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GSK-3 β /CREB axis mediates IGF-1-induced ECM/adhesion molecule expression, cell cycle progression and monolayer permeability in retinal capillary endothelial cells: Implication for Diabetic Retinopathy

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Key Words: IGF-1, GSK-3 β /CREB axis, ECM/adhesion molecule expression, cell cycle regulation, monolayer permeability

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ABSTRACT

Various growth factors and cytokines are implicated in endothelial dysfunction and blood retinal barrier (BRB) breakdown in early diabetic retinopathy (DR). However, cellular and molecular mechanisms that may underlie the pathology of DR are not fully understood yet. We therefore examined the effect of insulin-like growth factor (IGF)-1 on ECM/adhesion molecule expression, cell cycle regulation and monolayer permeability in an endothelial cell line (TR-iBRB2). We investigate whether the action of IGF-1 involves (1) glycogen synthase kinase 3beta (GSK-3 β) and cAMP responsive transcription factor (CREB) and (2) alters ECM/adhesion molecule gene expression. Treatment of TR-iBRB2 cell with IGF-1 (100 ng/ml for 0-24 h) increases phosphorylation of (i) Akt Thr308, and its substrates including GSK-3 β at Ser9, which inactivates its kinase function, and (ii) CREB at Ser133 (activation). These phosphorylations correlate positively with enhanced expression of CREB targets such as ECM protein fibronectin and cell cycle progression factor cyclin D1. However, stable transfection of a mutant GSK3 β (S9A), or a dominant negative K-CREB in TR-iBRB2 prevents IGF-1-induced fibronectin and cyclin D1 expression. Furthermore, IGF-1 reduces the level of intercellular adherence molecule VE-cadherin and increases monolayer permeability in TR-iBRB2 cells when measured by FITC-dextran leakage. The effect of IGF-1 on VE-cadherin and membrane permeability are absent in TR-iBRB2 cells expressing the GSK-3 β (S9A). Similarly, K-CREB reverses IGF-1 down-regulation of VE-cadherin and up-regulation of fibronectin. These results indicate that GSK-3 β /CREB axis alters ECM/adhesion molecule expression and cell cycle progression in retinal endothelial cells, and may potentially contribute to endothelial dysfunction and BRB leakage in DR.

Research Highlights:

- >GSK-3 β /CREB axis mediates aberrant ECM/cell adhesion molecule gene expression.
- >GSK-3 β /CREB axis regulates cell viability and cyclin D1 expression.
- >GSK-3 β mediates IGF-1-induced endothelial monolayer permeability.

>Stable expression of a constitutively active GSK3 β (Ser9Ala) or a dominant negative K-CREB blocks IGF-1's effects on aberrant gene expression and monolayer permeability.

> Modulation of GSK-3 β /CREB pathway may prevent microvascular dysfunction in diabetic retinopathy.

1. INTRODUCTION

Diabetic retinopathy (DR) is the most common cause of visual disorders leading to irreversible blindness. Chronic hyperglycemia-induced oxidative stress, decreased blood flow, hypoxia and growth factor-mediated altered gene expression are observed in the diabetic retina. The complications of DR are associated with capillary basement membrane thickening, blood-retinal-barrier (BRB) leakage, endothelial cell proliferation and neovascularisation, capillary cell apoptosis, and retinal detachment [1-3]. Among the various growth factors implicated in the development of DR, vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF) 2, and tumor necrosis growth factor (TNF)- α are well characterized [1]. Recent studies have shown that the insulin like growth factor (IGF)-1 and IGF-1 receptor system may be also involved in retinal endothelial cell proliferation and development of proliferative diabetic retinopathy (PDR) [3-5]. Grant and colleagues also demonstrated that knocking down the IGF-1 receptor by the expression of proliferating endothelial cell specific IGF-1 receptor ribosyme inhibits retinal neovascularization in mouse models of proliferative retinopathy [6]. Furthermore, in pig and rabbit models of DR, intravitreal injection of IGF-1 results in the breakdown of blood retinal barrier, basement membrane thickening and neovascularization similar to PDR [7, 8]. Transgenic mice overexpressing IGF-1 in the retina develop thickened vessel basement membrane and several similar clinical parameters of diabetic human eyes are also found [9].

Increased expression of extracellular matrix (ECM) proteins (e.g., fibronectin, laminin and collagen) and down regulation of intercellular adhesion molecules (e.g., VE-cadherin, claudins and occludins) are observed in the diabetic retina, which lead to altered cell-matrix and cell-cell interaction and hyper permeability of retinal capillaries [10-13]. In addition, an increased expression of

apical cell adhesion molecules such as ICAM1 and VCAM1 in diabetes can lead to leukocyte adhesion and endothelial inflammation of the retina [14].

Glycogen synthase kinase 3beta (GSK-3 β) is a signal mediator for the IGF-1 and PI3-K/Akt pathway that are required for cell proliferation and survival in many cell types including endothelial cells [15, 16]. Insulin and IGF-1 signaling involve activation of PI3K and the downstream Akt/PKB kinase, which in turn phosphorylates GSK-3 α at Ser 21 and GSK-3 β at Ser 9, and inhibits their kinase activities [17-19]. Both isoforms of GSK-3 have a similar range of substrate specificities and are regulated in response to growth factor stimulation. However, disruption of GSK-3 β gene in mice results in embryonic lethality, indicating that GSK-3 α may not completely substitute for a loss of GSK-3 β [15, 20]. Transcription factors are the signal convergent points for molecular signaling by growth factors and extracellular agents from where they mediate gene expression and signal diversification in target cells and organs. One such signal convergent site is the cyclic AMP responsive cis-element binding transcription factor CREB [21-23]. Most ECM genes contain cyclic AMP responsive element (CRE) sequences in their promoters and phosphorylation of CREB at Ser133 is considered to increase CREB/DNA interaction and gene expression [22]. We have previously shown that GSK-3 β is involved in IGF-1-induced CREB phosphorylation and downstream cyclin D1 and fibronectin expression in renal mesangial cells [15, 24].

In spite of the critical role(s) of GSK-3 β and CREB in cell proliferation, differentiation, survival and regulation of gene expression, putative involvement in endothelial dysregulation and DR remains unclear. Therefore, in the present investigation, we hypothesize that IGF-1 signaling in retinal endothelial cells involves the GSK-3 β /CREB axis and regulates ECM/adhesion molecule expression and monolayer hyperpermeability. Our results show for the first time that GSK-3 β and CREB mediate IGF-1-induced aberrant ECM/adhesion molecule gene expression and cell cycle progression, which may constitute a potential mechanism for endothelial dysfunction and BRB breakdown in DR.

2. MATERIAL AND METHODS

2.1. Materials. Insulin-like growth factor-1, and antibodies for cyclin D1, α -actin, and phosphorylated Akt at Thr308, total Akt antibodies, phosphorylated Akt substrate motif specific antibodies targeted at R-X-R-X-X-S*/T*p and total and phosphorylated CREB at S133 antibodies were purchased from Cell Signaling Technology (Danvers, MA). Anti-laminin γ 1, fibronectin, tubulin, phosphorylated and non-phosphorylated GSK-3 β antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Pan-cadherin and ICAM1 antibodies were obtained from Abcam (Cambridge, MA). SH-5 (Akt inhibitor) was from Alexis (San Diego, CA) while GSK-3 β inhibitor, SB216763, was obtained from Calbiochem (San Diego, CA). The enhanced chemiluminescence (ECL) system was obtained from Amersham (Arlington Heights, IL). DMEM and F-12 nutrient mixture (Ham's) were obtained from GIBCO (Grand Island, NY). Chicken anti-fibronectin antibodies were purchased from Molecular Probes (Invitrogen, Eugene, OR).

