IGFBP-3 reduces eNOS and PKCzeta phosphorylation, leading to lowered VEGF levels

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Purpose: In models of diabetic retinopathy, insulin-like growth factor binding protein-3 (IGFBP-3) is protective to the retina, especially retinal microvascular endothelial cells (RECs), but the underlying mechanisms are unclear. For this study, we hypothesized that IGFBP-3 may reduce vascular endothelial growth factor (VEGF) levels through reduced endothelial nitric oxide synthase (eNOS) activity, which may be protective against macular edema.

Methods: To test this hypothesis, we grew primary human retinal endothelial cells in normal glucose (5 mM) or high glucose (25 mM) for three days, treated with IGFBP-3 NB plasmid (a plasmid of IGFBP-3 that cannot bind IGF-1), followed by western blotting for eNOS, protein kinase C zeta (PKCzeta), and VEGF. Additionally, we treated some cells with recombinant eNOS or PKCzeta, after IGFBP-3 NB plasmid transfection to validate that these pathways regulate VEGF levels. Immunoprecipitation experiments were done with the eNOS antibody, followed by western blotting for PKCzeta, to determine if eNOS and PKCzeta interact directly.

Results: Our results suggest that 1) IGFBP-3 inhibits the endothelial nitric oxide synthase (eNOS) and protein kinase C zeta (PKCzeta) pathway, which in turn inhibits VEGF production, and 2) that eNOS plays a role in activating PKCzeta to increase VEGF levels in diabetic retinopathy.

Conclusions: In conclusion, IGFBP-3 may be a novel treatment for macular edema through the inhibition of eNOS and PKCzeta activation, leading to reduced VEGF levels.

Diabetic retinopathy is the leading cause of blindness in patients aged 20–74 years [1]. Retinal microvascular endothelial cell (REC) changes in permeability are key to the development of diabetic-related pathologies, including macular edema [2]. Progressive retinal endothelial cell inflammation, angiogenesis, and apoptosis are hallmarks of the disease [3-6]. Various mediators, such as endothelial nitric oxide synthase (eNOS), protein kinase C (PKC), the insulin-like growth factor 1 (IGF-1)/insulin-like growth factor binding protein (IGFBP) system, and vascular endothelial growth factor (VEGF), are known to regulate both angiogenic and macular edema pathways in response to hyperglycemia in various retinal cell types, including REC [7-10]; however, the specific pathways involved are unclear. It has been previously reported that IGFBP-3 can inhibit VEGF production, leading to an inhibition of both angiogenesis and macular edema [11-13]. Building on this important finding, the goal in the current study was to uncover the cellular signaling pathways that underlie IGFBP-3 regulation of VEGF, and specifically to determine if IGFBP-3 inhibition of eNOS and PKCzeta are involved in IGFBP-3's protective effects on REC.

IGFBP-3 binds to IGF-1 to elicit multiple functions physiologically [14-16]. We and others have previously shown that IGFBP-3 has separate IGF-1-independent actions to support cell survival [12]. In our work and the work of others, IGFBP-3 has been shown to reduce TNF-α levels, inhibit REC apoptosis, and mediate protective effects on blood retinal barrier integrity in diabetic retinopathy [17-20]. The studies described here use IGFBP-3 NB plasmid with an endothelial cell-specific promoter to focus on IGFBP-3 regulation of VEGF actions that are independent of IGF-1.

There is literature suggesting that specific IGFBP isoforms correlated with insulin sensitivity, vascular NO generation, and the development of diabetes [21]. Here, we hypothesize that eNOS may be a target of IGFBP-3, based on previous work by Jarajapu et al. [12]. Recent studies showed that endothelial nitric oxide synthase (eNOS) acts as an important mediator of vascular growth and maturation [22,23], and eNOS expression was shown to be upregulated in early diabetic diseases, especially in afferent and glomerular endothelium [24,25]. On the other hand, eNOS uncoupling caused by inflammation has been suggested to be a key mediator in the development and progression of diabetes [26].

