C-terminal is amidated; these modifications ensure proper cell entry and prevent degradation once inside the cell. A FLAG tag peptide sequence enables detection and quantification of these peptides (36, 37).

**HRP Transfection**—For siRNA transfection, hRP were cultured in 6-well dishes and 1 ml of fresh media was added to each well 30 min prior to treatment. For each well, 10 μM siRNA oligomers (negative control siRNA or Siah1-directed siRNA) (siRNA sequence identification sc-37495A, B and C, Santa Cruz, Dallas, TX), 9 μl of Targetec Solution A (Targeting systems, El Cajon, CA), and 18 μl of Virofect (Targeting systems) were added to 250 μl of Opti-MEM (Life Technologies) in a separate tube, and inverted between the addition of each reagent. Mixed reagents were incubated at 37 °C for 25 min before being added to cultured hRP. Cells were incubated with transfection reagents for 12 h, before being washed and treated with fresh media. Experimental treatments began 24 h post-transfection.

**Nuclear Fractionation and Western Blot Analysis**—HRP were treated as necessary. Cells were harvested using TrypLE Express (Life Technologies), and lysed using radioimmunoprecipitation assay (RIPA) buffer (Qiagen, Limburg, Netherlands). The NE-PER nuclear and cytoplasmic extraction reagents (Thermo Scientific, Nashville, TN) were used to separate lysates into cytosolic and nuclear fractions. Samples were equilibrated for total protein concentration, subjected to 10% SDS-PAGE, and gels were transferred to nitrocellulose membranes using the iBlot system (Life Technologies). Membranes were blocked in 5% milk (for β-actin (Thermo Scientific) and GAPDH (Abcam, Cambridge, UK) immunoblot) or 5% BSA (for Siah1 (Santa Cruz), H3 (Cell Signaling), and MEK (Cell Signaling) immunoblot) probed with appropriate primary antibody (anti-β-actin 1:3000, anti-GAPDH 1:1000, anti-Siah1 1:250, anti-Histone H3, and anti-MEK 1:750). Blots were then labeled with horseradish peroxidase-conjugated secondary antibodies diluted at 1:2000 (GAPDH, MEK, and Histone H3, anti-rabbit; Siah1, anti-goat; and β-actin, anti-mouse). MEK and Histone H3 served as cytoplasmic and nuclear fractionation control. β-Actin was used to determine total protein concentration. Membranes were incubated in Pierce ECL Western blotting substrate and developed using ChemiDoc MP.
HENS buffer and incubated with 1M methyl methanethiosulfate.

For 1 h at room temperature. Following this incubation, lactate dehydrogenase and additional cofactors were added as well for 2 h later.

Nitric Oxide Synthase Assay—

The manufacturer’s instructions were followed as stated in the kit manual. Briefly, samples were incubated with NAPDH and nitrate reductase solutions were followed as stated in the kit manual. Briefly, samples/antibody mixture at room temperature for 1 h. Beads were collected with a magnetic stand and eluted using 50 μl of 4× SDS-PAGE reducing sample buffer at 100 °C for 10 min. The immunocomplexes were then subjected to Western blot analysis. Siah1-depleted samples served as controls for total pulldown of Siah1 from each lysate. Independent quality control experiments were performed to validate efficiency of the Siah1 immunoprecipitation (data not shown).

Immunocytochemical Analysis—HRP were cultured on multiwell glass slides and cells were permeabilized with 0.1% Triton X-100 in PBS for 30 min and blocked with 1.5% BSA in PBST overnight at 4 °C. Cells were incubated with anti-GAPDH primary antibody (Abcam) overnight at 4 °C. After incubation with primary antibody (1:100), cells were washed and incubated with secondary antibody for 1 h at room temperature. Cells were then washed in PBST and 4,6-diamidino-2-phenylindole (DAPI) stain was applied (Sigma). Last, cells were washed and embedded using Fluorogel with Tris buffer (Electron Microscopy Science, Hatfield, PA) and examined by fluorescence microscopy (Olympus AX70, Tokyo, Japan).

Apoptosis Measurements—

All apoptosis measurements were taken after 72 h of appropriate treatment. Annexin V-FITC staining was one of the methods used to assay apoptosis. Briefly, cell pellets were resuspended in Annexin V binding buffer (Biolegend, San Diego, CA). Annexin V (Life Technologies) and 7-aminoactinomycin D viability stain (Biolegend) was added to each sample for 15 min at room temperature. Samples were quantified using flow cytometry analysis performed at Vanderbilt’s Flow Cytometry Shared Resource core laboratory. Apoptosis was also assayed by measuring caspase-3 enzymatic activity. Activity was quantified using the EnzChek Caspase-3 Assay Kit (Life Technologies). Samples were incubated with 7-amino-4-methylcoumarin-derived substrate, Z-DEVD-AMC, for 1 h. Fluorescence emission at 440 nm was measured 2 h later.