2.2. Cell culture. We used a temperature-sensitive rat retinal endothelial cell line (TR-iBRB2 for transgenic-eye blood-retinal barrier endothelial cell line 2), which differentiates normally at 37 °C but proliferates at 33 °C. TR-iBRB2 cells have been employed previously to investigate retinal endothelial cell permeability, drug transport across the inner BRB, and gene expression analysis under various conditions [25,26]. TR-iBRB2 cells express endothelial markers, such as von Willebrand factor (vWF), acetylated low density lipoprotein receptors, VEGF receptor 2 (KDF/Flk-1), glucose transporter Glut-1 and maintain other endothelial properties.

TR-iBRB2 cells were cultured in medium containing DMEM (low glucose, 5.5 mM) and Ham's F-12 (3:1 ratio) supplemented with 7.5% fetal calf serum (FCS) and 0.5 mg/ml gentamicin at 33 °C in a humidified chamber with a 5% CO₂-95% air mixture (26). Monolayers at 70-80% confluence were transferred to 37 °C and serum-deprived overnight (0.2% serum medium). As described previously in our manuscripts [15, 24], we performed an initial dose-dependent experiment of IGF-1 ranging from 0, 5, 25, 50, 100 and 150 ng/ml on TR-iBRB2 cell viability using MTT assay. We observed that 100 ng/ml IGF-1 gave

the optimal effect on TR-iBRB2 survival; therefore, chose this concentration of IGF-1 in subsequent experiments (Fig. S1). For the time course, IGF-1 with 24 h treatment was added first, then on the second day IGF-1 was added at 4 h and 0 (no addition) to respective cultures before harvesting. Thus, all cells maintain a similar condition and length of serum-deprivation during the entire experimental procedure [15]. Cells were harvested by scrapping, snap frozen and stored at -80°C until used.

2.3. SDS-PAGE, and Western blotting: Proteins were extracted in RIPA buffer containing protease inhibitors and their concentrations were determined using a Coomassie-based reagent from Pierce with BSA as the standard. Sodium dodecyl sulfate polyacrylamide gel electrophoresis and Western blot analysis of proteins were performed as described previously [15].

2.4. Real-time quantitative PCR: Total RNAs were isolated by TRIZOL method and first-strand cDNAs were synthesized in 20 μl volume using 1 μg RNA the Bio-Rad iScript cDNA synthesis kit [26]. mRNA expression was analyzed by real-time quantitative PCR using the MJ Bio-Rad Chromo 4 detection system and SYBR Green PCR Master Mix from Applied Biosystems (Foster City, CA). Primers were designed using Primer Express v 2.0 [Applied Biosystems] and synthesized by Invitrogen (Carlsbad, CA). Primers for the real-time Q-PCR are as follows:

1. Fibronectin: forward: 5'-CTGGGGTCACGTACCTCTTCA-3'
 Reverse: 5'-AGTCGGTAGCCTGCTATACGG-3';
2. Laminin γ 1: Forward: 5'- TGCTGTGATCTCAGACACTTACTTC -3'
 Reverse: 5'- TGAAGATGTACTTCACAGTGGTCTC-3'
3. Actin: Forward: 5'- GGCTGCCTCGAAATTACAACA -3'
 Reverse: 5'- CCAGCAGATTCCATACCAATGAAAG -3'

The real time PCR reaction mixture contained 1X SYBR Green PCR Master Mix, 400 nM forward and reverse primers, and 2 μl cDNA in a final volume of 25 μl . The PCR cycling was programmed as 95°C for 15s, 55°C for 30s and 72°C for 45s 40 cycles followed by the construction of a melting curve through increasing the temperature from 60°C to 95°C at a ramp rate of 2% for 20 min.

The real time PCR samples were evaluated using a single predominant peak as a quality control. Ct values were used to calculate the relative expression level of ECM mRNAs that were normalized to actin [26]

2.5. Determination of cell viability and DNA content: We used three approaches to measure cell viability and DNA content. (A) MTT assay for cell viability was performed in 24 well plates and with 0.5 mg/ml MTT in each well as previously described in our laboratory [24, 27]. TR-iBRB2 cells (1×10^4 cells) were grown to 70% confluence and serum-starved overnight. IGF-1 100 ng/ml was added for 4-24 h and MTT was added for 3 h. The media was removed and cells were kept in 100 μ l of DMSO for 10 minutes. The resulting color was diluted with 500 μ l of distilled H₂O and detected at 570 nm using a Gemini Microplate reader. (B) Cell Live/Death assay was measured with a fluorescence reagent from Molecular Probes as previously described [15, 27]. Briefly, to determine live and dead cells simultaneously, a fluorescent multiwell plate assay (Molecular Probes) was performed under conditions similar to the MTT assay using 24-well plates according to the manufacturer's instructions using calcein AM and ethidium homodimer-1. Live cells are measured by the conversion of cell-membrane permeable non-fluorescent calcein AM dye, which is converted by intracellular esterases to intensely fluorescent calcein at 515 nm when excited at 495 nm. On the other hand, EthD-1 enters cells with damaged membranes and undergoes a 40-fold increase in fluorescence upon binding to DNA (emission and excitation wavelengths at 528/617 nm). (C) DNA contents were determined with CyQUANT kit based on a fluorescence enhancement of dye binding to cellular nucleic acids as described recently [24]. After IGF-1 treatment, cells were washed with cold PBS three times and stored at -80 °C for 1 h. Plates were taken out of the freezer and incubated with DNase-free RNase (1.35 Units/ml) for 1 h at room temperature to digest RNA. DNA content was measured by CyQuant Dye binding at Ex480/Em520 with the bottom read and scanning device of a Spectra MAX Gemini EM Fluorescence microplate reader.

2.6. Stable transfection of GSK-3 β (S9A) and K-CREB expression vectors in TR-iBRB2

cells: Constitutively-active Ser 9 to Ala mutated GSK-3 β (S9A) and dominant negative K-CREB in pcDNA3.1 plasmids were used to transfect TR-iBRB2 cells. A corresponding empty pcDNA3.1 served as control cells. Expression vector for GSK-3 β (S9A) was kindly provided by Dr. Woodgett from the University of Toronto Health Sciences, Toronto, and K-CREB plasmid was purchased from Clontech. TR-iBRB2 cells were plated in 6-or 12-well plates and grown overnight to ~70-80% confluence and serum-starved. Cells were transfected with 2.0-4.0 μ g DNA per well in 100 μ l serum-free DMEM containing 20 μ l of Lipofectamine 2000 [Invitrogen] in duplicate wells. Six hours later, the media was replaced with serum containing DMEM:F-12 (3:1 ratio) medium and were further grown to reach 90-95% confluence at 33 $^{\circ}$ C. Stable cell lines were selected under the influence of G418 as previously described [15, 26]. Cell passages between 4-8 were used. The cells were transferred to 37 $^{\circ}$ C and further experiments were performed. The effect of GSK-3 β (S9A) and K-CREB was monitored by CRE promoter activity using a mammalian expression vector containing the CRE element with a reporter secretory alkaline phosphatase (CRE-SEAP) from Clontech. The CRE-SEAP vector was transiently transfected in pcDNA3.1, GSK-3 β (S9A) and K-CREB cells and SEAP activity was detected in the media using a fluorescence detection kit according to manufacturer's instructions.