One potential mechanism for eNOS actions in the retina is through interaction with other proteins involved in cellular signaling. In other tissues, PKC and NOS interact to regulate microvascular hyperpermeability [27-30].

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Hyperglycemia-induced PKC activation is linked to diabetes, and the prevention of excessive PKC attenuated cardiac diastolic dysfunction caused by diabetes [26]. In the retina, hyperglycemia-induced overactivation of PKCzeta is associated with outer blood retinal barrier breakdown, and the inhibition of PKCzeta restores the outer barrier structure [27,31]. PKCzeta serves as a potential target for early and underestimated diabetes-induced retinal pathology [27]. Thus, we wished to determine whether IGFBP-3 regulates VEGF actions through reduced eNOS and PKCzeta activation in REC cultured under hyperglycemia conditions. Our current findings suggest that IGFBP-3 regulates VEGF through eNOS and PKCzeta, which may be key to IGFBP-3 actions in both angiogenesis and macular edema.

METHODS

Reagents: Phospho-PKCzeta (T410) and phospho-eNOS (S1177), and eNOS antibodies, were purchased from Cell Signaling (Danvers, MA). VEG and actin antibodies and protein A/G plus for immunoprecipitation experiments were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The PKCzeta antibody and 3-nitrotyrosine ELISA were purchased from Abcam (Cambridge, MA). An Ultrasensitive Colorimetric Assay for Nitric Oxide Synthase kit was bought from Oxford Biomedical Research (Oxford, MI). PRKCzeta recombinant human protein was purchased from Life Technologies (Carlsbad, CA). Endothelial bovine nitric oxide synthase and N-acetyl cysteine (NAC) were purchased from Sigma-Aldrich (St. Louis, MO). The SuperFect transfection reagent was bought from Qiagen (Valencia, CA). Horseradish peroxidase (HRP) conjugated secondary anti-mouse and anti-rabbit antibodies were purchased from Promega (Madison, WI). Enhanced chemiluminescence for immunoblot development and signal detection was purchased from Amersham Biosciences (Piscataway, NJ). IGFBP-3 NB plasmid DNA was a gift from Dr. Maria B. Grant (University of Indiana).

IGFBP-3-NB plasmid: Since the IGF-1 binding site in IGFBP-3 is located in both the N-terminal hydrophobic domain and the C-terminal, this plasmid was made by mutations in Leu^{77}, Leu^{80}, and Leu^{81} in the N-terminus, and Gly^{217} and Gln^{223} in the C-terminus [32]. These mutants showed undetectable binding to IGF-1 [32]. This same mutant has been used by other groups for work with REC [12,33].

Cell culture: Primary human REC were acquired from Cell System Corporation (CSC, Kirkland, WA). Cells were grown in an M131 medium containing microvascular growth supplements (Invitrogen; MVGS), 10 µg/ml gentamycin and 0.25 µg/ml amphotericin B. In the high-glucose condition, cells were transferred to a high-glucose (25 mM; Cell Systems) medium, supplemented with MVGS and antibiotics for 3 d. Only primary cells within passage 6 were used. Cells were quiesced by incubating in high or normal glucose media without MVGS for 24 h before all experiments. For experiments to investigate eNOS signaling, we first treated REC with eNOS at a dose range from 0.25 to 2 µM. Based upon our findings, we chose 1 µM for all further experiments and treated the cells for 2 h. For experiments to investigate PKCzeta actions on VEGF, we first treated REC with PKCzeta in a dose range from 1 to 4 µg/ml. We chose 4 µg/ml to treat the cells for 2 h for all experiments based on our findings.

Transfection of plasmid DNA: REC were transfected with IGFBP-3 NB plasmid DNA or control plasmid DNA at 1 µg/ml using the SuperFect transfect reagent, according to the manufacturer's instructions. The cells were used for experiments 24 h after transfection. For cells in high-glucose conditions, cells were transfected on Day 2 in a high-glucose medium and were kept in the high-glucose medium during transfection.

