SGK1-mediated Fibronectin Formation in Diabetic Nephropathy

Yuxi Feng^{1,2}, Quansheng Wang², Yumei Wang², Benito Yard¹ and Florian Lang³

¹5th Medical Clinic, Faculty of Clinical Medicine, University of Heidelberg; ²Department of Nephrology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan; ³Department of Physiology, University of Tübingen

Key Words

Fibrosis • Mesangial cells • Streptozotocin • Fibronectin

Abstract

The serum and glucocorticoid inducible kinase SGK1 has been shown to be up regulated in fibrosing tissue including diabetic nephropathy. The present study has been performed to determine the time course of SGK1 transcription in mouse kidneys following induction of diabetes by streptozotocin (STZ). Moreover, the study aimed to explore whether SGK1 may play an active role in the stimulation of matrix protein formation during hyperglycemia. The induction of diabetes in 8 weeks old male C57Bl/6 mice was indeed followed by a significant ($p < 0.001$) increase of SGK1 transcript levels (up to 2.5-fold) and protein abundance (up to 2.8-fold) both peaking 4 weeks after STZ treatment. The SGK1 transcript levels and protein abundance declined thereafter but remained significantly elevated up to 12 weeks ($p < 0.05$). Exposure to high extracellular glucose concentration (25 mM) significantly increased SGK1 transcript levels in human mesangial cells (HMCs). At low extracellular glucose concentration (5.5 mM), transfection with constitutively

active ^{S422D}SGK1 and transdominant inhibitory ^{K127N}SGK1 did not significantly modify fibronectin formation by HMCs. Exposure to high extracellular glucose concentration stimulated fibronectin formation (by 2.2 fold), an effect abrogated by transfection with inactive ^{K127N}SGK1 (1.2 fold) and markedly enhanced by transfection with ^{S422D}SGK1 (4.7 fold). In conclusion, hyperglycemia of diabetes mellitus leads to partially transient increase of SGK1 transcription and translation. SGK1 overexpression alone has little effect on fibronectin formation but potentiates the effect of hyperglycemia. Thus, SGK1 is upregulated in diabetic nephropathy and actively participates in the stimulation of matrix protein deposition in this common deleterious complication of diabetic hyperglycemia.

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Introduction

Diabetic nephropathy is characterized by excessive matrix protein deposition eventually leading to end stage renal failure [1, 2]. Mediators triggering enhanced matrix

protein deposition include TGF β [3-10], which in turn has been shown to enhance the transcription of the serum and glucocorticoid inducible kinase SGK1 [11]. The kinase has originally been cloned as glucocorticoid inducible gene [12] and subsequently shown to be strongly upregulated by mineralocorticoids [13, 14]. SGK1 is expressed in a variety of fibrosing tissues such as in diabetic nephropathy [15], glomerulonephritis [16], Crohn's disease [17], lung fibrosis [18], liver cirrhosis [19] and fibrosing pancreatitis [20]. The functional significance of SGK1 in the stimulation of matrix protein formation has remained elusive.

The present study aimed to elucidate the possible participation of SGK1 in the stimulation of matrix protein expression.

Materials and Methods

Streptozotocin-induced diabetes in mice

The care and experimental use of all animals in our study were approved by the animal care and use committee and were in accordance with institutional guidelines. Male C57Bl/6 mice at an age of 8 weeks were used in this study. Mice were divided randomly into a control and a diabetic group. Mice were fasted for 18 h and then diabetes was induced by intraperitoneal injection of high dose streptozotocin (150 mg/kg body weight; Sigma Chemical, St. Louis, MO) freshly dissolved in 10 mM sodium citrate, pH 4.5. After 2 days, induction of diabetes was confirmed by measurement of the blood glucose using the glucose oxidase method (One Touch Blood Glucose Instrument, LifeScan, USA), and hyperglycemic mice with levels >16 mmol/L were used. At 4 weeks after streptozotocin treatment, the individual 24 h urine was collected, and the urinary protein was determined following a standard Bradford protocol. To determine the time course of SGK1 transcription, diabetic mice were sacrificed at 1, 3, 4, 6 and 12 weeks after the STZ injection. Kidneys were removed and renal cortex was dissected. For RNA isolation, renal cortex was homogenized in Trizol, and for protein extraction, renal cortex was homogenized in 0.1 % SDS lysis buffer containing 50 mM Tris.Cl, pH 8.0; 150 mM NaCl; 0.02 % Sodium azide; 1 % NP-40; 0.05 % NaVO₃; 100 μ g/ml PMSF; 1 μ g/ml aprotinin, followed by centrifugation at 12000 rpm. Protein concentration in the supernatant was measured using the Bradford method. Finally, the samples were immediately stored at -70°C for the subsequent analysis.