2.7. In vitro endothelial monolayer permeability assay: The effects of IGF-1 on endothelial monolayer permeability were measured using an in vitro Vascular Permeability Assay Kit (Chemicon International) according to manufacturer's instructions. Briefly, 24 well tissue culture plates with semi-porous membrane inserts of 1.0 μ m symmetrical pores are coated with collagen 1 α . TR-iBRB2 cells were grown in these inserts to form confluent monolayer for 2 days at 33 $^{\circ}$ C. Afterwards, the cells were transferred to 37 $^{\circ}$ C in serum-deprived media (0.2% serum) overnight. Subsequently, IGF-1 (100 ng/ml) was added to the cells for another 24 h. The extent of monolayer permeability was determined on day 4 by adding FITC-Dextran provided in the kit on top of the cells for 30 minutes. Hundred μ l of

the media from the lower chamber was measured for fluorescent detection in 96 wells with a SpectraMAX GeminiEM Fluoremeter at ex485/em530 nm to determine the extent of leakage.

2.8. Statistical Analysis: Results are expressed as means +/-SE. Student's t-test or one way ANOVA followed by Bonferroni Post Hoc Test was used to compare differences between treatment conditions. A preset P value of <0.05 was considered statistically significant.

3. RESULT

3.1. IGF-1 activates Akt/GSK-3 β /CREB pathway and induces cell cycle progression and ECM protein expression in retinal endothelial cells: To understand the signaling mechanism of IGF-1 in endothelial gene expression, we examined different downstream signaling targets of IGF-1 receptor including Akt/GSK-3 β /CREB axis as previously described for renal mesangial cells [15, 24]. TR-iBRB2 cells were treated with IGF-1 (100 ng/ml) for 0, 4, and 24 h and total cellular extracts were subjected to Western blotting with primary antibodies targeted against phosphorylated Akt at Thr308, and total Akt. In addition, a phosphorylated Akt peptide motif (R-X-R-X-X-S*/T*p) specific antibody, which recognizes most phosphoproteins that are targeted by Akt, was also used to determine Akt activation and substrate phosphorylation. As shown in Figure 1A, IGF-1 increases Akt phosphorylation at Thr308 (upper panel), and several Akt targeted phosphoproteins of different molecular sizes (as indicated by arrows, lower panel). This result demonstrates that IGF-1 increases Akt activity and induces phosphorylation of several proteins including a protein at 48-kDa. The 48-kDa protein is the predicted molecular size of an Akt target protein, GSK-3 β . In agreement, the extent of GSK-3 β (S9) phosphorylation is significantly increased by IGF-1 at 4 h and continues up to 24 h period (p<0.01 versus time 0 for both) (Figure 1B). However, total GSK-3 β level is not changed by IGF-1 treatment. Furthermore, downstream transcription factor CREB phosphorylation at Ser 133 is also increased whereas total CREB content is not altered (Figure 1C). We have previously shown that CREB is

involved in gene transcription for cell cycle regulation and ECMs (24, 28); therefore, we further examined the expression of cell cycle control protein cyclin D1 and sclerotic fibronectin. As shown in Figure 2, fibronectin (A) and cyclin D1 (B) expressions are increased by IGF-1 significantly at 4 and 24 h ($p < 0.05$ versus time 0, $n=3$). These data indicate that IGF-1 signaling leads to intracellular Akt/GSK-3 β /CREB pathway activation and correlates positively with cell cycle progression and ECM expression.

3.2. IGF-1 enhances endothelial cell viability and DNA synthesis: IGF-1 is a survival and proliferation factor for various cell types including the retina and Akt/GSK-3 β plays important roles in these processes. Therefore, we investigated further if IGF-1 treatment of TR-iBRB2 enhances cell viability and DNA synthesis. IGF-1 treatment of TR-iBRB2 increases viability in a time-dependent manner at 24 h ($p < 0.05$ versus time 0) as measured by MTT and Live/Death assays (Figure 3A-B). Addition of SH-5, an inhibitor of Akt, blocks IGF-1 induced cell survival both at 4 and 24 h and significantly at 24 h ($p < 0.05$ versus time 0, Supplementary Fig. S2, darker bars) while the addition of SB216763, an inhibitor of GSK-3 β , further increases IGF-1 effects on TR-iBRB2 survival (Fig. S2, lighter bars). GSK-3 β is a negative regulator of cell growth and viability, therefore, IGF-1 effect is synergistically enhanced by GSK-3 β inhibitor while an inhibition of Akt leads to blockade of IGF-1-induced cell viability (Fig. S2, darker bars). IGF-1 also increases DNA content in TR-iBRB2 as measured by fluorescent CYQUANT binding to deoxynucleic acids (significant at 24 h, $p < 0.05$ versus time 0, Figure 3C). The results indicate that IGF-1 is a survival and cell cycle progression factor for retinal capillary endothelial cells in culture.

3.3. GSK-3 β and CREB regulate IGF-1 effects on retinal cell viability and gene expression for ECM and cyclin D1 in endothelial cells: To further understand the contribution of GSK-3 β and transcription factor CREB in EC survival and gene expression, we employed cDNA expression vectors for a constitutively active GSK-3 β Ser9A and a dominant negative K-CREB lacking the DNA binding activity. We established stable TR-iBRB2 cells expressing GSK-3 β (S9A) and K-CREB using G419

pressure. The effect of stable expression of GSK-3 β (S9A) or K-CREB was first established by blockade of a CRE-reporter activity for secretory alkaline phosphatase (SEAP) in TR-iBRB2 cells as shown in Fig. S3A. We found that expression of GSK-3 β (S9A) or K-CREB blocks CRE-SEAP activity in these cells suggesting that these proteins are active and functional intracellularly. IGF-1-induced cell viability is significantly blocked by GSK-3 β (S9A) and K-CREB transfection (Figure 4A) and K-CREB expressing cells have significantly lower survival rates at 24 h. Our data further show that IGF-1 induces fibronectin mRNA expression significantly in pcDNA3.1 control cells (Figure 4B), which is reduced by both GSK-3 β (S9A) and K-CREB though GSK-3 β (S9A) is more efficient (Figure 4B). In addition, we have previously shown that IGF-1 increases laminin γ 1 expression, another ECM molecule in renal cells [15, 24]. In agreement, IGF-1 also enhances laminin γ 1 protein levels in TR-iBRB2 cells both at 4 and 24 h (Fig. S3B), and GSK-3 β (S9A) and K-CREB completely blocks laminin γ 1 mRNA expression as well (Fig. S3C). Both GSK-3 β (S9A) and K-CREB block IGF-1-induced cyclin D1 expression in TR-iBRB2 cells when compared to pcDNA3.1 overexpressing cells (Figure 4C). We did not observe a change in overall phosphorylation of the endogenous GSK-3 β or CREB in pcDNA3.1 control when compared with GSK-3 β (S9A) or K-CREB overexpressing cells (not shown). Therefore, we propose that the effects of GSK-3 β (S9A) or K-CREB on ECM and cyclin D1 expression may be due to an interruption of the endogenous GSK-3 β or CREB function.