NOS analysis: NOS activity was measured using the ultra-sensitive Colorimetric Assay for Nitric Oxide Synthase kit. Briefly, the assay is based on the enzymatic conversion of nitrate to nitrite by the enzyme nitrate reductase (NaR), followed by quantization of nitrite using the Griess reagent according to the kit. Equal amounts of cell proteins were suspended in PBS and added to a reaction buffer containing NADPH part A and part B. After 6 h incubation at 37 °C, reconstituted nitrate reductase was added to the mixture. The activity of NOS was measured at an excitation wavelength of 540 nm. The specific enzymatic activity of NOS was calculated as relative units.

3-Nitrotyrosine ELISA: 3-nitrotyrosine was used as marker of peroxynitrite in REC and an indicator of reactive oxygen species. 3-nitrotyrosine levels were measured by a quantitative ELISA in REC treated with IGFBP-3 NB, eNOS, and NAC.

Immunoprecipitation: After rinsing with phosphate-buffered saline (PBS), cells were lysed by freeze–thawing in lysis buffer containing protease and phosphatase inhibitors for 20 min on ice. The cell pellets were transferred into 1.5 µl. The cells containing an equal amount of protein from control and each treatment were incubated with the eNOS antibody overnight at 4 °C, at which time protein A/G PLUS agarose beads were added and incubation continued for 2 h with gentle rocking. The beads were washed three times with the lysis buffer and once with PBS, and the immunocomplexes were released by heating in a Laemmeli sample buffer and analyzed by western blotting for eNOS and PKCzeta.
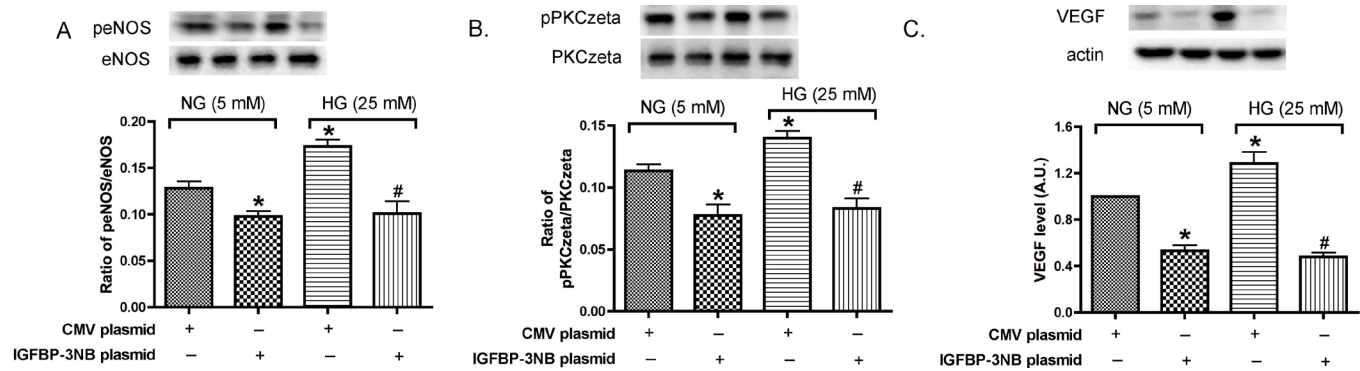
Figure 1. IGFBP-3 overexpression inhibited eNOS, PKCzeta, and VEGF in REC cultured in high ambient glucose. In all experiments, REC cells were treated with IGFBP-3 NB plasmid in a medium containing normal glucose (NG-5mM) or in a high-glucose (HG-25 mM) medium. **A**: Western blot result of the phospho-eNOS/eNOS ratio; **B**: Western blot result of phospho-PKCzeta/PKCzeta; **C**: Western blot of VEGF levels. *$p<0.05$ versus NG control plasmid DNA transfection. #$p<0.05$ versus HG control plasmid DNA transfection. n=3. Data are mean ± SEM.