Construction of h-SGK1

cDNA encoding human SGK1 was obtained *in vitro* from total RNA using a standard cloning protocol. Specific SGK1 primers for amplification were: forward 5'-ACG TCT TTG TGT CTC CCC G-3', reverse 5'-CCT TTAAAA CCAAGC CCT AAC A-3'.

Full length human SGK1 was inserted into the SrfI site of pPCRScript (Stratagene, Germany). The h-SGK1 active mutant

h-SGK1S422D (SGK1-SD) and transdominant negative mutant h-SGK1K127N (SGK1-KN) were made utilizing the site-directed mutagenesis kit (Stratagene, Germany). Primers used were: forward 5'-TTC CTA GGC TTT GAC TAT GCG CCT CCC-3', and 5'-ATG CAG TCA ACG TTT TAC AGA-3', respectively. A PSP1406 site was introduced into the SGK1-KN sequences during mutagenesis. The segment of pPCRscript-SGK1-WT and pPCRscript-SGK1-KN digested with NotI and EcoRI were inserted into SrfI and SmaI site of pGEMHJ, respectively. The segment of pPCRscript-SGK1-SD digested with EcoRI and ScaI was inserted into SmaI site of the pGEMHJ vector. Finally, the products of digestion with SacI and EcoRI in pGHJ-SGK1-WT/SD/KN were inserted into the same site of pIRES2-EGFP (Clontech, Germany). All constructs were verified by restriction site analysis and sequencing.

Cell culture and regulation of SGK1 under high glucose

Human mesangial cells (HMC), kindly provided by Prof. Liu and Prof. Wang, were maintained in DMEM Medium containing 5.5 mM D-glucose (Gibco, Germany), supplemented with 10 % heat inactivated fetal bovine serum (FCS), 100 U/ml penicillin, 100 μ g/ml streptomycin. The medium was changed every 3 d. For high glucose stimulation, cells were placed into 10 cm dishes and cultured to 80 % confluency. The cells were incubated in DMEM without FCS for 24 hrs. After incubation with 5.5 mM glucose, 25 mM D-glucose or 5.5 mM D-glucose plus 19.5 mM mannitol for 2 hrs or 8 h, the cells were homogenized in Trizol for analysis of transcriptional regulation of SGK1 under high glucose.

Transfection with h-SGK1 in HMCs and the effect of SGK1 overexpression on fibronectin formation

Human mesangial cells were seeded into dishes and grown to 90 % confluency at 37°C and 5 % CO₂ equilibration for 24 h. After 12 h incubation in serum free medium, cells were transfected by LipofectamineTM 2000 (Invitrogen, Carlsbad, CA USA) according to manufacturer's protocols with plasmid pIRES2-EGFP, pIRES2-EGFP-hSGK1-SD, pIRES2-EGFP-hSGK1-KN. The cells were transfected for 6 h, and then medium was aspirated and replaced with fresh DMEM (5.5 mM D-glucose) without FCS. The rate of transfection approached 50 %, as evident from FACS (Calibur Becton Dickinson, Mountain View, CA, USA) with GFP. The cells were washed with PBS after transfection, incubated in DMEM (5.5 mM D-glucose) without FCS overnight and stimulated with DMEM (25 mM D-glucose) for 8 hrs. To determine the production of fibronectin, the cells were scraped and homogenized in ice-cold buffer M-PER mammalian protein Extraction Reagent (PIERCE, Rockford, IL, USA) according to the manufacturer's instructions.