3.4. IGF-1 down regulates VE-cadherin and increases endothelial monolayer permeability: We next examined whether IGF-1 alters the expression of cell adhesion junction molecule VE-cadherin, which is involved in cell-cell adhesion and membrane permeability. As shown in Figure 5A, IGF-1 significantly reduces VE-cadherin expression (~2.5 folds, $p < 0.001$ versus control, $n=4$) in TR-iBRB2 cells. However, under these experimental conditions, we did not observe a significant effect of IGF-1 on ICAM1 expression (Figure 5A), which is an apical membrane protein involved in leukocyte adhesion. Our data show that IGF-1 increases TR-iBRB2 monolayer permeability significantly as determined by FITC-dextran leakage in a transwell membrane in culture

(Figure 5B, $p < 0.05$ versus control). In addition, we also measured the trans-endothelial/epithelial electrical resistance (TEER) using an EVOM² Epithelial Voltohmmeter fitted with a STX2 electrode after 4 days of TR-iBRB2 culture. A reduction in TEER value (~11.6%, $p < 0.05$) is observed after IGF-1 treatment for 24 h when compared with the control TEER (Fig. S4), which is in close agreement with the FITC-dextran leakage observed in Figure 5B. The effects of GSK-3 β in IGF-1-induced VE-cadherin down-regulation and endothelial monolayer permeability are shown in Figure 5C-D. Specifically, stable expression of GSK-3 β (S9A) in TR-iBRB2 cells blunts IGF-1-induced down-regulation of VE-cadherin and monolayer permeability. ICAM1 and tubulin levels are not affected by GSK-3 β (S9A) expression in TR-iBRB2 cells.

Involvement of CREB in IGF-1-induced VE-cadherin expression is also shown in Figure 5E. Stable K-CREB expression in TR-iBRB2 cell prevents IGF-1 down-regulation of VE-cadherin while in pcDNA 3.1 control cells IGF-1 still stimulates VE-cadherin suppression. However, due to the low viability of TR-iBRB2 cells upon K-CREB expression (Figure 4A), the monolayer permeability assay was not performed. Together, the data presented here demonstrate that IGF-1 causes VE-cadherin down regulation, enhanced ECM expression and cell cycle progression. These cellular effects may be responsible for the observed endothelial monolayer permeability and suggest a potential role for the GSK-3 β /CREB axis in vascular dysregulation in DR.

5. DISCUSSION

In this study, we demonstrate for the first time, that the GSK-3 β /CREB axis is involved in IGF-1-mediated retinal capillary endothelial cell survival, ECM/adhesion molecule gene expression, cell cycle regulation, and endothelial monolayer permeability in *in vitro* cultures. Specifically, (i) IGF-1 enhances phosphorylation of Akt at Thr-308 and its downstream target GSK-3 β at Ser 9, which increases TR-iBRB2 viability as measured by MTT and Live/Dead assays; (ii) IGF-1 increases the level of cell cycle progression factor cyclin D1 and DNA content; (iii) IGF-1 induces ECM expression

(fibronectin and laminin γ 1), (iv) down-regulates cell adhesion molecule VE-cadherin expression and GSK-3 β and transcription factor CREB may play a role in this process; and (v) finally, IGF-1 induces endothelial monolayer permeability, which is prevented by stable expression of a constitutively active form of GSK-3 β (S9A). Our findings provide new insights as to how growth factors may regulate intracellular signaling pathway(s) in retinal capillary endothelial cells and alter gene expression for cell-cell and cell-matrix interaction that lead to endothelial dysfunction and vessel leakage observed in diabetic ocular diseases. Furthermore, the intracellular Akt/GSK-3 β pathway is also used by different external stimuli including angiogenic growth factors such as VEGF-A, FGF-2, and BDNF [29, 30, 31] and integrins upon cell-matrix interaction [32] to regulate endothelial membrane permeability, cell viability, proliferation and angiogenesis further emphasizing the importance of this pathway in cellular physiology.

Our results further demonstrate that IGF-1 increases phosphorylation of cyclic AMP responsive element transcription factor CREB at Ser 133 in retinal endothelial cells and responsible for inducing ECM genes for fibronectin and laminin γ 1 and down-regulation of intercellular adhesion molecule VE-cadherin. Dominant-negative K-CREB expression in retinal endothelial cells blocks the IGF-1-induced VE-cadherin down-regulation. Furthermore, IGF-1-induced VE-cadherin down regulation and membrane permeability are reversed by stable expression of a constitutively active GSK-3 β in which Ser 9 is replaced by an alanine. The mechanism by which IGF-1 and/or GSK-3 β /CREB axis cause VE-cadherin degradation is not understood at present. However, it has been demonstrated that matrix metalloproteinase 2 (MMP2) is responsible for VE-cadherin degradation and retinal blood vessel leakage in diabetes rats [13]. In addition, IGF-1 induces membrane type 1 matrix metalloproteinase (MT1-MMP) expression in a PI3K/Akt dependent manner, which in turn activates extracellular MMP2 protease [33]. Furthermore, phosphorylation of CREB at S133 activates both MT1-MMP and MMP2 expression in various cell types including human vascular endothelial cells [34]. Our data showing that both GSK-3 β (S9A) and K-CREB block IGF-1-induced VE-cadherin degradation suggest the

participation of MT1-MMP and MMP2 in this process. [However, GSK-3 β and CREB might also directly affect VE-cadherin expression itself.] Currently, it is unknown how GSK-3 β inhibition leads to enhanced CREB phosphorylation at S133. GSK-3 β does not target CREB S133 directly but phosphorylates at S129, a motif created by phosphorylated S133 [35]. GSK-3 β inhibitors have been suggested as potential therapies for enhancing insulin-mediated glycogen storage in insulin-sensitive cells in diabetes [36-38]; however, a role for GSK-3 β and CREB phosphorylation in retinal capillary endothelial (dys)function and the development of diabetic retinopathy is yet to be determined. In case of cyclin D1 down-regulation by GSK-3 β , it is known that cyclin D1 phosphorylation by GSK-3 β leads to its nuclear export and proteasome degradation [39].

GSK-3 β is a negative regulator of growth factor signaling and cell survival. In general, GSK-3 β activity is higher in resting cells and its phosphorylation at Ser 9 is low. Growth factor addition leads to Akt-dependent phosphorylation of GSK-3 β at Ser 9 and inactivates its kinase function [18]. GSK-3 β inactivation up-regulates the function of several down-stream target proteins including transcription factor CREB (as demonstrated in this study), beta-catenin [40], NF- κ B [41], NFAT [42, 43], all of which are considered to involve in cell growth, survival, and proliferation of endothelial cells. We recently showed that both NF- κ B and CREB are involved in fibronectin and pro-inflammatory cytokine interleukin (IL-1 β) expression in primary cultures of Schwann cells and sciatic nerve injury [44]. This may also explain why stable K-CREB expression alone in TR-iBRB2 does not reduce the IGF-1-mediated fibronectin expression completely to the level of the K-CREB control cells without IGF-1 treatment (Figure 4B). We have previously shown that CREB is also involved in ECM gene induction in renal mesangial cells by IGF-1 and under high ambient glucose condition [23, 24, 45] and that CREB phosphorylation is increased in diabetic renal cortex, which correlated positively with ECM gene expression in the mesangium [15, 27]. In this study, we demonstrate that fibronectin and laminin γ 1 expressions are increased by IGF-1 in retina endothelial cells and that the GSK-3 β /CREB pathway is involved in this process (Figure 4B and Figure S3C). In concert with this observation, we also found

that the level of CREB phosphorylation and fibronectin and laminin $\gamma 1$ expressions are elevated in the retina of STZ-induced diabetic rats (Fig. S5). Thus, excessive ECM accumulation (enhanced expression/decreased degradation) and capillary basement membrane thickening could potentially disturb endothelial cell-matrix interaction, integrin signaling and cell-junction function, leading to capillary leakage [10, 12, 13] and clinical vasculopathy in DR..

