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Glucose-induced serum- and glucocorticoid-regulated kinase activation in oncofetal fibronectin expression

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Abstract

Preferential expression of oncofetal extra domain-B fibronectin (EDB^+ FN), a proposed angiogenic marker, has been shown in proliferative diabetic retinopathy. High levels of glucose also increase EDB^+ FN expression in endothelial cells (ECs) via transforming growth factor-β1 (TGF-β1) and endothelin-1 (ET-1). The present study was aimed at elucidating the role of serum- and glucocorticoid-regulated kinase (SGK-1) in glucose-induced EDB^+ FN expression. Using human macro- and microvascular ECs, we show that high levels of glucose, TGF-β1, and ET-1 increase the EDB^+ FN expression via SGK-1 alteration at the mRNA, protein, and activity levels. Inhibition of TGF-β1 and ET-1 prevented glucose-induced SGK-1 activation and the EDB^+ FN expression. Furthermore, using siRNA-mediated SGK-1 gene silencing, we show that glucose-induced EDB^+ FN expression can be completely prevented. These findings provide first evidence of glucose-induced SGK-1 activation in altered EDB^+ FN expression and provide novel avenues for therapeutic modalities.

Keywords: Serum- and glucocorticoid-regulated kinase; Oncofetal fibronectin; Extracellular matrix; ET-1; TGF-β1

Chronic diabetic complications manifest as vascular anomalies in a number of target organs [1,2]. Sustained hyperglycemia leads to the alteration of a number of biochemical pathways resulting in cellular dysfunction, non-perfusion, increased permeability, and capillary basement membrane thickening [1–3]. The structural hallmark of these secondary complications is the increased expression of basement membrane proteins, namely collagen and fibronectin (FN) [4–7]. We and others have shown that diabetes in vivo and high levels of glucose in vitro cause increased expression of FN in the target organs and the endothelial cells, respectively [4–9]. Endothelial cells are the major cell types which exhibit early biosynthetic features of diabetic complications. In diabetic retinopathy, a condition which represents the most profound effect of chronic diabetes, FN has been shown to be the major contribution of capillary endothelial cells compared to the bystander cells [10].

FN plays key roles in various cellular events which include proliferation of endothelial cells [11]. We have previously demonstrated that FN mediates endothelial cell proliferation by undergoing alterative splicing [8,12]. Such processing of FN mRNA produces embryonic variants, EDB^+ oncofetal FN species, that were traditionally believed to be exclusively expressed by neoplastic blood vessels [13]. We, however, provided first clear

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evidence that EDB$^+$ oncofetal FN is preferentially expressed in diabetic retinopathy which entails increased proliferation of endothelial cells and unregulated angiogenesis [8]. We have further shown that EDB$^+$ oncofetal FN induces endothelial-derived vascular endothelial growth factor expression, increased proliferative capacity, and endothelial morphogenesis [12]. These findings provide evidence of functional significance of FN and specifically, EDB$^+$ oncofetal FN.

The mechanisms which regulate the expression of FN and the preferential inclusion of EDB$^+$ oncofetal domain in the FN molecules are still elusive. We have previously shown that glucose-induced expression of EDB$^+$ oncofetal FN is mediated by fibrogenic growth factors, transforming growth factor-β1 (TGF-β1), and endothelin-1 (ET-1) [8]. However, intracellular proteins which transduce the high glucose, TGF-β1, and ET-1 signals are not fully identified. We and others have also previously shown that protein kinase pathways, such as protein kinase C (PKC) and mitogen-activated protein kinase (MAPK), may upregulate FN expression [9,7,14]. A novel protein kinase, which may also mediate glucose-induced FN expression, is serum- and glucocorticoid-regulated kinase-1 (SGK-1). SGK-1 is a recently identified protein kinase which exhibits structural homology with protein kinase B (PKB). SGK-1 is regulated by transcription, activity, and sub-cellular distribution [15,16]. An expanding number of cell surface receptors and cellular stress pathways have been shown to induce SGK-1 [17]. In addition, SGK-1 has been identified as an important mediator in lung fibrosis [18]. Further prompting the investigation of SGK-1 in glucose-induced FN and EDB$^+$ oncofetal FN expression are findings which demonstrate that this kinase is a downstream messenger of TGF-β1 signaling [15,16,18].