RNA isolation and RT-PCR

Total RNA was extracted with Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. First-strand cDNA synthesis was performed using 2 μ g of RNase-free DNase-treated RNA sample with RevertAidTM First strand cDNA synthesis kit (MBI, Fermentas, USA). All specific primers for mouse SGK1, fibronectin and GAPDH were designed

Table 1. PCR primers and conditions for amplification.

	sequences	Annealing temperature	cycles	Product length
m SGK1 forward	5'- CTG TTC TAC CAT CTC CAG AG -3'	51 °C	35	329 bp
m SGK1 reverse	5'- CCG TAG AGC ATC TCA TAC AG -3'			
h SGK1 forward	5'- CAA TCC TCA TGC TAA ACC -3'	56 °C	35	322 bp
h SGK1 reverse	5'- TTC CAG GAA GCA GCG TTC -3'			
m FN forward	5'- ACG ATG GGA AGA CCT ACG ATG TA -3'	60 °C	35	253 bp
m FN reverse	5'- TCA GCT TGC ACA TCT AAC GGC AT -3'			
m GAPDH forward	5'- CAT CAC CAT CTT CCA GGA GCG -3'	55 °C	28	357 bp
m GAPDH reverse	5'- GAG GGG CCA TCC ACA GTC TTC -3'			
h β -actin forward	5'- GGT CAG AAG GAT TCA TAT GTG -3'	55 °C	26	615 bp
h β -actin reverse	5'- ATT GCC AAT GGT GAT GAC CTG -3'			

according to the sequences in the GenBank. The PCR conditions were standardized to observe the linear amplification of the PCR products. RNA treated with DNase without reverse transcription served as negative control. PCR conditions are indicated in table 1. PCR was performed in a Gene Amp PCR System 9700 (Perkin-Elmer, Applied Biosystems, Foster City, CA, USA) thermal cycler. PCR products were subjected to electrophoresis on 2.0 % agarose gels, and the bands were visualized by ethidium bromide. Finally, analysis was performed by densitometric gel scanning utilizing a model GS-700 imaging densitometer (Bio-Rad laboratories, Hercules, Calif, USA). The results were expressed as a ratio of SGK1 to GAPDH or β -actin.

Western Blot analysis

40 μ g protein were denatured and separated on 10 % SDS-PAGE, and then transferred to nitrocellulose membranes (Micron Separations, Westborough, MA, USA) according to standard procedures. Following incubation in a blocking buffer (5 % nonfat milk in TBS/0.1 % Tween 20, pH 7.4) for 1 hr, the membranes were incubated with rabbit anti-human SGK1 (diluted 1:500, Upstate, USA) at room temperature for 1 h. After washing in PBS, horseradish peroxidase conjugated second antibody, sheep anti rabbit IgG (diluted 1:7000 PIERCE, Rockford, IL, USA) was used. An enhanced chemiluminescence kit (PIERCE, Rockford IL, USA) was used for immunodetection, and the blots were exposed to Kodak X-OMAT film (Eastman Kodak, Rochester, NY, USA). To assess the equality of loading, the membranes were reprobed with goat anti actin (diluted 1:500, Santa Cruz Biotechnology, Inc) and then with its secondary antibody rabbit anti-goat IgG in a dilution of 1:5000 (PIERCE, Rockford, IL, USA). For fibronectin determination, the primary antibody mouse anti-human fibronectin (diluted 500-fold, Sigma, St. Louis, MO, USA) and the secondary antibody sheep-anti-mouse Ig conjugated to horseradish peroxidase (diluted 5000-fold) were used. The bands were scanned with a laser densitometer (model PD1211; Molecular Dynamics, USA) to assess the density.

Statistical analysis

Values are expressed as means $\pm$ SEM. Statistical differences between groups were evaluated with ANOVA. $P < 0.05$ denotes significant difference to the respective control.

Results

Development of diabetes

Four weeks after the streptozotocin (STZ) treatment fasting blood glucose levels were significantly ($p < 0.001$) higher in treated animals (24.5 ± 3.8 mM, $n = 6$) than in control animals (4.2 ± 0.5 mM, $n = 6$). Accordingly, HbA1c approached 7.9 ± 0.7 % ($n = 6$) in the STZ treated animals, a value again significantly ($p < 0.01$) higher than in control animals (4.0 ± 0.7 %, $n = 6$).