Western blot analysis: After appropriate treatments and rinsing with cold phosphate-buffered saline, REC were scraped into a lysis buffer containing protease and phosphatase inhibitors. Equal amounts of protein from the cell extracts were separated on the pre-cast tris-glycine gel and blotted onto a nitrocellulose membrane. After blocking in TBST (10 mM Tris-HCl buffer, pH 8.0, 150 mM NaCl, 0.1% Tween-20) containing 5% (w/v) BSA, membranes were treated with anti-phospho-PKCzeta, PKCzeta, phospho-eNOS, eNOS, and VEGF antibodies, followed by incubation with HRP-conjugated secondary antibodies. The antigen-antibody complexes were detected using a chemi-luminescence reagent kit (Thermo Scientific).

Statistics: All the experiments were repeated at least three times, and the data are presented as mean ± SEM. Data were analyzed by the Kruskal–Wallis test, followed by Dunn's testing, with p values <0.05 considered statistically significant. In the case of western blotting, one representative blot is shown. Normal glucose was normalized to 1, with all treatments compared to normal glucose, followed by normalization to actin levels.

RESULTS

When grown in the presence of 25 mM glucose (diabetic-like conditions) and transfected with IGFBP-3NB plasmid, REC expressed decreased levels of phosphorylated eNOS and phosphorylated PKCzeta, as well as decreased levels of VEGF, compared to untreated cells cultured in high glucose. For our experiments, we transfected REC with IGFBP-3 NB plasmid DNA at 1.0 μg/ml for 24 h in either normal or high glucose (Figure 1). We compared changes in eNOS and PKC activation in cells receiving control plasmid versus cells

transfected with IGFBP-3 plasmid. Since the phosphorylated form of eNOS and PKCzeta is regarded as the activated form, we used western blots to monitor changes in the ratio of phospho-eNOS/eNOS, as shown in Figure 1A. We found that high glucose increased the ratio by approximately 50% above baseline, while IGFBP-3 transfection returned the ratio to near control levels (Figure 1A).

To determine if IGFBP-3 inhibition of eNOS expression leads to expected downstream effects, we also monitored changes in phosphorylated PKCzeta. Since eNOS is known to stimulate PKC, we predicted similar changes in REC cells after IGFBP-3 inhibition of eNOS. As shown in Figure 1B, PKCzeta activation was significantly higher in samples from cells cultured in high glucose compared to normal glucose-treated cell samples. IGFBP-3 transfection restored PKCzeta activation to near-normal glucose levels.

VEGF is well known, regulating both angiogenesis and macular edema in diabetic retinopathy. VEGF levels were significantly increased in response to high glucose, which were decreased after IGFBP-3 transfection (Figure 1C). These results support our hypothesis that high glucose activates the eNOS/PKC pathway, leading to increased VEGF levels, and that IGFBP-3 can inhibit its activity in the pathway to near normal levels.

IGFBP-3 blocked eNOS-induced VEGF changes in REC grown under high glucose conditions: To detect the optimal dose of recombinant eNOS to regulate VEGF in REC, we first performed a dose-range analysis from 0.25 to 2 μM and found NOS activity was significantly increased at 1 or 2 μM compared to control levels (Figure 2). Therefore, we chose 1 μM for future experiments. To verify whether IGFBP-3 is required for NOS actions in REC, we treated cells with
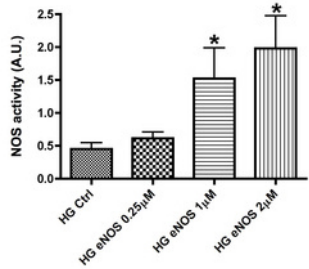
Figure 2. Dose curve of treatment with recombinant eNOS on NOS activity. Quiescent REC were treated with different doses of recombinant eNOS under high glucose conditions and analyzed by ELISA for NOS activity. *p<0.05 versus HG control. n=3. Data are mean ± SEM.

