Effects of hyperglycemia on the TGF-β pathway in trabecular meshwork cells

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Research Article

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Abstract

Background: Hyperglycemia, which can lead to apoptosis, hypertrophy, and fibrosis, induces hyperinflammation through inflammasome activation. A major cause of diabetic vascular complications is increased oxidative stress due to hyperglycemia. In order to elucidate the potential dual roles and regulatory signal transduction of TGF-β1 and TGF-β2 in human trabecular meshwork cells (HTMCs) in vitro, we established an oxidative cell model in HTMCs using 5.5, 25, 50, and 100 mM D-glucose-supplemented media and characterized the TGF-β-related oxidative stress pathway.

Methods: Further analysis was conducted to investigate oxidative damage and protein alterations in the trabecular meshwork caused by the signal transduction. This was done through a series of qualitative cell function studies, such as cell viability/apoptosis analysis, intracellular reactive oxygen species (ROS) detection, analysis of calcium release concentration, immunoblot analysis to detect the related protein expression alteration, and analysis of cell fibrosis to study the effect of different severities of hyperglycemia. We also illustrated the role of TGF-β1 and TGF-β2 in oxidative stress-induced injury by shRNA-mediated knockdown or stimulation with recombinant human TGF-β1 protein (rhTGF-β1).

Results: Results from the protein expression analysis showed that p-JNK, p-p38, p-AKT, TGF-β1, TGF-β2, and related SMAD family members were upregulated in HTMCs under hyperglycemia. In the cell functional assays, HTMCs treated with rhTGFβ-1 (1 ng/mL) and under hyperglycemic conditions showed higher proliferation rates and lower ROS and calcium levels than those with higher concentration or without rhTGFβ-1 treated. Furthermore, TGF-β1 and TGF-β2 were found to be associated with the activation of fibrosis-response proteins, α-SMA, collagen I, and laminin, which may lead to HTMC damage.

Conclusions: To summarize, mechanistic analyses in HTMCs showed that hyperglycemia-induced oxidative stress activated TGF-β1 along with its associated pathway: at low concentrations, TGF-β1 protects cells from antioxidation, whereas at high concentrations, it accumulates in the extracellular matrix, causing further HTMC dysfunction.

1. Introduction

According to the World Health Organization, diabetes mellitus (DM) is one of the four major non-communicable diseases, a risk factor for premature mortality, and the third leading cause of death (Vicas et al., 2020). The disease is caused by a pancreatic insulin secretion disorder or by the resistance of peripheral cells to insulin action. Hence, it is considered a metabolic condition that results in the disruption of glucose and insulin homeostasis. The global epidemic of insulin resistance is caused by hyperglycemia, followed by several different complications based on the form of DM. Hyperglycemia and metabolic imbalance, which lead to hyperlipidemia, inflammation, and ultimately oxidative stress, are the consequences of impaired insulin action (Maritim, Sanders, & Watkins, 2003). In addition, during long and ineffective antidiabetics treatments, elevated blood glucose levels contribute to numerous complications
linked to endothelial dysfunction, such as retinopathy, kidney failure, neuropathy, and macrovascular disease (Loghmani, 2005). Overproduction of reactive oxygen species (ROS), caused by hyperglycemia, potentially induces oxidative stress and stimulates the production of cytokines. Oxidative stress is a significant cause of complications associated with diabetes (Fiorentino, Priolotta, Zuo, & Folli, 2013). An imbalance between oxidants and antioxidants, such as NAD(P)H quinone dehydrogenase-1 (NQO1), heme oxygenase-1 (HO-1), catalase, superoxide dismutase, (SOD), and glutathione peroxidase (GPx) results in oxidative stress (Newsholme, Cruzat, Keane, Carlessi, & de Bittencourt, 2016; Turkmend, 2017). Increased pathogen invasion due to the hyperglycemia-induced oxidative stress disrupt the normal signaling of cells and contribute to hyperinflammation and apoptosis (Habib, 2013).

In addition, the generation of advanced glycemic end products (AGE) and activation of AGE-receptor under hyperglycemic conditions directly cause renal inflammation by increasing oxidative stress (Bakker et al., 2015). AGEs are heterogeneous compounds that accumulate in various human tissues during normal aging, particularly under hyperglycemic conditions (K. H. Huang et al., 2019; Ramis et al., 2019). Furthermore, caspases mediate apoptosis via the extrinsic and intrinsic pathway. In diabetes, ROS-related apoptosis is caused by an increase in the Bax/Bcl-2 ratio, which is associated with the extent of pro-apoptotic caspase-3 activation (Balasescu, Ion, Cioplea, & Zurac, 2015; Wagener, Dekker, Berden, Scharstuhl, & van der Vlag, 2009). ROS also stimulate protein kinase C (PKC), which activates the transcription of transforming growth factor-β (TGF-β) (Zhu, Usui, & Sharma, 2007). TGF-β is typically involved in fibroblast proliferation and stimulates alpha-smooth muscle actin (alpha-SMA), which in turn promotes the production of collagen and cellular hypertrophy. It also stimulates mesangial cell proliferation, which results in cellular fibrosis (S. Chen et al., 2001). This multifunctional cytokine has recently been reported to be strongly associated with fibrosis in the trabecular meshwork (TM) (Inoue-Mochita et al., 2018; Sakata et al., 2008; Verrecchia & Mauviel, 2007). A variety of polypeptides, including cytokines and growth factors, coordinate the tissue repair process. TGF-β also controls a broad range of essential biological activities, including cell formation, proliferation, apoptosis, migration, immune cell activity, transcription of extracellular matrix (ECM) proteins, and the contraction of collagen gel (Kita et al., 2007; Yao et al., 2019). Therefore, TGF-β plays a critical role in many fibrotic diseases and is also assumed to be essential to pathways associated with the pathology of proliferative vitreoretinal diseases (Montecchi-Palmer et al., 2017). Additionally, TGF-β activity is high in the eyes of patients with proliferative vitreoretinopathy and proliferative diabetic retinopathy and is implicated in intraocular fibrosis (Tellios et al., 2017; Tsukamoto, Kajiwara, Nada, & Okada, 2019). However, the downstream mediators of this effect are poorly understood.