An important aspect of endothelial permeability barrier is the integrity of the intercellular adhesion molecules between adjacent cells. Permeability of endothelial junctions is affected by various vasoactive agents and adhesion of circulating monocytes at apical cell surface adhesion molecules such as ICAM1, VCAM1, etc., during inflammation [14]. In this study, we observed that IGF-1 down-regulates VE-cadherin, and induces ECM, and cell cycle progression factor cyclin D1 expression, but not of ICAM1. On the other hand, treatment of TR-iBRB2 cells with high glucose (25 mM versus 5 mM glucose) for 3 days also decreases VE-cadherin and increases ECM expression (Fig. S6); however, there is no endothelial membrane leakage under these experimental conditions. Addition of IGF-1 and high glucose together produced marginal enhancement of IGF-1 effects on endothelial monolayer permeability in transwell cultures but not significant suggesting that various signaling mechanisms operate to induce endothelial barrier opening in addition to cell adhesion molecule suppression alone. Busik et. al. [47] demonstrated that hyperglycemia itself is not sufficient to induce oxidative stress and activation of pro-inflammatory and apoptotic pathways in human retinal endothelial cells but requires glucose-induced cytokine expression from adjacent cells such as Muller glia. We could consider here that IGF-1 and growth factors/cytokines induce cell growth and alter ECM/adhesion molecule expression in endothelial cells under diabetic conditions, which result in morphology changes and cell rounding [48, 49], leading intercellular junction loosening and membrane permeability as depicted in Figure 6. VE-cadherin itself also plays an important role in the regulation of both adherens junction and tight junction by upregulating claudin 5 in a PI3K/Akt and FOXO1 dependent pathway [50]. Thus, down-regulation of VE-cadherin by IGF-1 and GSK-3 β /CREB could potentially lead to further

inhibition of other adhesion molecules such as claudins. In such a scenario, maintaining an optimal GSK-3 β activity may be advantageous for upholding the differentiated state of retinal endothelial cells and maintaining the endothelial cell membrane integrity. We demonstrate in this study that the expression of an active form of GSK-3 β (S9A) in retinal capillary endothelial cells may be sufficient to prevent IGF-1-induced ECM and VE-cadherin expression and monolayer hyperpermeability. In addition, we can't rule out that increased IGF-1 in the vitreous humor in diabetes [3-5] may interact further with insulin receptor and further amplify its action on Akt/GSK-3 β and CREB phosphorylation. Retina EC cells express both IGF-1 and insulin receptors and high glucose may increase insulin receptor expression (Fig. S7). Further studies will delineate the putative role of IGF-1 and insulin receptors in retinal endothelial cells under high ambient glucose and the signaling mechanisms.

In conclusion, our results provide a potential role of the GSK-3 β /CREB axis in IGF-1/growth factor-mediated aberrant ECM/adhesion molecule expression, cell cycle regulation and endothelial monolayer hyperpermeability. Alterations in ECM and adhesion molecule expression in retinal endothelial cells and blood vessel leakage in diabetes have been well studied [11, 51, 52]. However, further investigation is necessary to establish a potential role of the GSK-3 β /CREB/cyclin D1 and altered ECM expression and vessel thickening in microvascular dysfunction and blood retinal barrier breakdown in models of diabetic retinopathy as well as diabetic patients.

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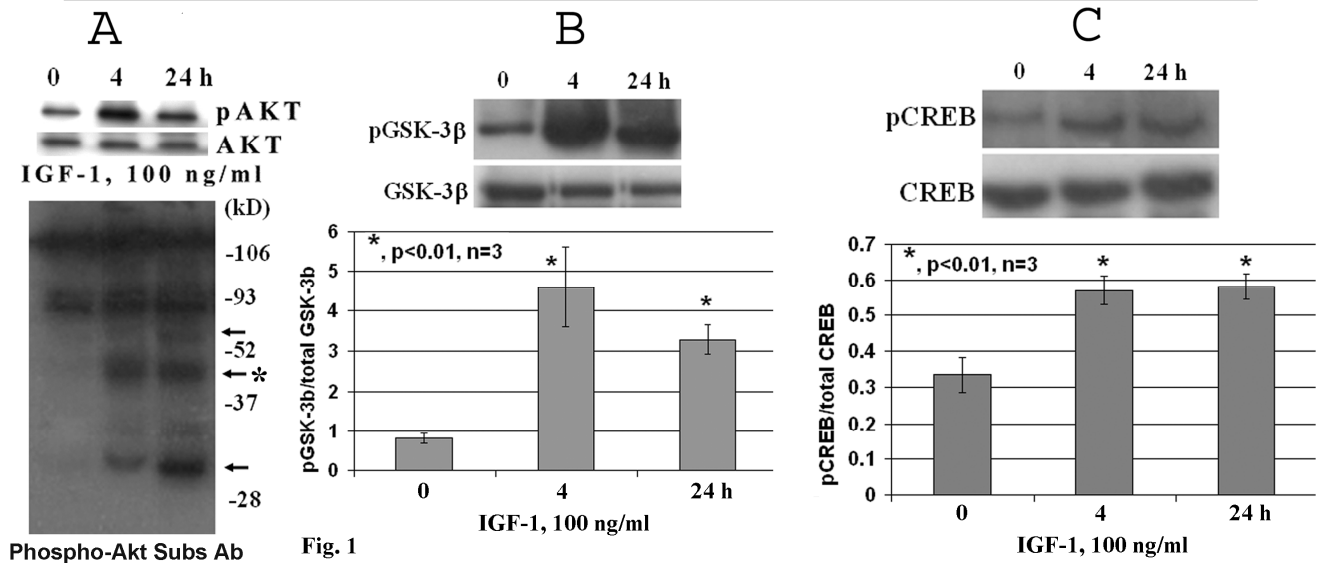


Fig. 1

Figure 1. IGF-1 activates AKT/GSK-3 β /CREB signaling in retinal capillary endothelial cells in culture. TR-iBRB2 cells were treated with IGF-1 (100 ng/ml) for 0, 4, 24 h and proteins were extracted in RIPA buffer and 30 μ g proteins were subjected to Western blotting. (A) IGF-1 (100 ng/ml) induces Akt phosphorylation at Thr 308 in TR-iBRB2 cells (upper panel). IGF-1 also increases phosphorylation of several Akt targets at 4 and 24 h including hyperphosphorylated proteins at 48- (* molecular size of GSK-3 β) and 32-kDa (arrow, lower panel) using an antibody that recognizes phosphorylated Akt substrates. The blot is a representative of 3 different experiments. (B) IGF-1 increases GSK-3 β Ser 9 phosphorylation at 4 and 24 h ~4.5 and 3.3-folds, respectively, when compared with controls at 0 time (*, $p < 0.05$ for both). Densitometric analysis of Western blots for phosphorylated GSK-3 β at Ser 9 and CREB at Ser 133 were performed and normalized by total GSK-3 β and CREB, respectively. Representative blots of phosphorylation of GSK-3 β at Ser 9, total GSK-3 β and actin are shown ($n = 3$). (C) IGF-1 increases phosphorylation of CREB at Ser 133 approximately ~3-folds at 4 and 24 h (*, $p < 0.05$ versus time 0, $n = 3$). Phosphorylated CREB at Ser 133 and total CREB were measured on Western blots and their densitometric ratio of CREB_p/CREB was determined.