recombinant eNOS after IGFBP-3NB plasmid DNA transfection. Recombinant eNOS significantly increased phosphorylation of PKCzeta. We found that treatment of REC with IGFBP-3 alone lowered phosphorylation of PKCzeta by approximately 25% (Figure 3A), similar to what was observed in Figure 1B. When IGFBP-3 and eNOS were added together, the net effect was a significant reduction of PKCzeta phosphorylation compared to eNOS alone, which suggests that IGFBP-3 must reduce eNOS to regulate PKCzeta or levels would be more similar to eNOS alone. Similarly, increased eNOS levels led to expected downstream effects, including increased VEGF levels (Figure 3B). However, when IGFBP-3 and eNOS were added together, the net effect was not significantly different than IGFBP-3 actions alone, again hinting that IGFBP-3 requires a reduced eNOS level to reduce VEGF levels.

IGFBP-3 inhibited the interaction of PKCzeta, which is reduced in the presence of the antioxidant NAC: To determine whether eNOS and PKCzeta interact directly, we performed

immunoprecipitation with the eNOS antibody, followed by western blotting for phosphorylated eNOS (as a control) and PKCzeta. We also treated some cells with NAC to determine if IGFBP-3 actions are mediated in part through reduced reactive oxygen species. Figure 4A demonstrates that eNOS levels are increased following the immunoprecipitation, which can be inhibited by IGFBP-3. NAC was able to reduce the IGFBP-3-induced inhibition of eNOS activation. PKCzeta directly interacts with eNOS, as immunoprecipitation with eNOS antibodies resulted in significantly increased phosphorylation of PKCzeta, which was reduced with eNOS when combined with IGFBP-3 (Figure 4B). Similar to eNOS, the addition of NAC to samples with eNOS and IGFBP-3 significantly increased PKCzeta phosphorylation when compared to cells with eNOS and IGFBP-3 only (Figure 4B). Finally, to demonstrate whether IGFBP-3 and eNOS have actions on reactive oxygen species, we performed a 3-nitrotyrosine ELISA. Figure 4C demonstrates that eNOS significantly increased 3-nitrotyrosine, used as a marker of reactive oxygen species. IGFBP-3 was able to reduce 3-nitrotyrosine, but was not as effective in the presence of NAC (Figure 4C). NAC was highly effective in reducing 3-nitrotyrosine.

IGFBP-3 blocked PKCzeta-induced VEGF changes in REC grown under high glucose conditions: To detect the optimal dose of PKCzeta to regulate VEGF in REC, we first determined the best dose of recombinant PKCzeta using a dose analysis from 1 to 4 μg/ml first, and found that PKCzeta was increased significantly at 2 or 4 μg/ml compared to controls (Figure 5). We chose 4 μg/ml for the following experiment. Recombinant PKCzeta alone significantly increased VEGF levels in high glucose (Figure 6B). IGFBP-3 NB reduced VEGF levels (Figure 6B), which is similar to findings in Figure 1C. When IGFBP-3 NB and PKCzeta were added
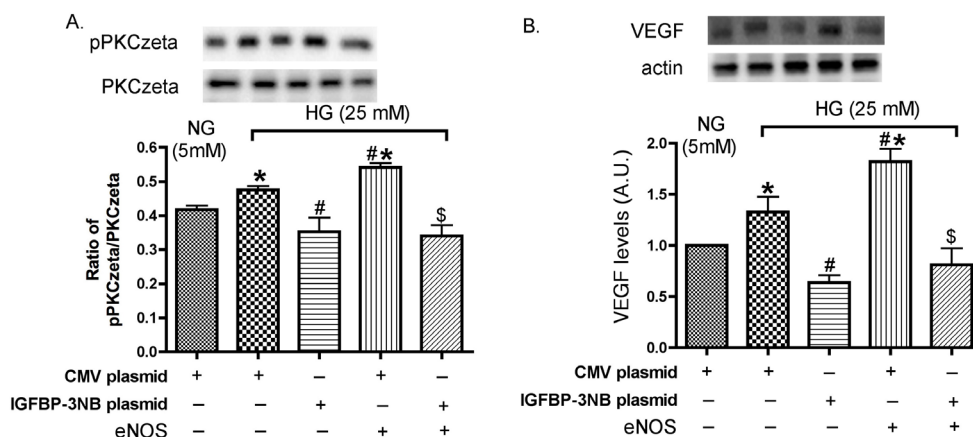