In the present study, we have examined the role of SGK-1 in preferential EDB$^+$ oncofetal FN expression in vascular endothelial cells. Furthermore, we have tested the hypothesis that glucose-induced TGF-β1 and ET-1 mediate differential FN expression via alteration and activation of SGK-1. In such an attempt, we have utilized both macrovascular and microvascular endothelial cells to delineate the role of SGK-1.

Materials and methods

Endothelial cell cultures. Human umbilical vein endothelial cells (HUVECs; American Type Culture Collection, Rockville MD) and human dermal microvascular endothelial cells (HDMECs; Clonetics, Walkersville, MD) were cultured as previously described [8,14]. We

Table 1
Oligonucleotide sequences for real-time RT-PCR and siRNA synthesis

Gene	Sequence [5′ → 3′]	PCR temperatures[a]	
β-Actin	CCTCTATGCCAACACAGTGC	Denaturation	95 °C-0 s
	CATCGTACTCCTGCTTGCTG	Annealing	58 °C-5 s
		Extension	72 °C-8 s
		Signal	83 °C-1 s
EDB$^+$ FN	CCGCCATTAATGAGAGTGAT	Denaturation	95 °C-0 s
	AGTTAGTTGCGGCAGGAGAAG	Annealing	55 °C-5 s
		Extension	72 °C-8 s
		Signal	81 °C-1 s
Total FN	GATAAATCAACAGTGGGAGC	Denaturation	95 °C-0 s
	CCCAGATCATGGAGTCTTTA	Annealing	50 °C-6 s
		Extension	72 °C-10 s
		Signal	81 °C-1 s
SGK-1	TCTCTGGAAGCTTAGCAATC	Denaturation	95 °C-0 s
	GCTTTGGAGCTAACACAATC	Annealing	55 °C-7 s
		Extension	72 °C-14 s
		Signal	81 °C-1 s
Oligonucleotide sequences[b] [5′ → 3′]		Nucleotide[c]	
SGK-1 siRNA-1	***AATTCTCA TCGCTTTCAT GAA***CCTGTCTC	123–143	
	AA***TTCATGAAAGCGATGAGAATT***CCTGTCTC		
SGK-1 siRNA-2	***AATGCCAACCCTTCTCCTCCA***CCTGTCTC	265–285	
	AA***TGGAGGAGAAGGGTTGGCATT***CCTGTCTC		
SGK-1 siRNA-3	***AATGGTGGAGAGTTGTTCTAC***CCTGTCTC	595–615	
	AA***GTAGAACAACTCTCCACCATT***CCTGTCTC		
SGK-1 siRNA-4	***AAGGAGAACATTGAACACAAC***CCTGTCTC	790–811	
	AA***GTTGTGTTCAATGTTCTCCTT***CCTGTCTC		

[a] Initial denaturation was carried out at 95 °C-1 min. Ramp rate for all PCR phases was 20 °C/s.
[b] Targeted sites in SGK-1 are italicized.
[c] Nucleotide position in reference to Human SGK-1 sequence [Accession No. NM_005627].

have previously demonstrated that the greatest increase in total FN and EDB^+ oncofetal FN is achieved by exposing cells to 25 mM glucose for 24 h [8,14]. Hence, all treatments were carried out for 24 h with either 5 mM glucose (low glucose; LG) or 25 mM glucose (high glucose; HG). Following incubation, cells were subjected to experimental analyses. Cells in LG were also treated with 1 ng/mL recombinant human TGF-β1 (Sigma–Aldrich, St. Louis, MO) or 5 nM ET-1 (Peninsula Laboratories, Belmont, CA). In addition, we incubated cells in (HG) with either 10 ng/mL TGF-β1 neutralizing antibody (Sigma–Aldrich) or 10 μM dual ET receptor antagonist, bosentan (Courtesy of M. Clozel, Actelion, Allschwill, Switzerland). Concentrations of peptides and inhibitors were in accordance with our previous studies in endothelial cells [8].

Real-time RT-PCR. Real-time RT-PCR was performed in LightCycler (Roche Diagnostics, Canada, Quebec, Canada) as previously described [8,9]. The reaction mixture (20 μL final volume) consisted of 10 μL SYBR Green Taq ReadyMix (Sigma–Aldrich), 1.6 μL of 25 mM $MgCl_2$, 1 μL of each forward and reverse 10 μM primers (Table 1), 4.4 μL H_2O, and 1 μL cDNA template. The data were normalized to housekeeping gene (β-actin) to account for differences in reverse transcription efficiencies and amount of template in the reaction mixtures.