Nitrile Oxide Synthase Assay—

The manufacturer’s instructions were followed as stated in the kit manual. Briefly, samples were incubated with NAPDH and nitrate reductase solutions for 1 h at room temperature. Following this incubation, lactate dehydrogenase and additional cofactors were added as well for 20 min. Griess reagents R1 and R2 were added to each sample and absorbance (540 nm) was read using a plate reader 10 min later.

S-Nitrosylation Western Blot Kit—

Cells were lysed with HENS buffer and incubated with 1 M methyl methanethiosulfonate for 30 min at room temperature. 1 μl of iodo-Tandem Mass Tag labeling reagent was added to each sample as well as 1 M sodium ascorbate. Ultra pure water was used for negative controls instead of sodium ascorbate. Samples are reduced with 4× Laemmli sample buffer and analyzed via Western blot analysis. Anti-Tandem Mass Tag antibody was diluted in 5% milk (1:1000) and anti-mouse IgG-HRP-conjugated antibody was used as a secondary (1:20,000).

Statistics—Data were analyzed with commercial software (GraphPad Prism 6) using analysis of variance with Fisher’s LSD post hoc analysis. Values of p < 0.05 were considered statistically significant.

Results

High Glucose Increases Siah1 Protein Levels in hRP—

HRP were treated with normal glucose (5 mM d-glucose), osmotic control (25 mM l-glucose), or high glucose (25 mM d-glucose) for 48 h. Siah1 total protein increased 2-fold in cultures treated with high glucose compared with those treated with the osmotic control (p = 0.0136). There was no significant difference between osmotic control and normal glucose-treated cells (Fig. 1A). Quantification of three independent Western blots is demonstrated in Fig. 1B.
FIGURE 3. Inhibition of the GAPDH/Siah1 pathway with Siah1 siRNA prevents high glucose induced GAPDH/Siah1 association. Siah1 knock-down (KD) efficiency. A, Siah1 expression and B, protein levels are significantly reduced with 10 μM Siah1-directed siRNA oligomers. Expression levels are measured by RT-PCR and protein levels are measured by Western blot analysis. 10 μM Siah1 siRNA (C) inhibits high glucose-induced GAPDH/Siah1 association. Quantification of three independent experiments is demonstrated in D. Siah1 siRNA also inhibits GAPDH/Siah1 association in HRP nuclear fractions (E). IP, immunoprecipitates.

TABLE 1

| Name         | Sequence                                                                 | Function                           |
|--------------|--------------------------------------------------------------------------|------------------------------------|
| Control peptide | N terminal acetylation-YGRKKRRQRRRDYKDDDDK-C terminal amidation          | Positive cell entry control        |
| GAPDH peptide   | N terminal acetylation-YGRKKRRQRRRDYKDDDDKVIPELNGKTLTGMAFRVPTA-C terminal amidation | Blocks GAPDH binding site on Siah1 |
| Siah1 peptide   | N terminal acetylation-YGRKKRRQRRRDYKDDDDKGNLGINVTISMC-C terminal amidation | Blocks Siah1 binding site on GAPDH |

FIGURE 4. Inhibition of the GAPDH/Siah1 pathway with GAPDH/Siah1 blocking TAT-FLAG peptides prevents high glucose-induced GAPDH/Siah1 association. A, immunocytochemistry analysis of anti-FLAG (red) staining in hRP. Top left panel demonstrates hRPs cultured in control medium with no peptide treatment. This condition serves as a measure of background FLAG fluorescence. All four panels are stained with anti-FLAG antibody. Nuclei are stained blue with DAPI. Western blot analysis of anti-FLAG immunoprecipitates (IP). FLAG-BAP fusion protein is used as a positive control to confirm the functional integrity of anti-FLAG monoclonal antibody. B, cell viability assay of hRPs treated with a corresponding peptide. Cells were treated with 70% methanol for 30 min as a positive control (C). D, 1 μM GAPDH/Siah1 blocking TAT-FLAG peptides inhibits high glucose-induced GAPDH/Siah1 association. Quantification of three independent experiments is demonstrated in E.
High Glucose Increases the Association between GAPDH and Siah1 in hRP—HRP were treated with normal glucose (5 mM), high glucose (25 mM), or l-glucose (25 mM) for 48 h. Pulldown assays were performed and are described as follows: immunoprecipitation with anti-Siah1, followed by Western blot analysis of the immunocomplexes with anti-GAPDH, revealed a 1.5-fold increase in GAPDH/Siah1 association in high glucose-treated cells compared with those treated with the osmotic control ($p = 0.0292$) (Fig. 2A). Quantification of three independent Western blots is demonstrated in Fig. 2B.