Figure 3. IGFBP-3 regulates PKCzeta activation and VEGF levels through eNOS. REC were treated with IGFBP-3 plasmid and recombinant eNOS in REC in both NG and HG media. **A**: Western blot data of phosphorylated and total PKCzeta levels; **B**: Western blot results of VEGF levels; *p<0.05 versus NG control plasmid DNA transfection. #p<0.05 versus HG control plasmid DNA transfection. *p<0.05 versus HG IGFBP-3 plasmid DNA transfection. $p<0.05 versus HG control DNA transfection + eNOS. n=3. Data are mean ± SEM.
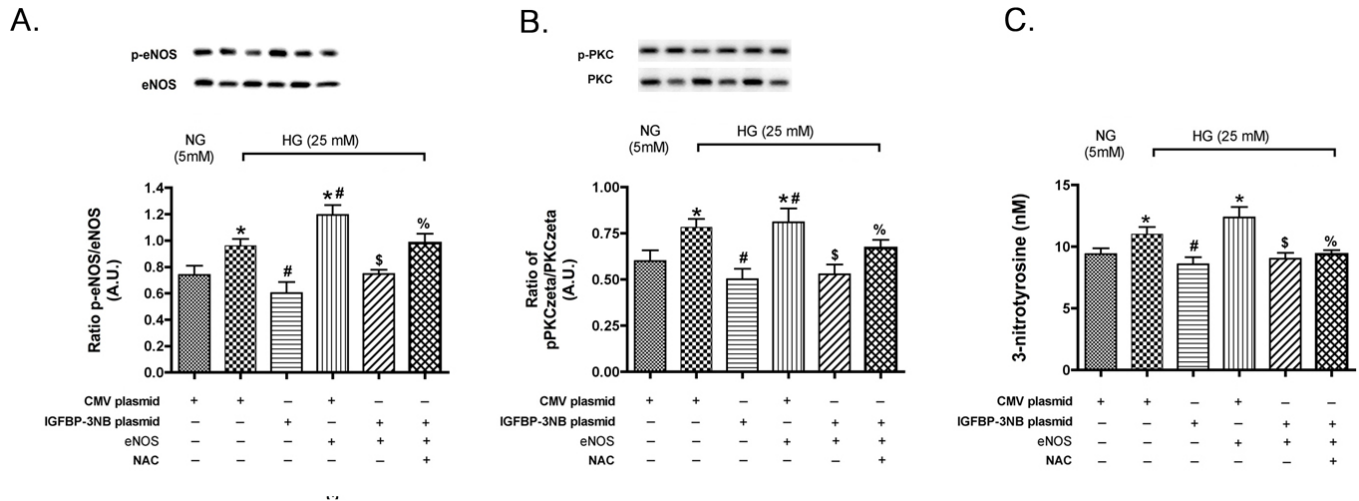
A.

B.

C.