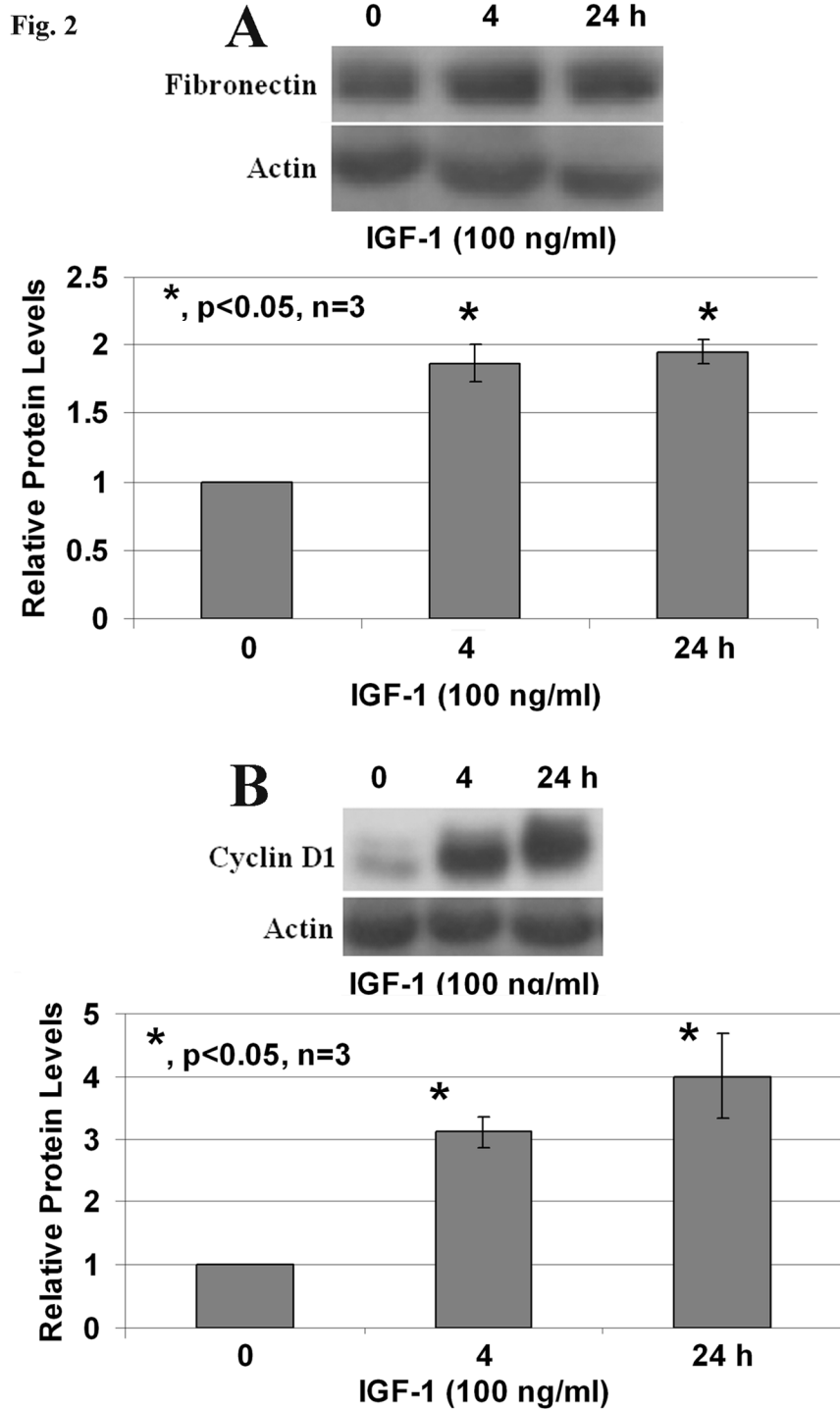


Figure 2. IGF-1 increases fibronectin and cyclin D1 expression in retinal capillary endothelial cells. (A) Treatment of TR-iBRB2 cells with IGF-1 increases fibronectin levels significantly at 4 and 24 h (~2-fold; $p<0.05$ versus time 0; $n=3$). Actin was not changed. Panel below shows densitometric analysis of fibronectin bands in gels. (B) Similarly, cyclin D1 is also increased by IGF-1 at 4 and 24 h significantly ($p<0.05$; $n=3$). Panel below shows cyclin D1 densitometric analysis.

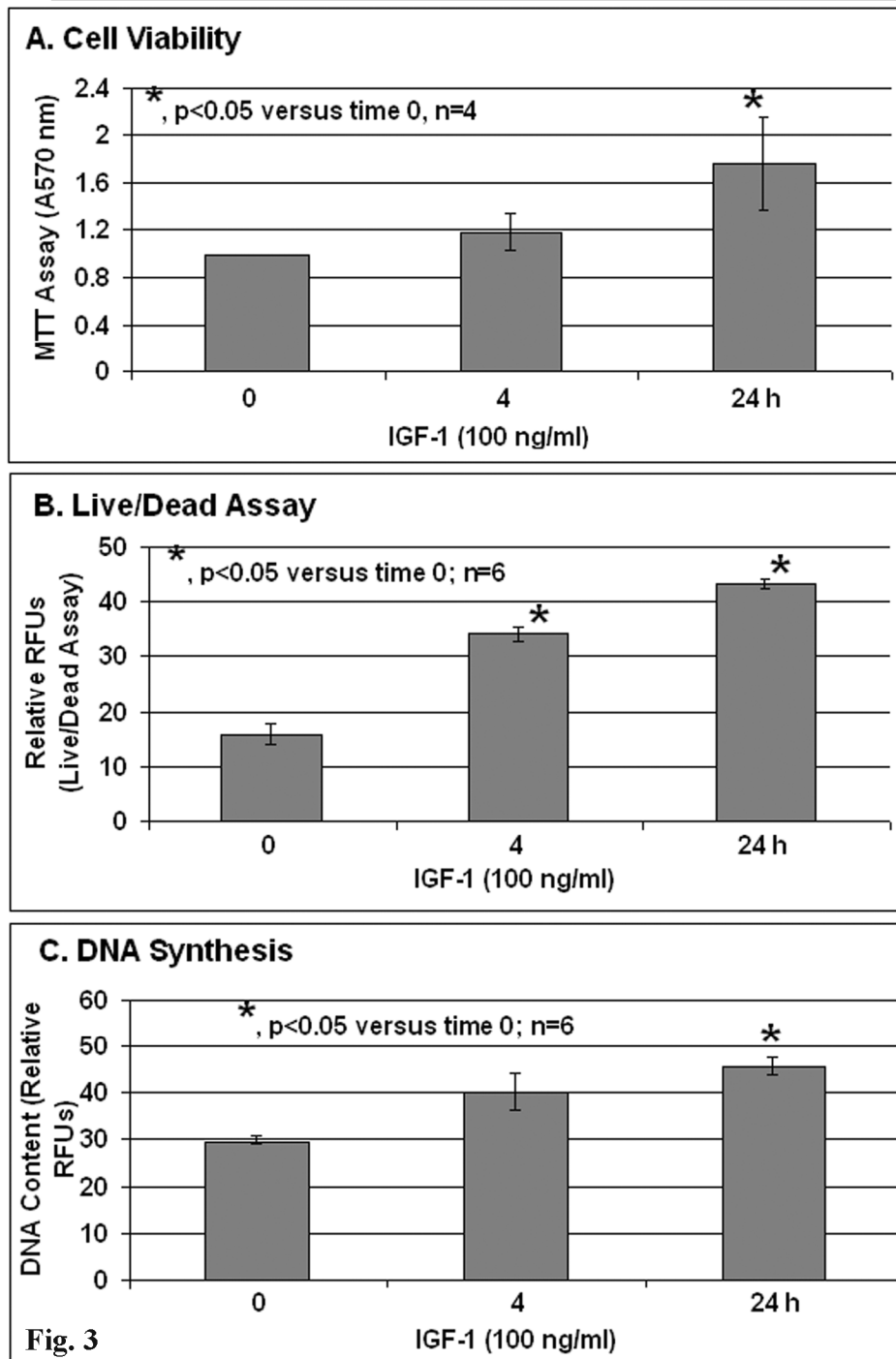
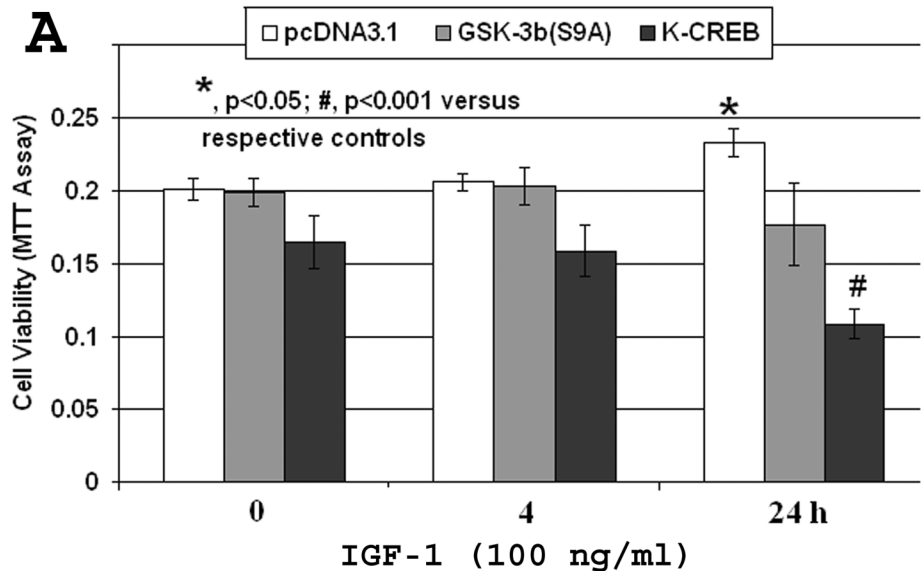