Siah1 Knockdown and Site-specific Blocking Peptides Mitigate High Glucose-induced GAPDH/Siah1 Association—HRP were treated with normal, osmotic control, or high glucose plus 10 μM negative control siRNA, 10 μM Siah1-directed siRNA, 1 μM TAT-FLAG control, 1 μM TAT-FLAG GAPDH peptide, 1 μM TAT-FLAG Siah1 peptide, or 1 μM GAPDH + 1 μM Siah1 peptides. Pulldown assays were performed as described above. Knockdown efficiency and other quality control aspects of our siRNA experiments are shown in Fig. 3, A and B. Our GAPDH peptide was designed to block the GAPDH binding site on Siah1 and the Siah1 peptide was designed to block the Siah1 binding site on GAPDH (Table 1). High glucose significantly increased GAPDH/Siah1 association ($p = 0.0390$) and this association was significantly reduced by Siah1 siRNA ($p = 0.0461$) (Fig. 3, C and D). High glucose-induced GAPDH/Siah1 association is also increased in hRP nuclear fractions and nuclear accumulation can be blocked by treating cells with Siah1-directed siRNA (Fig. 3E). TAT peptide identification and cell toxicity is shown in Fig. 4, A–C. The GAPDH ($p = 0.0194$) or Siah1 peptide ($p = 0.0066$), or a combination of both ($p = 0.0146$), significantly inhibited high glucose-induced GAPDH/Siah1 association as well (Fig. 4, D and E).

High Glucose Increases GAPDH Nuclear Translocation in hRP, Siah1 siRNA, or GAPDH/Siah1-specific Peptides Block High Glucose-induced GAPDH Nuclear Translocation—After 48 h of treatment with normal glucose, osmotic control, or high glucose, cell lysates were prepared and separated into cytoplasmic and nuclear fractions. Each fraction was then subjected to GAPDH, MEK, and Histone H3 Western blot analysis. MEK and Histone H3 were used as control antigens to assess the purity of the cytoplasmic and nuclear fractions, respectively. High glucose treatment caused significant accumulation of nuclear GAPDH when compared with either normal glucose or osmotic control ($p = 0.0061$) (Fig. 5, A and B). Siah1 siRNA (10 μM) inhibited high glucose-induced nuclear accumulation of GAPDH ($p = 0.0227$) (Fig. 5, C and D). The GAPDH ($p = 0.0142$) or Siah1 peptide ($p = 0.0221$) or combination of both peptides ($p = 0.0100$) significantly inhibited high glucose-induced GAPDH nuclear translocation (Fig. 5, E and F). GAPDH nuclear translocation was also assayed by immunocytochemical analysis, which demonstrated that translocation was...
induced by high glucose (Fig. 6D) and this induction was inhibited by 1 μM GAPDH peptide (Fig. 6F).

**High Glucose Causes Human Retinal Pericyte Apoptosis by a GAPDH/Siah1-dependent Pathway**—HRP were treated with normal glucose, L-glucose, or high glucose for 48–72 h. Cell death is evident after 48 h of high glucose treatment and significantly increased after 72 h. Treatment with 25 mM D-glucose for 72 h resulted in a 3-fold increase in caspase-3 enzymatic activity, a common marker of apoptosis \((p < 0.0001)\) (Fig. 7A). High glucose exposure also caused a significant increase in Annexin V levels, another measure of apoptosis-specific cell death \((p < 0.0001)\) (Fig. 7B). Siah1 siRNA significantly blocked this high glucose-induced apoptosis \((p = 0.0009)\) (Fig. 7C). Furthermore, GAPDH and Siah1 blocking peptides inhibited high glucose-induced hRP apoptosis (Fig. 7D, control Peptide, \(p = 0.0019\); GAPDH peptide, \(p = 0.0090\); Siah1 peptide, \(p = 0.0053\)).

**Discussion**

It is widely recognized that high glucose results in retinal cell apoptosis, but the specific molecular mechanisms responsible for cell death remains under active investigation. Some mechanisms that are hypothesized to play a role in high glucose-induced retinal pericyte apoptosis include activation of nuclear factor-κB \((38)\), activation of protein kinase C-δ \((17)\), and increased expression of the polyol pathway \((39, 40)\). High glucose has also been shown to increase the amount of intracellular NO and superoxide levels, resulting in impaired endothelial cell function, mitochondrial dysfunctions, and ultimately pericyte apoptosis \((41, 42)\).