Figure 4. eNOS directly interacts with PKCzeta, with both regulated by IGFBP-3. REC were transfected with IGFBP-3 NB, followed by recombinant eNOS only or eNOS and NAC in NG or HG. **A**: Immunoprecipitation results with eNOS beads, followed by western blotting for phosphorylated and total eNOS to demonstrate that eNOS treatment was effective. IGFBP-3 is not as effective in reducing eNOS when NAC is present. **B**: Immunoprecipitation results with the eNOS antibody, followed by western blotting for phosphorylated to total PKCzeta, demonstrating that eNOS interacts directly with PKCzeta. IGFBP-3 actions on PKCzeta are mediated in part through reduced peroxynitrite, as NAC increased PKCzeta compared to eNOS and IGFBP-3 only. **C**: ELISA for 3-nitrotyrosine on samples' immunoprecipitated results with the eNOS antibody to demonstrate that IGFBP-3 reduces eNOS, likely through reduced peroxynitrate actions. *$p < 0.05$ versus NG, # $p < 0.05$ versus HG+ IGFBP-3 plasmid DNA transfection. $^{\$}p < 0.05$ versus HG control DNA transfection + eNOS. % $p < 0.05$ versus eNOS+IGFBP-3. n=3.
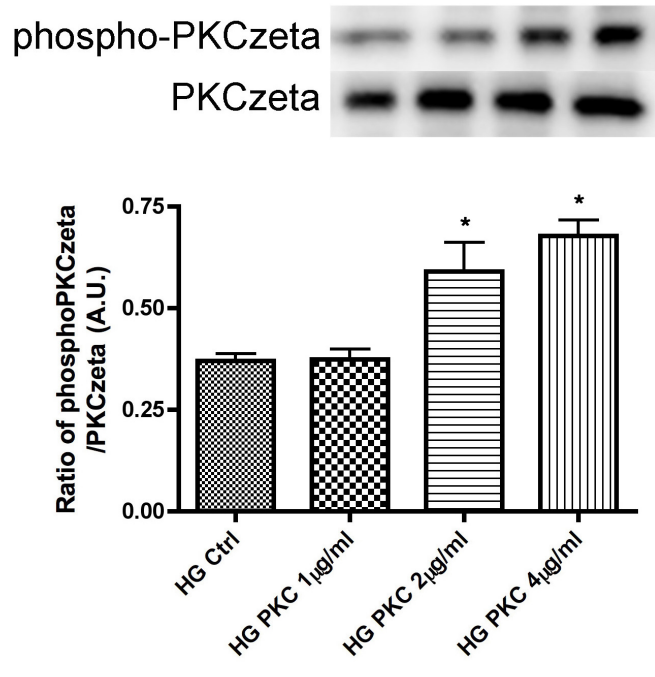

Figure 5. Dose curve of recombinant PRKCzeta on PKCzeta activity. Quiescent REC were treated with indicated doses of PRKCzeta in high-glucose conditions and analyzed by western blotting for phosphorylated PKCzeta/PKCzeta ratios. *$p < 0.05$ versus HG control. n=3. Data are mean ± SEM.

together, PKCzeta was not able to significantly increase VEGF, suggesting that IGFBP-3 actions lie upstream of PKCzeta.

DISCUSSION

Our results propose a novel pathway to show the IGF-1-independent actions of IGFBP-3 in the regulation of eNOS and PKCzeta cascades under conditions of high ambient glucose. Our data suggest that IGFBP-3 has a direct inhibition on VEGF levels in cells cultured under diabetic-like conditions. Using IGFBP-3NB transfection to increase intracellular IGFBP-3NB levels, we found a significant decrease in eNOS and PKCzeta activation. In addition, we also identify the relationship between PKC and NOS. Endothelial NOS enzymes led to the activation of PKCzeta phosphorylation, which may be dependent on the uptake/transport properties of REC. Therefore, we suggest that there are multiple signaling pathways contributing to the pathophysiological regulation of diabetic endothelial complications. One pathway involves a rapid elevation in NO synthesis, likely leading to activation of PKCzeta. Our data suggest that eNOS and PKCzeta interact in REC cultured in high glucose.

Our findings also support the work of others demonstrating that IGFBP-3 can decrease VEGF levels [13]. Our results presented here demonstrate that IGFBP-3 inhibition of VEGF under high glucose conditions is mediated through a reduction of the eNOS/PKCzeta signaling pathway.