Figure 3. IGF-1 increases cell viability and DNA synthesis in retinal capillary endothelial cells. (A) Treatment of TR-iBRB2 cells with IGF-1 for 24 h increases cell survival significantly (*, $p < 0.05$ versus 0 time, $n = 4$) in MTT assays. (B) IGF-1-induced TR-iBRB2 survival is also measured by Live/Death Fluorescence assays ($p < 0.05$ at both 4 and 24 h vs. 0 time; $n = 6$). (C) IGF-1 increases DNA content (relative fluorescence units) significantly (*, $p < 0.05$ versus 0 time, $n = 6$) after 24 h but not at 4 h.



B Fibronectin

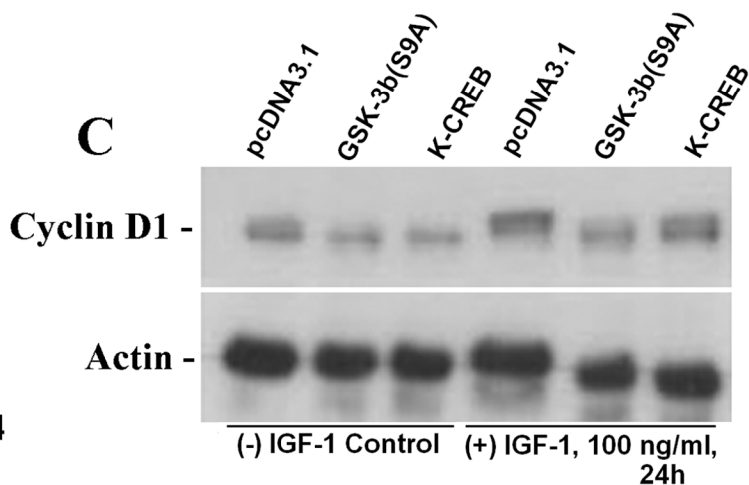
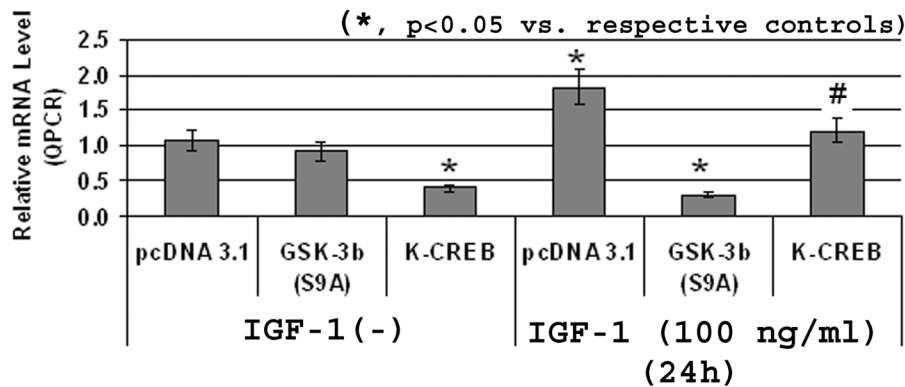


Fig. 4

Figure 4. Stable GSK-3 β (S9A) and K-CREB expression prevents IGF-1-induced endothelial cell survival and fibronectin and cyclin D1 expression. TR-iBRB2 cells were stably transfected with pcDNA3.1 (control), GSK-3 β (S9A) or K-CREB under the pressure of antibiotic G418. (A) IGF-1 increases TR-iBRB2 cell survival in pcDNA3.1 controls at 24 h as revealed by MTT

assays (*, $p < 0.05$ versus pcDNA3.1 at 0 time, $n=6$); however, GSK-3 β (S9A) expression blocks IGF-1's ability to induce cell survival at 24 h (grey bar). Furthermore, K-CREB expression itself reduces cell viability at 0 time and significantly blunts IGF-1 effects at 24 h (~50% reduction at 24 h; #, $p < 0.001$ versus pcDNA 3.1+IGF-1, $n=6$, dark bar). (B) Total RNA was isolated by TRIZOL from stably transfected cells with or without IGF-1 treatment and mRNA levels for fibronectin was measured by RT-qPCR. They were normalized to actin. IGF-1 increases fibronectin mRNA expression significantly in pcDNA3.1 cells (~1.7-fold; *, $p < 0.05$ versus control, $n=4$). However, GSK-3 β (S9A) completely blocks IGF-1-induced fibronectin mRNA expression without a significant effect on basal expression. On the other hand, K-CREB affects both basal and IGF-1-induced fibronectin expression in TR-iBRB2 cells (#, $p < 0.05$, IGF-1+pcDNA3.1 versus IGF-1+K-CREB, $n=4$). (C). Stable GSK-3 β (S9A) and K-CREB expression in TR-iBRB2 also blocks Cyclin D1 expression. A representative of $n=2$ is shown.