Other groups have shown that inhibiting GAPDH activity activates this high glucose-induced increase in reactive oxygen species \((43)\). Interestingly, it is hypothesized that NO-induced S-nitrosylation abolishes GAPDH catalytic activity suggesting a connection to studies showing an increase of GAPDH nuclear accumulation as a result of reactive oxygen species accumulation.
In accordance with previous work done in ocular and non-ocular systems, in this study we demonstrate that high glucose causes human retinal pericyte apoptosis and this is in part dependent on the association between GAPDH and Siah1. Our findings also demonstrate that dissociation of the GAPDH/Siah1 pro-apoptotic complex blocks high glucose-induced apoptosis widely considered a hallmark feature of DR. Annexin V staining and caspase-3 enzymatic activity are both common markers of apoptosis; therefore we suggest that high glucose specifically causes apoptotic cell death in human retinal pericyte cultures by an increase in GAPDH/Siah1 association (20, 44).

To inhibit the GAPDH/Siah1 pro-apoptotic pathway we employed the use of three antisense, non-overlapping Siah1-directed siRNA oligomers to block Siah1 expression. We show that blocking Siah1 using siRNA inhibits GAPDH nuclear translocation as well as high glucose-induced hRP apoptosis, suggesting that Siah1 is an essential player in high glucose-induced GAPDH nuclear translocation. To specifically examine the association between GAPDH and Siah1 as a result of high glucose treatment, we designed FLAG-TAT peptides to competitively block the reciprocal GAPDH and Siah1 binding sites. We also used a control peptide that lacks specificity for either GAPDH or Siah1 binding sites and is
used as a positive cell entry control. Using these inhibitors, we prevented high glucose-induced GAPDH nuclear accumulation and ultimately prevented high glucose-induced apoptosis. These results suggest that the association between GAPDH and Siah1, at least in part, mediates GAPDH nuclear translocation and cell death secondary to prolonged high glucose exposure.

Although there are no pharmacological agents available to specifically inhibit the association between GAPDH and Siah1, R-(-)-Deprenyl (Selegiline), is currently used as a treatment for Parkinson disease (45) and has been shown to be neuroprotective (45–49) and anti-apoptotic (49–52) and to inhibit the association between GAPDH and Siah1 (53, 54). Others have shown that GAPDH is a target of R-Deprenyl, and that R-Deprenyl prevents GAPDH nuclear translocation (55). Finally, R-Deprenyl has been shown to prevent high glucose-induced cell death and high glucose-induced nuclear GAPDH translocation in retinal glia (56). These studies suggest that inhibiting the association between GAPDH and Siah1 and ultimately preventing GAPDH nuclear translocation could be an effective method to prevent pericyte apoptosis during early stages of DR disease progression. It is also possible that R-Deprenyl, already in the clinic, will be useful in this regard, and we have begun to examine its effectiveness in high glucose-treated human retinal pericytes.

To our knowledge this study marks the first attempt to elucidate a role for the pro-apoptotic GAPDH/Siah1 in human retinal pericyte cell death. Based on previously published data demonstrating high glucose-induced nuclear accumulation of GAPDH (54) and the findings of the present experiments, we propose a model that is initiated by an increase in pericyte NO levels as the result of high glucose (Fig. 8A). This increase in NO leads to an increase in S-nitrosylated GAPDH (Fig. 8B) (57–59) facilitating association of GAPDH with Siah1 (24). Increased levels of Siah1 could also result from high glucose-induced Siah1 new protein synthesis. Yego and Mohr (54) reported an increase in Siah1 total protein as well as mRNA levels as a result of high glucose treatment in retinal glial cells. Thus, the association between GAPDH and Siah1 in turn results in nuclear translocation and accumulation of the complex in the nucleus, leading to cell death. We also suggest that in the retina this pathway may be specific to pericytes and glial cells (54), because high glucose does not cause significant up-regulation of Siah1 protein levels in human retinal microvascular endothelial cells or human dermal fibroblasts (Fig. 9). The proposed GAPDH/Siah1 model is illustrated in Fig. 10.

Although significant progress has been made in the understanding of DR pathogenesis, mechanisms that occur during early disease progression remain poorly understood. To develop therapeutics aimed at earlier stages of disease, better understanding of the mechanisms underlying early disease progression are warranted. Future in vivo studies will be necessary to evaluate the therapeutic potential of targeting the GAPDH/Siah1 pathway in early stages of DR. Future studies are also needed to further understand adverse side effects associated with inhibiting the retinal GAPDH/Siah1 signaling.

FIGURE 10. Proposed model of the pro-apoptotic pathway GAPDH/Siah1 in high glucose-induced human retinal pericyte apoptosis. Cell stress, such as high glucose, causes an increase in nitric-oxide synthesis (NOS) activity. This increase in NOS activity results in elevated cytosolic nitric oxide (NO), which causes S-nitrosylation of GAPDH. Nitrosylated GAPDH associates with Siah1, stabilizing the complex and facilitating its translocation to the nucleus. Once in the nucleus, Siah1 degrades target proteins and/or GAPDH undertakes other non-glycolytic functions resulting in cell instability and ultimately cell death. This model is an adaptation from Hara et al. (31).
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