Western blotting. Total proteins from endothelial cells were isolated and quantified as described previously [8,9]. SGK-1 and phospho-SGK-1 protein levels were measured by polyclonal anti-human SGK-1 (1:500; Upstate Cell Signaling Solutions, Waltham, MA) and polyclonal anti-human phospho-SGK-1 (1:500; Upstate Cell Signaling Solutions, Waltham, MA) antibody followed by secondary antibody conjugated with horseradish peroxide (1:10,000). ECL-PLUS Western Blotting Detection kit (Amersham Pharmacia Biotech, Piscataway, NJ) was used for detection.

Immunofluorescent analyses. Endothelial cells were cultured in 12-well plates (Fisher Scientific, Ontario, Canada) with coverslips. Following 24 h serum starvation, cells were treated with 25 mM glucose and fixed using anhydrous ethanol. SGK-1 and phospho-SGK-1 antibodies were used with secondary antibody conjugated with Texas Red (Vector Laboratories, Ontario, Canada) for detection.

SGK-1 gene silencing. siRNAs were constructed to target four SGK-1 mRNA sites (Table 1) using Silencer siRNA construction kit (Ambion, Austin, TX, USA). Endothelial cells were transfected with SGK-1 siRNAs (100 nM final concentration) using siPORT *Lipid* siRNA transfection reagent. Seventy-two hours following transfection, gene expression analyses were performed. All siRNA experiments included negative transfection of cells (negative control siRNAs, Ambion). In order to assess transfection efficiency, we have used real-time RT-PCR technique which allows detection of a single template.

Statistical analysis. The data are expressed as means ± SEM and were analyzed by ANOVA followed by Student's t test. Differences were considered significant at values of $p < 0.05$.

Results

Glucose- and fibrogenic factor-induced upregulation of FN and EDB^+ oncofetal FN is mediated by SGK-1

We have previously demonstrated that high levels of glucose increase mRNA levels of both total FN and relative EDB^+ oncofetal FN by elaboration of TGF-β1 and ET-1 [8]. We have re-produced the results and show that glucose and aforementioned growth factors upregulate total FN and relative EDB^+ oncofetal FN [ratio of EDB^+ FN to total FN] in both HUVECs and HDMECs (Figs. 1A–D). In order to determine whether such increases are mediated by alteration of SGK-1, we have performed real-time RT-PCR analysis to measure SGK-1 mRNA levels. Our results show that glucose, TGF-β1, and ET-1 upregulate SGK-1 mRNA levels in both HUVECs and HDMECs (Figs. 1E and F). Interestingly, SGK-1 upregulation was found to be more pronounced in HUVECs as compared to HDMECs. These findings are in complete agreement with total FN and EDB^+ oncofetal FN data. To confirm that SGK-1 upregulation by glucose is mediated, at least in part, by TGF-β1 and ET-1, we treated cells exposed to 25 mM glucose with TGF-β1 neutralizing antibody and ET receptor antagonist. Our results indicate that inhibition of either TGF-β1 or ET-1 activity prevents the high glucose-induced SGK-1 upregulation (Figs. 1E and F).

High levels of glucose increase SGK-1 protein expression and activation via TGF-β1 and ET-1

To assess whether SGK-1 mRNA exhibits concurrent protein upregulation, we have performed Western blotting with SGK-1 and phospho-SGK-1 (activated form) antibodies. Our results indicate, similar to mRNA data, that high levels of glucose and fibrogenic factors (TGF-β1 and ET-1) increase protein levels of SGK-1 (Figs. 2A and B) and activated SGK-1 (Figs. 2A and C). Furthermore, inhibition of TGF-β1 and ET-1 activity in cells exposed to high glucose reduced SGK-1 expression and activation. These results provide support for the regulation of SGK-1 by glucose, TGF-β1, and ET-1 at the transcriptional and activity levels.

As activation/phosphorylation of SGK-1 is expected to cause translocation to the nucleus, we performed immunofluorescent analysis to determine the localization of both SGK-1 and activated phospho-SGK-1 protein. Our results indicate cytoplasmic staining of SGK-1 which increased in intensity with exposure of cells to 25 mM glucose (Figs. 2D and E). Interestingly, immunodetection of activated phospho-SGK-1 also revealed cytoplasmic localization (data not shown). These results provide interesting cues as to the role of SGK-1 in glucose-induced FN expression.