Herein, we investigated the hypothesis that TGF-β pathways are involved in hyperglycemia-induced HTMC cellular oxidative stress and characterized the etiopathology of the diabetes. We further investigated whether TGF-β deficiency in TM cells causes intracellular ROS accumulation and analyzed the involved signal transduction pathways. We have shown that the activation of injurious signaling pathways by TGF-β and the resulting oxidative stress have a detrimental impact on normal cell processes. TM cells cultured under various glucose concentrations, along with shTGF-β-knockdown or the addition of recombinant TGF-β protein in the presence or absence of TGF-β protein were used as in
vitro models. Low TGF-β concentrations, which influence the antioxidative protection or delay of TM cell pathology and diabetes, were found to reverse certain deleterious effects.

2. Materials And Methods

Cell line and cell culture

HTMCs, which were isolated from the juxtacanalicular and corneoscleral regions of the human eye, were purchased from ScienCell Research Laboratories (Carlsbad, CA, USA). Cells were cultured in a 5% CO₂ incubator. The culture medium was trabecular meshwork cell medium (TMCM) supplemented with fetal bovine serum (FBS), trabecular meshwork cell production supplement, and penicillin/streptomycin solution at 37 °C. Cells were exposed to D-glucose at final glucose concentrations of 5.5, 25, 50, and 100 mM for various glucose concentrations (equivalent blood glucose concentrations of 100, 454, 910, and 1600 mg/dL, respectively). 25, 50, 100 mM glucose concentrations were equal to 2 h post-meal plasma glucose concentrations in diabetic patients and glucose concentrations in unregulated diabetic patients and were correlated with cultures subjected to 5.5 mM D-glucose for monitoring purposes (corresponding to fasting plasma glucose levels of diabetes-free control) (Candiloros et al., 1995; Jouven et al., 2005; Newsholme et al., 2016; Saydah et al., 2001). According to a previous study, mannitol was used to regulate osmolarity at differential glucose concentrations to prevent hyperosmotic stress at high glucose concentrations (Cai et al., 2002). The long-term hyperglycemia-state monolayer cultured cells were used for further study following treatment for at least 20 days.

For H₂O₂ sufficiency, 30% hydrogen peroxide was purchased from Sigma-Aldrich (Thermo Fisher Scientific Inc., Waltham, MA, USA). Before the treatment, we first prepared the solution with a final concentration of 20 mM in serum-free medium. The reagent was diluted to specific concentrations in serum-free medium before addition to the cells. After the duration time, we stopped the H₂O₂ treatment by washing twice with 1× PBS, followed by ROS reagent staining, an apoptosis assay, western blot sample preparation, or other functional assays.

Proliferation assay

The MTT solution was used to measure proliferation rates (USB Corp., Cleveland, OH, USA). The cells were seeded into 96-well plates at a density of 2 × 10³ cells per well. The medium was extracted after 24-h of adhesion (Day 1) and the cells were incubated in 100 μL of MTT solution (1 mg/mL) per well for 3.5 h in the dark at 37 °C. The supernatant was then extracted and each well was filled with 100 μL of dimethyl sulfoxide (DMSO). The absorbance at 570 nm was determined by a plate reader after the plates were shaken for 30 s to completely dissolve the insoluble purple formazan. The cell growth rate estimation for each group was measured on days 2, 3, and 4 using the same method. A standardized ratio normalized to day 1 was estimated for proliferation ability.

Cell viability assay
Using MTT solution (USB Corp., Cleveland, OH, USA), cell viability rate was detected. Cells were seeded into 96-well plates at a density of $8 \times 10^3$ cells per well. The media were discarded after 24 h of incubation and the cells were stimulated with various hydrogen peroxide concentrations and exposure durations for the specified time courses. The media containing hydrogen peroxide was removed and the cells were then further incubated in 100 μL of MTT solution per well (1 mg/mL) for 3.5 h at 37 °C in the dark. Then, the MTT solution was extracted and dimethyl sulfoxide (DMSO) was applied at 100 μL per well. To completely dissolve the insoluble purple formazan, the 96-well plates were shaken for 30 s, and the absorbance of each well was measured at 570 nm.

Detection of ROS

ROS generation was measured using aminophenyl fluorescein (APF; Goryo Chemical, Sapporo, Japan). To prepare the solution with a final concentration of 5 mM, 1 mg of APF powder was dissolved in 0.47 mL N, N-dimethylformamide (DMF). Before applying the reagent to the cells, PBS or serum-free medium was diluted to 5 μM. The cells were incubated in the dark for 15 min after the addition of APF, and then the fluorescence emission at 490 nm was measured at 515 nm after excitation by using Accuri CFlow® and CFlow Plus tools (BD Biosciences, San Jose, CA, USA) (Tamai et al., 2018). The intensity of APF fluorescence was quantified and the fluorescence shift percentage was normalized to that of the control (5.5 mM) group.