The favorable metabolic phenotypes of IGFBP-3 observed here are accordant with other murine models in which IGFBP-3 overexpression had a protective role in diabetes [9,11,15]. A critical novel finding of the current study is that IGFBP-3 has a direct effect on the vascular endothelium, resulting in decreased eNOS activation. Research has revealed that free radicals or reactive oxygen species (ROS) create oxidative stress, which leads to a variety of pathological lesions in diabetes [34]. High glucose may lead to the production of ROS to cause endothelial injury and apoptosis [35]. Moreover, previous studies have found that radicals destroy the endothelium by changing NO production [36]. Indeed, in the present study, the data showed that NOS activation in retinal endothelial cells was markedly reduced by IGFBP-3 in high ambient glucose. The underlying mechanism by which IGFBP-3 protects the retinal endothelium from hyperglycemia-induced oxidative damage is an important question raised by the results presented in this study. Data with NAC suggest that some of IGFBP-3 actions are mediated through the reduction of peroxynitrite. Further experiments on IGFBP-3 and reactive oxygen species need to be completed.

PKCzeta was originally discovered as a unique PKC isotype and is classified into the atypical PKC subfamily based on its structure [37]. Here we have shown that activation of PKCzeta in high ambient glucose can be regulated by the overexpression of IGFBP-3, which was associated with decreased levels of VEGF. The molecular mechanism behind the inhibitory effect of IGFBP-3 on PKCzeta is intriguing. It has been reported that IGFBP-3/IGFBP-3 receptors interact with specific proteins involved with inflammation [18,19], suggesting that IGFBP-3 may regulate PKCzeta through the IGFBP-3 receptor. Additionally, in this work, immunoprecipitation with eNOS suggests a direct interaction of eNOS with PKCzeta. However, our findings are likely highly dependent on the unique uptake/transport of REC for these proteins.

Retinal endothelial cell dysfunctions are known to play a pivotal role in many complications of diabetic retinopathy.
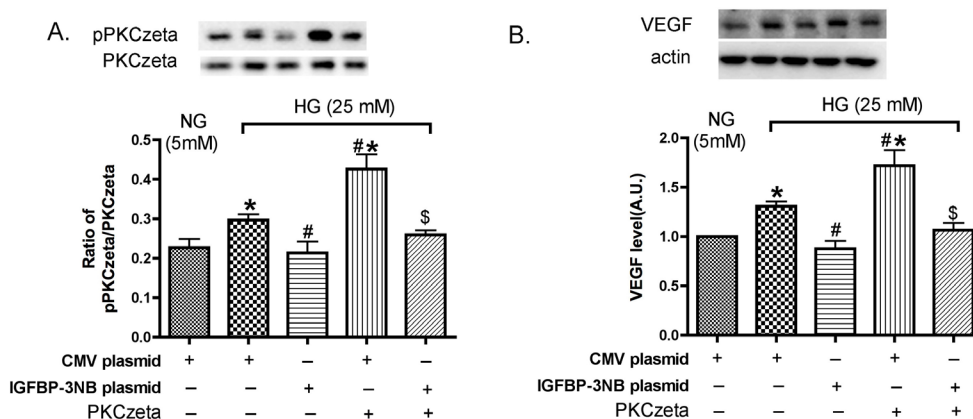

Figure 6. IGFBP-3 regulated VEGF level through PKCzeta activation. REC were treated with IGFBP-3 plasmid and PRKCzeta in REC in NG and HG media. **A**: Western blot data of the ratio of phosphorylated to total PKCzeta; **B**: Western blot results of VEGF levels; *p<0.05 versus NG control plasmid DNA transfection. #p<0.05 versus HG control plasmid DNA transfection. *p<0.05 versus HG IGFBP-3 plasmid DNA transfection. $p<0.05 versus HG IGFBP-3 control DNA transfection + PRKCzeta. n=3. Data are mean ± SEM.

Previous studies have demonstrated that IGFBP-3 demonstrates retinal endothelial cell protection in vivo and in vitro [17,38,39]. Given the importance of REC in diabetic retinopathy, the discovery of cellular mechanisms that offer protection to REC against the effects of high glucose may offer new strategies for understanding the overall disease process. Future studies detecting the regulation of IGFBP-3 systems will be important for creating effective treatments for diabetic retinopathy.

ACKNOWLEDGMENTS

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