SGK-1 gene silencing prevents glucose-induced FN and EDB^+ oncofetal FN expression

To obtain a definitive and clear indication that SGK-1 may mediate glucose-induced FN and EDB^+ oncofetal FN expression, we performed gene silencing experiments. siRNA-mediated degradation of mRNA represents the most effective way of inhibiting the activity of the kinase [8,12]. Our real-time RT-PCR results show a significant reduction in SGK-1 mRNA following siRNA transfection (84.4% reduction; Fig. 3A). Follow-

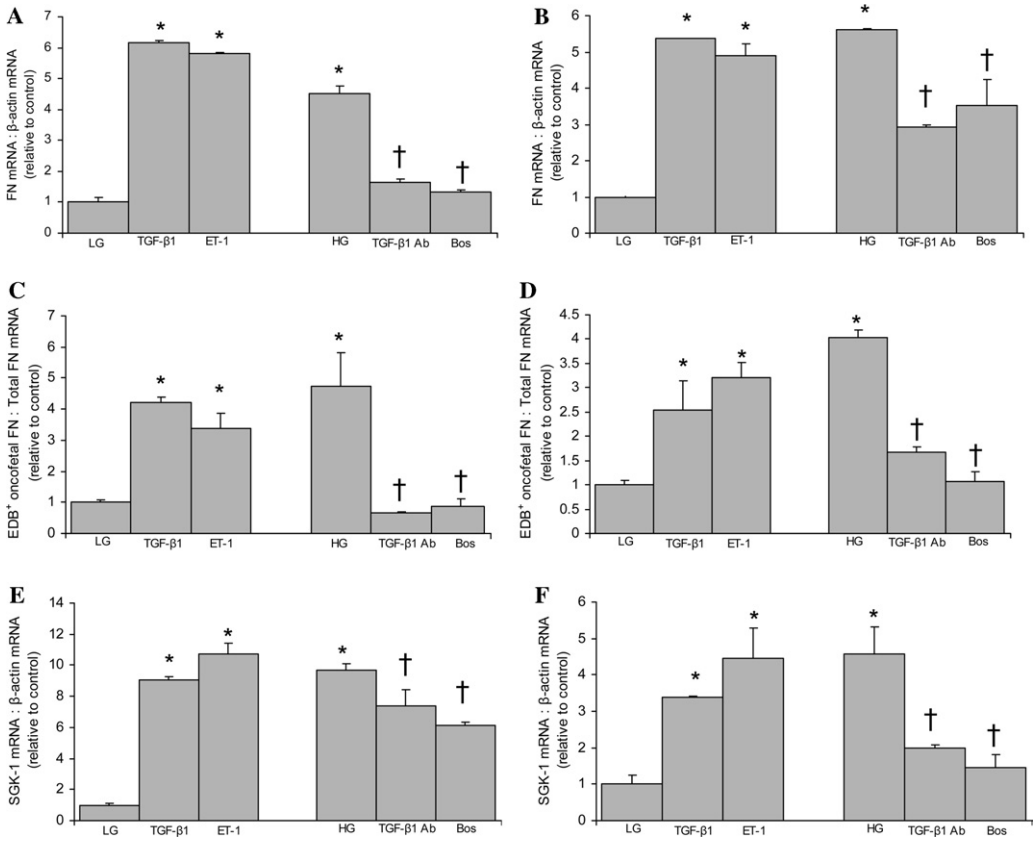

Fig. 1. Effect of high glucose concentration, TGF-β1, and ET-1 peptide on mRNA expression of (A) total FN in HUVECs, (B) total FN in HDMECs, (C) EDB$^+$ FN in HUVECs, (D) EDB$^+$ FN in HDMECs, (E) SGK-1 in HUVECs, and (F) SGK-1 in HDMECs [mRNA expression was determined by real-time RT-PCR and expressed as ratio of target mRNA to β-actin (relative to control); For EDB$^+$ FN, transcript levels are expressed as ratio of target to total FN; LG = 5 mM glucose, TGF-β1 = 1 ng/mL TGF-β1, ET-1 = 5 nM ET-1, HG = 25 mM glucose, TGF-β1 Ab = HG + 10 ng/mL TGF-β1 neutralizing antibody, and Bos = HG + 10 μM bosentan; *$p < 0.05$ compared to LG; $^{\dagger}p < 0.05$ compared to HG; and $n = 4$/treatment].

ing efficient transfections, cells were exposed to high glucose levels and EDB$^+$ oncofetal FN mRNA levels were quantified. Our results show that glucose increase both the total FN and the relative EDB$^+$ oncofetal FN mRNA levels in negative control transfected cells (siRNA with no sequence homology) (Figs. 3B and C). However, SGK-1 siRNA transfection, completely, inhibited glucose-induced FN and EDB$^+$ oncofetal FN expression.