Intracellular calcium-level measurement

Fluo-8® dye was used to quantify intracellular free calcium (AAT Bioquest, Sunnyvale, USA). A total of 0.25 mg of Fluo-8® dye powder was dissolved in 0.48 mL of DMSO to prepare the solution with a final concentration of 5 mM. The reagent was diluted to 5 μM in PBS or serum-free medium prior to being added to the cells. After the addition of Fluo-8® dye solution, the cells were incubated for 20 min in the dark, and the fluorescence emission was estimated at 520 nm after excitation at 490 nm. To test the intensity of APF fluorescence, Accuri CFlow® and CFlow Plus tools were used (BD Biosciences). The fluorescence shift percentage was normalized to that of the control group.

Immunoblotting

The quantified protein samples were separated on a 12% gel and transferred to polyvinylidene difluoride (PVDF) membranes (Pall Corp., Port Washington, NY, USA). The primary antibodies were diluted 1:2000 after membranes were blocked with 5% (w/v) skimmed milk or bovine serum albumin in Tris-buffered saline with Tween-20 [TBST; 50 mM Tris, 150 mM NaCl, and 0.1% Tween-20 (v/v); pH 8.0] for 1 h and applied to the membranes that were incubated overnight at 48 h at 4 °C. The membranes were subsequently washed five times in TBST (10 min/wash) and then incubated in a TBST solution containing suitable horseradish peroxidase-coupled secondary antibodies (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) at a 1:10000 dilution for 1 h with gentle agitation. The membranes were then washed six times for 10 min at a time in TBST, and the analyzed proteins were visualized using an improved chemiluminescence (ECL) technique (Visual Protein Biotech Corp., Taipei,
Taiwan). The ImageJ program analyzed the data for densitometric quantification based on the ratio to the household protein (LDH) (Li et al., 2018).

**Immunofluorescence**

These cells were seeded on 12 mm coverslips (VWR International Corp., Radnor, USA) in 24-well plates at a density of $4 \times 10^4$ cells and incubated overnight. The cells were then set at 37 °C for 25 min with PBS containing 4% (v/v) paraformaldeyde (PFA) and washed with PBS three times. The coverslips were incubated in PBS containing 0.1% (v/v) Triton X-100 for 5 min to permeabilize the cell membrane. Coverslips were washed and blocked in PBS containing 5% (w/v) BSA for 1 h before incubation with phalloidin tagged with F-actin selectively binding fluorescent dyes diluted in 2.5% BSA/PBS for optimized length at a ratio of 1:40. The coverslips were washed three times with PBS, slipped onto glass slides with DAPI staining, and then deposited overnight at 4°C, using Prolong™ Antifade Mounting Medium (Thermo Fisher Scientific. Chemistry, Waltham, MA, USA). Cells were examined using a 200 M fluorescent microscope (Zeiss Axiovert, Carl Zeiss, Oberkochen, Germany). Pictures were exported using Zeiss Axioversion 4.0 as .zvi files and edited with the program Adobe Photoshop V.7.0.

**Treatment of cells with recombinant human TGF-β protein1 (rhTGF-β 1)**

The cells were treated in a serum-free medium for 24 h before being exposed to a 1 and 5 ng/mL rhTGF-β 1 protein 24-h or were left untreated in the Ctrl condition. Further data obtained were immunoblotted using whole-cell extracts in an NP40 cell lysis buffer or ROS identification and calcium content using fluorescence probe and MTT analysis.

**Statistical analysis**

Data and figures are presented as means ± standard error (SEM). Differences between study groups were tested using a paired Student's t-test or a two-way variance analysis (ANOVA). Statistical significance was set at $p < 0.05$. (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$)

3. Results

3.1. Hyperglycemia effects on HTMCs

To investigate the effects of hyperglycemia on HTMCs, different concentrations of glucose were added to the medium (5.5, 25, 50, and 100 mM glucose), and proliferation assays were performed on HTMC cell cultures at variable glucose concentrations after 3 weeks. As shown in Figure 1A, HTMCs with four different glucose concentrations were observed for 4 days. As a result, glucose levels decreased the rate of cell proliferation in a concentration-dependent manner. Moreover, the proliferation rate was markedly lower for cells with a higher glucose concentration (100 mM). ROS levels were identified by aminophenyl fluorescein (APF) and flow cytometry using Accuri Cflow and Cflow Plus (BD BioSciences, San Jose, CA, USA) tools, used to assess the increase in ROS through altered percentages of signal as
compared with the peak of the control sample (5.5 mM). Furthermore, glucose concentrations of 50 and 100 mM increased the ratio of fluorescence shift to 3-fold and 4-fold, respectively (Figure 1B). The state of hyperglycemia directly influenced the production of HTMC cell ROS in a concentration-dependent manner. Accumulation of iROS was increased in HTMCs under high glucose concentration-induced oxidative stress. Additionally, cytosolic Ca$^{2+}$ ([Ca$^{2+}$]i) was estimated using Fluo-8® dyes, which were specifically Ca$^{2+}$-binding fluorescent probes. The data revealed that at 100 mM glucose concentration, calcium accumulation increased by two times compared to the 5.5 mM glucose concentration (Ctrl) (Figure 1C).

### 3.2. Immunoblot analysis of hyperglycemia effects on HTMCs

HTMCs were treated with a medium at different concentrations (5.5, 25, 50, and 100 mM glucose), and the related signaling molecules were examined by immunoblotting after 3 weeks at the aforementioned specific glucose concentrations. The protein levels of p-JNK, p-p38, and p-Akt were slightly elevated with the increasing glucose concentration, whereas PI3K, calreticulin, and calmodulin levels remained unchanged. Moreover, the level of p-smad2 and p-smad3 increased only at 100 and 25 mM glucose concentrations, respectively. No significant change was observed in Smad2/3 and smad4 levels, whereas smad7 levels increased at 25 mM but decreased at 50 and 100 mM concentrations. Additionally, we examined the levels of TGF-β1, TGF-β2, and the ECM markers, vimentin, α-SMA, and collagen I. The level of TGF-β1 increased continuously at 50 and 100 mM; thus, TGF-β2 levels increased only in 100 mM. The protein levels of vimentin, collagen I, and α-SMA slightly increased with high glucose treatment (Figure 2).