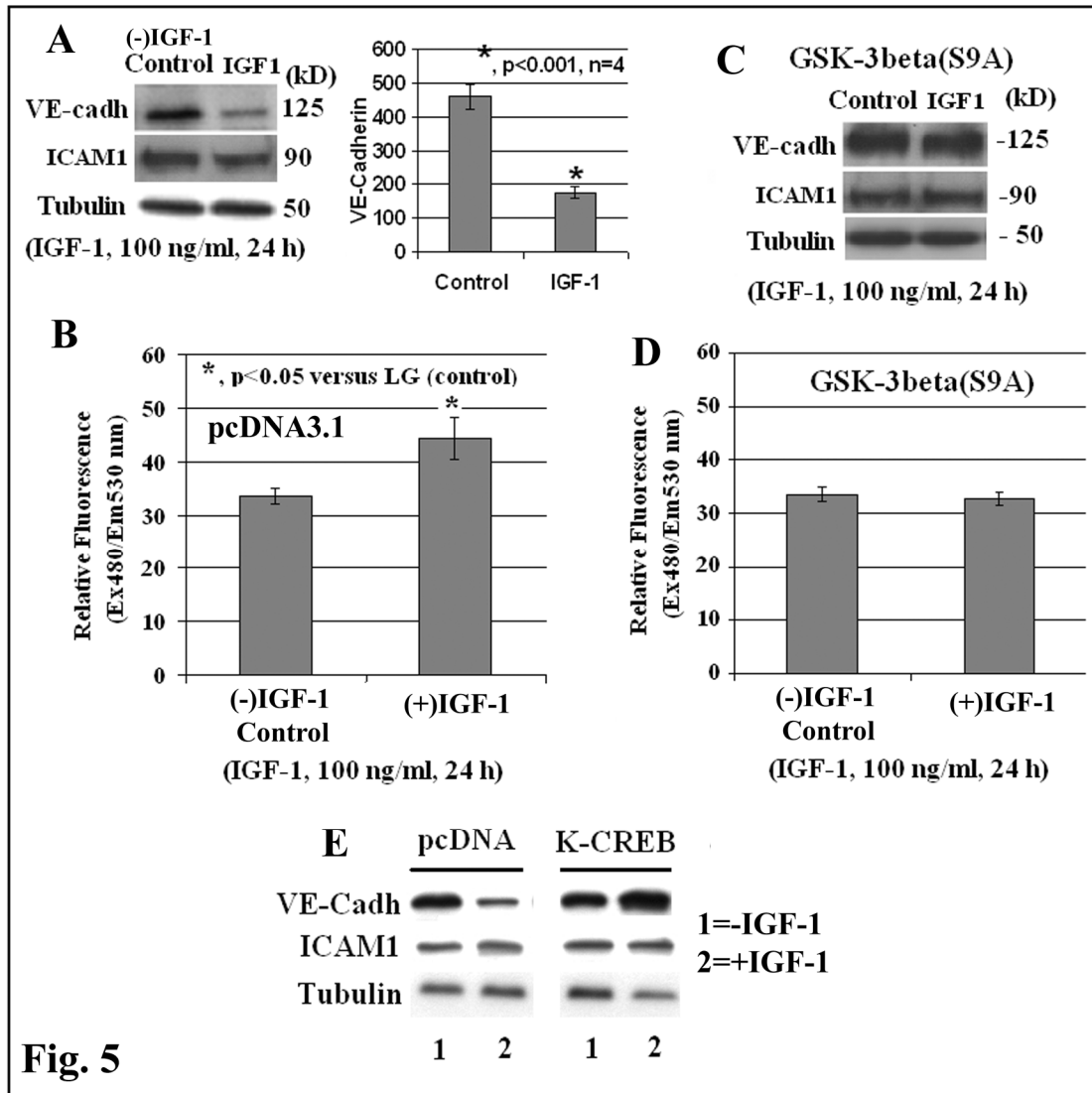


Fig. 5

Figure 5. IGF-1 down regulates VE-cadherin expression and increases endothelial monolayer permeability. (A) VE-cadherin expression was detected using pan-cadherin antibodies in control (no IGF-1) or IGF-1 (100 ng/ml, 24 h) treated pcDNA 3.1 cell extracts. IGF-1 down regulates VE-cadherin expression significantly (~2.7 folds less than the control; *, p<0.001, n=4). Left panel show a representative blot and right panel show the densitometric analysis. IGF-1 has no effect on ICAM1 expression, an apical membrane adhesion molecule (p=0.2; n=4). (B) IGF-1 increases endothelial monolayer permeability in pcDNA 3.1 transfected TR-iBRB2 cells using semi-permeable trans-well cultures (~12.5*, p<0.05 versus control, n=6) and determined by FITC-dextran leakage as described in Methods. (C) Stable overexpression of the constitutively active GSK-3 β (S9A) in TR-iBRB2 cells impedes IGF-1 down regulation of VE-cadherin (p=0.2 for control versus IGF-1, n=4). No change in ICAM1 is observed (middle panel). (D) GSK-3 β (S9A) overexpression blocks IGF-1-induced monolayer leakage of FITC-dextran (p=ns, not significant between control and IGF-1 treated cells, n=6). (E) In pcDNA 3.1 cells, IGF-1 decreases VE-cadherin expression (left panel). However, in

K-CREB expressing cells, IGF-1 is unable to down-regulate VE-cadherin expression (right panel). ICAM1 and tubulin levels are not affected by IGF-1 either in pcDNA 3.1 or K-CREB expressing cells. A representative n=3 is shown.

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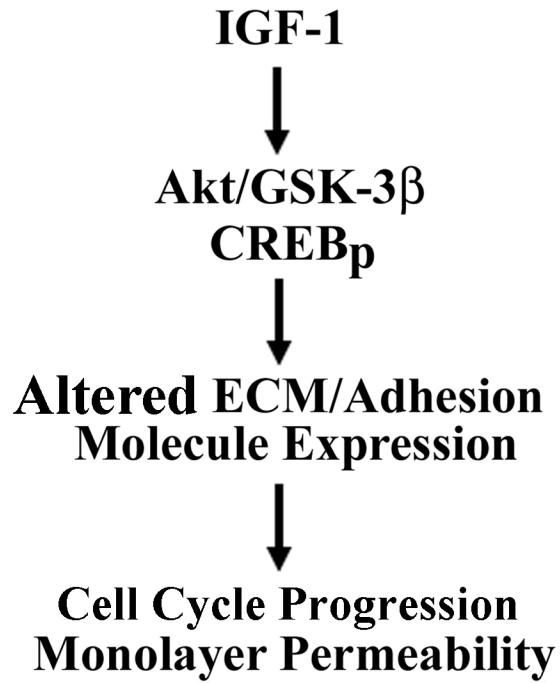
Retinal Capillary EC

Fig. 6

Figure 6. A schematic presentation of IGF-1 signaling, ECM/adhesion molecule expression and retinal endothelial dysregulation. IGF-1 increases Akt/GSK-3 β signaling leading CREB phosphorylation and altered ECM/adhesion molecule expression and cell cycle progression (cyclin D1 expression). This potentially leads to retinal endothelial monolayer hyperpermeability in trans-well cultures. These *in vitro* findings provide a potential mechanism for retinal capillary endothelial dysfunction, BRB breakdown and vessel leakage in diabetic retinopathy.