Discussion

In the present study, we have for the first time demonstrated the role of SGK-1 in glucose-induced EDB$^+$ oncofetal FN alteration in vascular endothelial cells. We have shown that glucose and fibrogenic growth factors, TGF-β1 and ET-1, increase SGK-1 at the mRNA, protein, and activity levels. Such augmented expression and activity coincided with increased EDB$^+$ oncofetal FN expression. In addition, using specific siRNA-mediated SGK-1 inhibition, we have demonstrated complete normalization of glucose-induced EDB$^+$ oncofetal FN expression in endothelial cells.

Differential FN expression is regulated in a highly restrictive pattern [13,19,20]. Isoform switching of FN in the extracellular matrix may represent an important phenomenon in altering the behavior of vascular endothelial cells. It is increasingly being realized that the extracellular matrix provides positional and environmental information. This information is important for proper tissue function; a phenomenon evident in studies which demonstrate heterogeneity in extracellular composition and architecture in a tissue-specific manner [21]. The biochemical mechanisms that regulate the composition of matrix proteins, including FN, are not fully understood. We have shown that SGK-1 may represent one signaling pathway which could not only contribute to increased FN expression but also the regulation of EDB$^+$ inclusion in FN molecules. Interestingly, exposure of endothelial cells to high levels of glucose, TGF-β1, or ET-1 did not cause redistribution and nuclear localization of activated SGK-1. Such a finding suggests that, in the context of high glucose

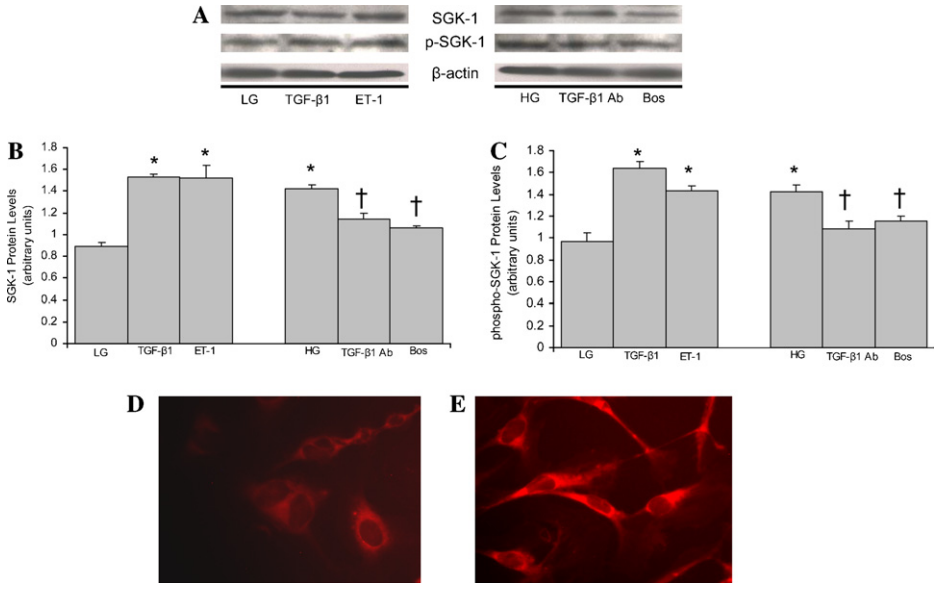

Fig. 2. SGK-1 protein expression in endothelial cells. Quantitative protein analysis showing (A) representative immunoblots, (B) semi-quantitative analysis of SGK-1 protein levels in HUVECs, and (C) semi-quantitative analysis of phospho-SGK-1 levels in HUVECs. Immunofluorescent analysis of SGK-1 protein in cells exposed to (D) 5 mM glucose and (E) 25 mM glucose. [β-Actin was used as loading control; LG = 5 mM glucose, TGF-β1 = 1 ng/mL TGF-β1, ET-1 = 5 nM ET-1, HG = 25 mM glucose, TGF-β1 Ab = HG + 10 ng/mL TGF-β1 neutralizing antibody, and Bos = HG + 10 μM bosentan; *$p < 0.05$ compared to LG; †$p < 0.05$ compared to HG; and $n = 4$/treatment; original magnification 400×.]