### 3.3. Immunofluorescence analysis of the effects of hyperglycemia on HTMCs

We performed immunofluorescence analysis to analyze the fibers and actin dots in HTMCs to assess whether high glucose concentration-induced oxidative stress can cause trabecular meshwork cell fibrosis. Data revealed that more and thicker stress fibers formed in 50 and 100 mM glucose-cultured HTMCs compared to 5.5 and 25 mM glucose-cultured cells that complied with previous dose-dependent results (Figure 3A). In addition, we established immunofluorescence analysis experiments to track ECM protein markers, specifically collagen I (Figure 3B upper) and laminin (Figure 3B lower). Our findings revealed a much higher intensity of collagen I and laminin in cells with higher glucose concentrations, which suggests a significant progression of fibrosis.

### 3.4. Hyperglycemia effects on TGF-β1/2 knockdown HTMCs

To identify the role of TGF-β in high glucose concentration-induced oxidative damage, we knocked down TGF-β in cells and cultured them for at least 3 weeks with 5.5, 25, 50, and 100 mM glucose supplementation, and further detected the levels of ROS and calcium. Our findings (Figure 4A) revealed that the increased ROS levels in HTMCs treated with hyperglycemia was markedly accelerated by the knockdown of TGF-β1 and TGF-β2; shTGF-β1 and shTGF-β2 knockdown raised the fluorescence shift ratio from 5.5 to 7.3 and 15.1, respectively, at 25 mM glucose concentration. Data regarding shTGF-β1
and shTGF-β2 increased the fluorescence shift ratio from 7.1 to 8.5 and 15.3 at a 50 mM glucose concentration, respectively, whereas that of shTGF-β1 and shTGF-β2 increased the fluorescence shift ratio from 7.6 to 18.6 and 20.4, respectively, at 100 mM glucose concentration. The calcium level increased markedly in the shTGF-β1 and shTGF-β2 groups (Figure 4B), independent of glucose concentration. These findings indicated that the expression of TGF-β protects HTMCs against oxidative damage caused by hyperglycemia and that TGF-β1 knockdown aggravates oxidative damage caused by hyperglycemia in HTMCs.

3.5. Immunoblot analysis to determine the effects of hyperglycemia on TGF-β1/2 knockdown HTMCs

In order to determine the TGF-β-related downstream pathway with elevated glucose concentrations, as observed in Figure 5, we cultivated HTMCs at different medium-containing glucose concentrations for at least 3 weeks to determine their long-term hyperglycemic status, and further observed alterations in protein expression. TGF-β1/2 downregulated the protein expression of p-JNK, JNK, p-p38, p38, and p-AKT linked to cell self-protection and antioxidation, whereas TGF-β1/2 upregulated PI3K, AKT, p-smad2, p-smad3, smad2/3, smad4, vimentin, alpha-SMA, and collagen I. Collectively, these results suggested that excessive TGF-β induces downstream signal transduction, which can trigger severe oxidative damage in the absence.

3.6. Hyperglycemia effects of on HTMCs with or without rhTGF-β1 protein (1 and 5 ng/ml)

In order to further explore the function and physiological significance of TGF-β, we conducted experiments with rhTGF-β1 protein at 1 and 5 ng/mL, and then detected the effects of TGF-β-upregulation on oxidative damage caused by hyperglycemia. Our findings (Figure 6A) revealed that increased cell proliferation of HTMCs treated with high glucose concentrations resulted in the upregulation of TGF-β1 (1 ng/mL), whereas those in the Ctrl group presented lower cell proliferation rates. In particular, the cell proliferation rate of the 50 mM glucose concentration with TGF-β1 (1 ng/mL)-treated group was markedly increased to the highest, whereas it was slightly lower than the rate of 25 mM under normal conditions. Therefore, we selected TGF-β1 (1 ng/mL) as the treatment condition for the following experiments. Thereafter, the production of HTMCs with hyperglycemia with or without recombinant TGF-β1 protein (1 ng/mL) in iROS was observed. Our findings (Figure 6B) revealed a substantial decrease in ROS output of hyperglycemic HTMCs by TGF-β1 in the case of low concentrations (1 ng/mL). At these four glucose concentrations, regardless of the group, treatment with TGF-β1 protein (1 ng/mL) revealed a remarkable decrease in the ROS levels. In addition, we investigated the calcium content in HTMCs incubated at 5.5, 25, 50, and 100 mM concentrations with or without recombinant TGF-β1 protein (1 ng/mL; Figure 6C). We found that with rhTGF-β1 (1ng/mL)-treated cells, the data presented lower cellular calcium content in each glucose concentration, particularly at 50 and 100 mM. Collectively, these results suggested that low levels of TGF-β1 protected HTMCs against hyperglycemia-induced oxidative damage and promoted cell proliferation.

3.7. Immunoblot analysis to determine the effects of hyperglycemia on HTMCs with or without recombinant TGF-β1 protein (1 ng/ml)
In Figure 7, for further analysis of TGF-β-related high glucose pathway involvement, we first cultured HTMCs in four separate medium glucose concentrations for at least 3 weeks in order to assess long-term hyperglycemic status and then treated them with recombinant TGF-β1 protein (1 ng/mL) and observed improvements in the p-JNK/JNK, p-p38/p38, PI3K, p-AKT/AKT, calreticulin, and protein expressions. The expressions of p-JNK, p-p38, p-AKT, p-smad2, p-smad3, smad4, and α-SMA upregulated in the rhTGF-β1 (1 ng/mL)-treated condition. These results suggest that a low concentration of TGF-β1 induces downstream signal transduction, which can trigger the antioxidation pathway in HTMCs, and this signaling pathway promotion in HTMCs with hyperglycemia treatment was beneficial for antioxidation.

3.8. Hydrogen peroxide-induced oxidative stress and effects of hyperglycemia on HTMCs

We conducted experiments with the aforementioned two oxidative stress induction factors to assess the additional effects of oxidative damage caused by hyperglycemia and H$_2$O$_2$ and subsequently detected the effects such as cell viability, ROS production, and calcium level. Thus, the glucose concentration affects hydrogen peroxide (H$_2$O$_2$) tolerance in a concentration-dependent manner (Figure 8A). Higher glucose concentration leads to greater sensitivity toward oxidative stress induced by H$_2$O$_2$. The cell viability in 100 mM glucose concentration with H$_2$O$_2$ treatment was consistently lower than that of other glucose cell concentrations (5.5, 25, and 50 mM). Moreover, iROS accumulation was observed in HTMCs with or without H$_2$O$_2$ (0.5 mM) hyperglycemia for 1 h. Our results (Figure 8B) revealed that ROS production remarkably increased in hyperglycemic conditions, particularly at 50 and 100 mM, followed by H$_2$O$_2$ treatment. Furthermore, in Figure 8C, apoptotic cells were measured and the apoptosis rate was significantly increased in 25, 50, and 100 mM glucose with H$_2$O$_2$ treatment. In general, these findings indicate that oxidative damage caused by hyperglycemia and H$_2$O$_2$ may lead to more iROS and cell death in HTMCs, which indicates a risk for severe tissue damage in patients with diabetes retinopathy in combination with glaucoma.

3.9. Immunoblot analysis to determine the effects of hyperglycemia on HTMCs with or without H$_2$O$_2$ (0.5 mM) for 1 h

In order to further study the TGF-β-related pathway involvement with high glucose concentrations, as illustrated in Figure 9, we first cultured HTMCs at different medium-containing glucose concentrations for at least 3 weeks to assess the long-term hyperglycemic status and then treated them with H$_2$O$_2$ (0.5 mM) for 1 h; subsequently, we observed changes in the protein expression of p-JNK/JNK, p-p38/p38, PI3K, p-AKT/AKT, calreticulin, calmodulin, p-smad2/3, smad2/3, smad4, smad7, TGF-β1, TGF-β2, vimentin, α-SMA, and collagen I. The expression of p-JNK and p-p38 was related to apoptosis and inflammation; moreover, α-SMA and collagen I, the fibrosis markers, were upregulated with H$_2$O$_2$ (0.5 mM) treatment for 1 h and revealed additional oxidative effects.

3.10. A hypothetical model detailing the effects of hyperglycemia on TGF-β pathway in trabecular meshwork cells

Accumulated ROS facilitates TGF-β conversion from the latent form to the active form in a hyperglycemic state, causing downstream signaling. Upregulated TGF-β in HTMCs subsequently induces
JNK-p38 pathway, PI3K-AKT, and Smad family phosphorylation. Activation accelerates the expression of TGF-β, which facilitates the production of ECM markers. Thus, low TGF-β1 levels prevent HTMCs from oxidative damage caused by hyperglycemia by promoting cell antioxidation, but TGF-β signaling may turn out to be dangerous when overexpressed beyond the cell tolerance threshold (Created in BioRender.com).

Discussion

In this study, we examined the hypothesis of TGF-β pathways involving oxidative hyperglycemia and characterized the etiopathology of TM. Moreover, we investigated whether TGF-β in HTMCs induces increased intracellular ROS levels and analyzed possible related signal transduction. We demonstrated that HTMC cell processes are adversely affected by the activation of deleterious signaling pathways. By shTGF-β knockdown or addition of TGF-β recombinant protein, which indicated the presence and absence of TGF-β as a model system, the role of TGFβ and oxidative stress with high glucose concentration have been elucidated. Low concentration TGF-β levels have reversed these deleterious processes, a finding that has implications for treating or delaying TM cell pathobiology and diabetes.

Malik et al. stated that oxidative stress caused by hyperglycemia due to the overproduction of mitochondrial reactive oxygen species (ROS) is involved in this process (Brownlee, 2001; Czajka & Malik, 2016; Nishikawa et al., 2000). Excessive ROS production mediates oxidative stress in DM by high glucose and/or fatty acid oxidation (Germoush et al., 2019). Lu et al. demonstrated that the main mediator against diabetic complications is oxidation stress (Wang et al., 2019). Furthermore, hyperglycemia triggers cell and macrophage mitochondrial dysfunction and aberrant activation of cytoplasmic NADPH oxidases (NOX), which collectively intensify ROS production (X. Huang et al., 2011). Our data demonstrated that the hyperglycemic state directly influences the production of HTMC cell ROS in a concentration-dependent manner. Treatment with higher glucose concentrations resulted in increased ROS production. Previous studies have reported that several affected TGF-βs are regulated by ROS during tumorigenesis, as they control the downstream TGF-β signal transduction involving Smads, MAPKs, and NF-κB, as well as increase cell motility (G. Liu et al., 2010; R. M. Liu & Gaston Pravia, 2010). Moreover, TGF-β can control ROS levels by increasing their production and reducing the activity of antioxidant/scavenging systems (Ishikawa et al., 2014). In addition, elevated ROS levels may in turn increase the expression of TGF-β and promote the release of TGF-β from the secreted latent complex, making the growth factor bioavailable and active (Annes, Munger, & Rifkin, 2003). Furthermore, the protein expression of TGF-β1 & 2, particularly TGF-β1, obviously increased in high glucose concentration (100 mM).