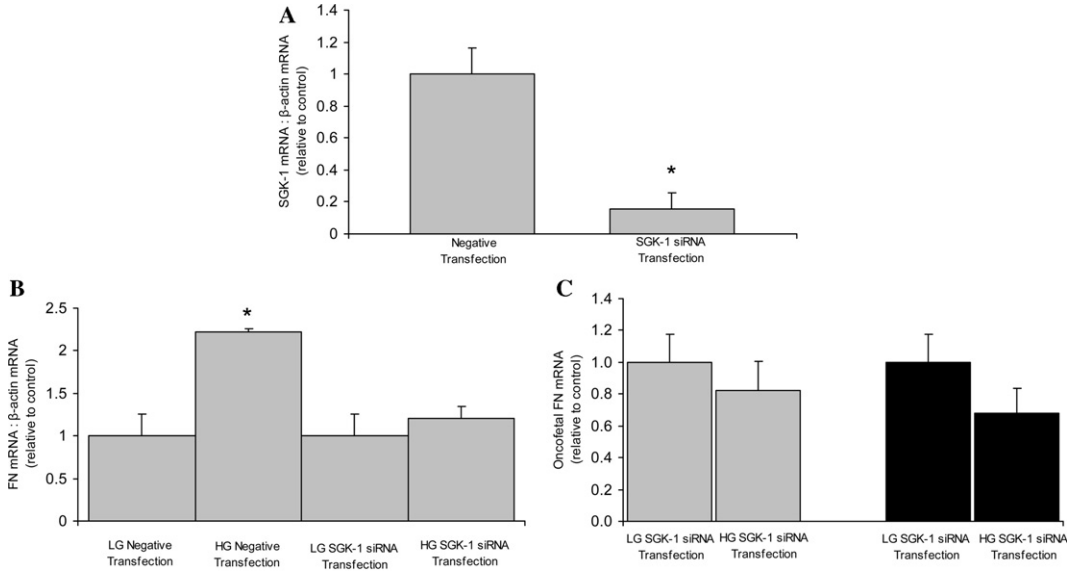

Fig. 3. Effect of SGK-1 siRNA-mediated gene silencing. Real-time RT-PCR analysis of (A) siRNA efficiency as assessed by SGK-1 mRNA levels in HUVECs, (B) high glucose-induced FN mRNA levels, and (C) high glucose-induced EDB^+ oncofetal FN mRNA expression in HUVECs. [mRNA levels are expressed as ratio of target mRNA to β-actin (relative to control); EDB^+ oncofetal FN mRNA levels are shown in both absolute (ratio of EDB^+ FN to β-actin (grey bars)) and relative (ratio of EDB^+ FN to total FN (black bars)) manner; LG = 5 mM glucose; HG = 25 mM glucose; negative transfection = negative control; *$p < 0.05$ compared to negative transfection; and $n = 4$/treatment].

levels, phosphorylated SGK-1 localizes to the cytoplasm, possibly sequestered or associated with a partner protein. Further elucidation of such partner proteins would greatly enhance our understanding of the mechanisms which orchestrate the preferential FN expression in diabetic complications.

Previous studies have demonstrated upregulation of SGK-1 mRNA in kidneys of diabetic patients and animal models [22]. Furthermore, high levels of glucose have also been shown to increase SGK-1 mRNA in microvascular endothelial cells [23]. However, SGK-1 is regulated at both the transcriptional

and posttranslational levels [15–17]. In addition, the function of SGK-1 may be modified by cellular localization. Our studies have shown for the first time that SGK-1 is upregulated in both the microvascular and the macrovascular endothelial cells by glucose via TGF-β1 and ET-1. Such mRNA upregulation paralleled protein levels and activation/phosphorylation status. Furthermore, our results indicate that SGK-1 upregulates EDB^+ oncofetal FN. These studies indicate that SGK-1 may be involved in endothelial cell proliferation and angiogenesis. However, such notions require further investigation.

In conclusion, our studies provide first clear evidence of a novel role of SGK-1 in high glucose-induced EDB^+ oncofetal FN expression. We have also demonstrated that SGK-1 upregulation and activation is mediated via fibrogenic proteins, ET-1 and TGF-β1. These findings provide great insight into the pathogenesis of diabetic vascular complications and may introduce novel avenues for the development of therapeutic modalities.

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