TGF-β is one of the main factors causing excess ECM deposition in certain fibrotic environments (Pohlers et al., 2009). Cell signaling pathways that cause excess ECM deposition by TGF-β are therefore well investigated (Meng, Nikolic-Paterson, & Lan, 2016). In contrast, TGF-β plays pleiotropic functions in natural tissues and does not induce fibrosis in preserving tissue homeostasis. The TGF-β-mediated mechanism for signaling causing fibrosis is possible, as TGF-β-induced increased matrix
metalloproteinase activity that degrades ECMs is suppressed or balanced (Han, Kampik, Grehn, & Schlunck, 2013; Tellios et al., 2017). Therefore, further research is required to identify specific regulatory feedback pathways to avoid TGF-β fibrosis without disrupting its ordinary homeostatic functions in tissues. These signaling pathways can also be used to inhibit disease-associated fibrosis as effective therapeutic targets. The proposed IOP elevation mechanism is an improved aquatic outflow resistance through the trabecular mesh, assisted by findings of lower cellularity and altered ECM protein expression in glaucoma TM (Wahlig, Lovatt, & Mehta, 2018). Excessive ECM protein accumulation (e.g., collagen, alpha-SMA, and laminin) in the TM can cause IOP elevations. Typical POAG-like modifications (ECM aggregation, cell death, cytoskeletal disorders, inflammatory marker activation, etc.) contribute to \textit{in vitro} induction of oxidative stress in HTMCs, which can be substantially decreased by pretreatment with antioxidants and vasopressors (prostaglandin analogs and carbonic anhydride inhibitors) (Welge-Lussen & Birke, 2010; Yu, Birke, Moriniere, & Welge-Lussen, 2010). Considering POAG, the levels of the ECM portion have been substantially increased. Higher concentrations of ECM components can not only induce HTMC dysfunction, but also decrease the number of HTMCs, affecting normal aqueous filtration (Hogg, Calthorpe, Batterbury, & Grierson, 2000). Our findings revealed remarkably higher intensity of collagen I and laminin in cells with higher glucose concentrations, which suggests a significant progression of fibrosis. The imbalance of oxidants and antioxidants leads to accumulation of ROS and structural remodeling of TM, enlargement, or failure. In comparison, oxidative stress induces \textit{in vitro} migration of HTMCs, resulting in TM thickening, expansion, and fusion.

Flow cytometry and immunoblotting were performed to measure ROS levels, calcium contents, and the expression of related downstream pathway molecules, which are responsible for TGF-β signaling. As aforementioned in the Results section, HTMC shTGF-β1 and shTGF-β2 knockdown cells increased ROS production in HTMCs. Our results indicated that the increased calcium content in HTMCs treated with high glucose levels was markedly accelerated by TGF-β1 and TGF-β2 knockdown. These findings revealed that the expression of TGF-β protects HTMCs against oxidative damage caused by hyperglycemia and that TGF-β1 knockdown aggravates oxidative damage caused by hyperglycemia in HTMCs. Furthermore, upregulation of TGF-β1 (1 ng/mL) resulted in an increase in cell proliferation of HTMCs with high glucose concentrations; whereas, TGF-β1 (5 ng/mL) presented an even lower proliferation rate than the Ctrl group, and ROS production in HTMCs treated with hyperglycemia decreased significantly with low-concentration TGF-β1 (1 ng/mL) therapy, as well as calcium content in the Ctrl group. The protein expression of p-JNK, JNK, p-p38, p38, and p-AKT was downregulated with TGF-β1/2 knockdown, which are related to self-protection of cells and antioxidation; whereas PI3K, AKT, p-smad2, p-smad3, smad2/3, smad4, vimentin, α-SMA, and collagen I were upregulated with TGF-β1/2 knockdown. In contrast, the same protein marker with TGF-β1 protein (1 ng/ml) treatment revealed an opposite trend and implied the signaling pathway of TGF-β in hyperglycemia. Eventually, we illustrated the experiments with these two oxidative stress induction factors to investigate the additional effects of oxidative damage caused by hyperglycemia and \textit{H}_2\textit{O}_2, and subsequently, detected the effects that exhibited lower cell viability and increased ROS production at high glucose concentrations. Moreover, it has been found that Col1A2 in the liver is upregulated through ROS generation and calcium influx by
TGF-β1 (Yang, Chang, Hong, Hung, & Chuang, 2017); however, calreticulin, a Ca^{2+}-binding/buffering chaperone typically residing in the lumen of the endoplasmic reticulum (ER) of eukaryotic cells (Krstic, Trivanovic, Mojsilovic, & Santibanez, 2015), and calmodulin, a calcium-binding protein, both revealed no change in our data; however, the intracellular calcium influx increased remarkably.

In our previous study, we demonstrated that TGF-β1 has defended against hyperglycemia-induced oxidative damage via promotion of cell antioxidation and neuroprotection pathways, including the signaling of Nrf2/Keap1/ALDH3A1/HO-1. These findings suggest that in the RGC antioxidant system, TGF-β1 plays an important role. A candidate therapeutic strategy for diabetic retinopathy could be to target TGF-β1 to facilitate Nrf2/Keap1/ALDH3A1/HO-1 signaling, provided that antioxidant signal transduction in retinopathy diseases is a promising therapeutic target (H. Y. Chen et al., 2020). In this study, the beneficial concentration of TGF-β1 was 5 ng/ml in RGCs, whereas in HTMC, this concentration aggravates the damage caused by induced oxidative stress. Based on these findings, the amount of protein expression changes due to IR damage was coincidentally comparatively lower in the retina compared with other tissues, presumably because its resistance is similar to that of other tissues. Retina seems to be the most important tissue that is damaged at a later stage in the eye ball; however, this damage cannot be reversed.

**Conclusions**

In summary, our studies indicated that hyperglycemic effects in HTMCs caused lower proliferation and increased ROS production and calcium influx, leading to cell dysfunction and fibrosis. By promoting cell antioxidation pathways, including JNK/p38/PI3K/AKT pathway and TGF-β-related Smad family, TGF-β1 may play a pivotal role in the HTMC antioxidant system. Considering the dual effect of TGF-β, the underlying molecular mechanisms that regulate the progression of diseases, the possibility of treatment for these antioxidant benefits and the selection of treatment objectives will be better understood. This research would also help in detecting eye pathologies and diabetic care as eye disorders occur.

**Declarations**

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- **Competing interests:** The authors declare that they have no conflicts of interest.

- **Ethical Approval and Consent to participate:** Not applicable

- **Consent for publication:** Not applicable

- **Availability of data and material (data transparency):** The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.
-**Code availability:** Not applicable

-**Author Contributions:** All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Hsin-Yi Chen, Mei-Lan Ko and Hong-Lin Chan. The first draft of the manuscript was written by Hsin-Yi Chen, and revised by Mei-Lan Ko and Hong-Lin Chan and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Figures
Figure 1

Hyperglycemic effects on human trabecular meshwork cells (HTMCs). (A) Upper: MTT assay was used to determine cell viability; cell proliferation rates were determined using different glucose concentrations from Day 1 to Day 4. Data for Days 2, 3, and 4 are standardized to Day 1. Lower: Day 4 results have been isolated to illustrate differential proliferation rates at four glucose concentrations. Data are presented as mean ± SEM; ***, p < 0.001, in contrast with the 5.5 mM group. (B) Upper: increased intracellular reactive oxygen species (iROS) levels in HTMCs treated with media containing high glucose concentrations. Using an aminophenyl fluorescein (APF) assay, iROS levels were determined in HTMC cultures treated with various glucose concentrations (5.5, 25, 50, and 100 mM glucose) for 3 weeks. After exposure, 106 cells were stained with diluted APF (5 μM). iROS generation was quantified using flow cytometry. Lower: statistic bar chart shows the quantified and normalized 5.5-mM groups relative to the fluorescence shift percentage. Data are presented as mean ± SEM; *, p < 0.05 and **, p < 0.01, when compared to the 5.5 mM group (n = 3). (C) Calcium concentrations in HTMCs in a hyperglycemic environment. Cells were treated with 5.5, 25, 50, and 100 mM glucose medium and dose responses of complete calcium inflow were assayed. Data are presented as the mean ± SEM; *, p < 0.05; **, p < 0.01 and ***, p < 0.001 when compared to the 5.5 mM group (n = 3).
Figure 2

Immunoblot analysis of hyperglycemia effects on HTMCs. Immunoblot analysis of TGFβ pathway-associated proteins (p-JNK/JNK, p-p38/p38, PI3K, p-AKT/AKT, calreticulin, calmodulin, p-smad2/3, smad2/3, smad4, smad7, TGFβ-1, TGFβ-2, vimentin, α-SMA, and collagen I) in 5.5, 25, 50, and 100 mM glucose medium. Protein expression is quantified in relation to the loading control (n = 3).
Figure 3

Immunofluorescence analysis of the effects of hyperglycemia on HTMCs. (A) Photomicrographs of HTMCs treated with 5.5, 25, 50, and 100 mM glucose-supplemented medium for at least 3 weeks. Cells were found to express abnormal stress fibers, which were much denser and thicker compared to that in the control group treated with 5.5 mM glucose. HTMCs were treated with four different glucose concentrations on 12-mm coverslips and fixed and stained with phalloidin and DAPI. A similar exposure was used for each set of fields and the images are reflective of three different fields (scale bar = 20 μm). (B) HTMCs were treated with specific glucose concentrations on 12-mm coverslips and then fixed and stained. The colors represented in the upper figure are as follows: collagen I (green), lower figure: laminin (green), phalloidin (red), and DAPI (blue). A similar exposure was used for each set of fields and the images are indicative of three different fields (scale bar = 20 μm).
Figure 4

Effects of high glucose levels on TGF-β1/2 knockdown HTMCs. (A) Upper: APF assay in TGF-β1/2 knockdown HTMCs after prolonged exposure to glucose for the determination of intracellular ROS (5.5, 25, 50, and 100 mM glucose). Approximately 106 cells were treated with APF (5 μM) in phosphate-buffered saline after exposure. Flow cytometry was used to quantify intracellular ROS (n = 3). Lower: in relation to the shPLKO.1 (5.5 mM) group, the relative fluorescence shift percentage is quantified and normalized. Data are presented as mean ± SEM *, p < 0.05 and ***, p < 0.001, when compared to the 5.5-mM shPLKO.1 control group. (B) Upper: Calcium influx in TGF-β1/2 knockdown HTMCs with hyperglycemia. Lower: Dose responses of intracellular calcium levels of TGF-β1/2 knockdown cells treated with 5.5, 25, 50, and 100 mM glucose medium were determined. Data are presented as means ± SEM ***, p < 0.001 when compared to the 5.5-mM shPLKO.1 (Ctrl). (n = 3)
Figure 5

Immunoblot analysis to determine hyperglycemic effects on TGF-β1/2 knockdown HTMCs. Immunoblot analysis of TGF-β pathway-associated proteins (p-JNK/JNK, p-p38/p38, PI3K, p-AKT/AKT, calreticulin, calmodulin, p-smad2/3, smad2/3, smad4, smad7, vimentin, α-SMA, and collagen I) after treatment of cultured TGF-β1/2 knockdown HTMC with medium containing 5.5, 25, 50, and 100 mM of glucose. Protein expression is quantified in relation to the loading control (n = 3).
Hyperglycemia effects on HTMCs with or without rhTGF-β1 protein (1 & 5 ng/mL). (A) Upper: MTT assay to determine cell viability; cell proliferation rates under different glucose concentrations from Day 1 to Day 4 were determined. Data obtained on Days 2, 3, and 4 are standardized to Day 1. HTMCs were incubated for a long-term exposure to 5.5, 25, 50, and 100 mM glucose and plated on 96-well plates to observe rhTGF-β1 protein (1 & 5 ng/mL) affected cell proliferation rates for 24 h at various glucose concentrations. Lower: Isolated Day 4 data show the differential proliferation rate of HTMCs with recombinant TGF-β1 protein (1 & 5 ng/mL) at four glucose concentrations. Data are presented as mean ± SEM; *, p < 0.05, **, p < 0.01 and ***, p < 0.001, when compared to the control. (B) Upper: Increased intracellular reactive oxygen species (iROS) levels in hyperglycemic HTMCs with the rhTGF-β1 protein (1 ng/mL). An aminophenyl fluorescein (APF) assay was performed using HTMC cultures treated with different glucose concentrations (5.5, 25, 50, and 100 mM glucose) for a duration of 3 weeks with or without the rhTGF-β1 protein (1 ng/mL) to determine iROS levels. About 106 cells were stained with diluted APF (5 μM) after exposure. Intracellular ROS generation was quantified using flow cytometry. Lower: The statistic bar chart shows the quantified and normalized 5.5-mM group relative to the fluorescence shift percentage. Data are presented as mean ± SEM; ***, p < 0.001 compared to the 5.5 mM group (n = 3). (C) Upper: calcium influx in HTMCs with or without rhTGF-β1 protein (1 ng/mL). Lower: Dose responses of intracellular calcium levels of cells treated with 5.5, 25, 50, and 100 mM glucose with or without rhTGF-β1 protein treated were determined. Data are presented as means ± SEM; ***, p < 0.001 compared to the 5.5-mM control group (n=3).
Figure 7

Immunoblot analysis of hyperglycemia effects on HTMCs with or without recombinant TGF-β1 protein (1 ng/mL). Immunoblot analysis of TGFβ pathway-associated proteins (p-JNK/JNK, p-p38/p38, PI3K, p-AKT/AKT, calreticulin, calmodulin, p-smad2/3, smad2/3, smad4, smad7, vimentin, α-SMA, and collagen I) under 5.5, 25, 50, and 100 mM glucose medium with or without recombinant TGF-β1 protein (1 ng/mL). Protein expression is quantified in relation to the loading control (n = 3).
Figure 8

Hydrogen peroxide-induced oxidative stress in HTMCs treated with high glucose concentrations. (A) HTMCs were incubated (long-term exposure) with 5.5, 25, 50 and 100 mM glucose and seeded in 96-well plates and treated with different concentrations of H2O2. MTT assay was used to determine cell viability after treatment with different concentrations of H2O2 for 1 h; ***, p < 0.001, when compared to the 5.5 mM group. (B) Upper: Increased level of intracellular reactive oxygen species (iROS) in HTMCs incubated in a hyperglycemic environment with or without H2O2 (0.5 mM) treatment for 1 h. An aminophenyl fluorescein (APF) assay was used to determine iROS levels of HTMC cultures treated with different glucose concentrations (5.5, 25 M, 50 and 100 mM) and with or without H2O2 (0.5 mM) treatment for 1 h for 3 weeks. Approximately 106 cells were stained using dilute APF (5 μM) after exposure. iROS generation was quantified using flow cytometry. Lower: Statistic bar chart shows the quantified and normalized 5.5-mM group relative to the fluorescence shift percentage. Data are presented as mean ± SEM; **, p < 0.01 and ***, p < 0.001 when compared to the 5.5 mM group (n = 3). (C) Upper: Analysis of apoptosis in HTMCs treated with media containing 5.5, 25, 50, and 100 mM glucose and further treatment with or without H2O2 (0.5 mM) for 1 h. Lower: Dose responses involving cell death in HTMCs in a medium containing high glucose levels and with or without H2O2. Data are presented as means ± SEM; ***, p < 0.001 when compared to the 5.5-mM control (n = 3).
Figure 9

Immunoblot analysis of the effects of hyperglycemia on HTMCs with or without hydrogen peroxide (0.5 mM) treatment for 1 h. Immunoblot analysis of TGF-β pathway-associated proteins (p-JNK/JNK, p-p38/p38, PI3K, p-AKT/AKT, calreticulin, calmodulin, p-smad2/3, smad2/3, smad4, smad7, TGFβ-1, TGFβ-2, vimentin, α-SMA, and collagen I) after treatment of cells with 5.5, 25, 50, and 100 mM glucose with or without hydrogen peroxide (0.5 mM) for 1 h. Protein expression is quantified in relation to the loading control (n = 3).
Figure 10

A hypothetical model detailing the effects of hyperglycemia on the TGF-β pathway in trabecular meshwork cells.