The hyperglycemia was paralleled by proteinuria averaging 6.47 ± 0.66 mg/24 h ($n = 6$) in STZ treated animals and 0.29 ± 0.03 mg/24 h ($n = 6$) in control animals. Fibronectin transcript levels, an indicator of renal fibrosis, were significantly ($p < 0.01$) higher in kidneys from STZ treated animals (1.25 ± 0.15 , $n = 6$) than from renal tissue of control animals (0.96 ± 0.08 , $n = 6$).

SGK1 expression in mice with experimental diabetic nephropathy

To investigate the potential role of SGK1 in diabetic nephropathy, we evaluated the mRNA levels of SGK1 in the renal cortex of STZ-treated mice. Figure 1A shows a representative experiment illustrating the transcriptional up regulation of SGK1 in the renal cortex following induction of diabetes. Figure 1B summarizes the quantification of SGK1 mRNA levels in the diabetic and

Fig. 1. Upregulation of SGK1 transcript levels in diabetic nephropathy. RT-PCR analysis of SGK1 and GAPDH mRNA abundance in murine renal cortex 1, 3, 4, 6 or 12 weeks (w) after induction of diabetes with streptozotocin. Values from diabetic animals (DC) are compared to age matched non treated normal animals (NC). A: Original blots, B: Arithmetic means $\pm$ SEM (n = 5). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Fig. 2. Upregulation of SGK1 protein abundance in diabetic nephropathy. Western blot analysis of SGK1 and β -actin protein abundance in murine renal cortex 1, 3, 4, 6 or 12 weeks (w) after induction of diabetes with streptozotocin. Equal loading of the lanes was verified by staining with β -actin. Size of the band detected by SGK1 was 49 kDa. Values from diabetic animals (DC) are compared to age matched non treated normal animals (NC). A: Original blots, B: Arithmetic means $\pm$ SEM (n = 5). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

non-diabetic mice. The expression of SGK1 mRNA in the diabetic renal cortex was markedly increased after 1 week of diabetes. At 4 weeks of diabetes, the SGK1 mRNA level was increased 2.5-fold ($P < 0.001$ vs. control mice), the up regulated SGK1 mRNA was maintained up to 12 weeks after induction of diabetes, although the SGK1 transcript levels declined again after the first 4 weeks of diabetes induction.

Fig. 2A shows a representative Western blot of SGK1 protein abundance in renal cortex before and 1-12 weeks after the induction of diabetes, illustrating the up regulation of SGK1 protein levels in the renal cortex during STZ induced diabetes. Figure 2B summarizes the quantification of the SGK1 expression levels. The expression of SGK1 protein in the diabetic kidney was significantly increased after 3 weeks of diabetes, and increased up to 2.8-fold ($p < 0.001$) 4 weeks after induction of diabetes. The transcript levels declined thereafter but

remained significantly elevated up to 12 weeks after STZ treatment.

Glucose sensitive SGK1 expression in mesangial cells

As shown in Fig. 3A and 3B, the SGK1 mRNA levels in human mesangial cells (HMCs) were increased by exposure to high extracellular glucose concentrations. The transcript levels increased significantly within 2 hrs ($230 \pm 22\%$, $n = 9$) and remained significantly enhanced up to 8 hrs ($200 \pm 16\%$, $n = 9$). Similarly, SGK1 transcription in HMCs was upregulated by a 2 hrs hyperosmotic incubation in mannitol, indicating that increased osmolarity triggers SGK1 expression. However, the comparison of the experiments with glucose and mannitol revealed that glucose exerts a significant additional effect not explained by increased osmolarity alone.

Fig. 3. Glucose sensitive SGK1 transcript levels in human mesangial cells (HMCs). RT-PCR of SGK1 transcript levels in mesangial cells exposed for 2 hours to 5.5 mM glucose (normal glucose NG), 25 mM glucose (high glucose, HG) or 25 mM mannitol (MA). A: Original blots, B: Arithmetic means $\pm$ SEM (n = 9). * P<0.05 MA vs NG and HG vs MA.

SGK1 and glucose sensitive fibronectin formation in mesangial cells

To gain some insight into the functional significance of SGK1 in renal cells, we inserted wild type, constitutively active and inactive (dominant negative) SGK1 into the mammalian vector pIRES-EGFP. To explore the functional significance of SGK1 for matrix protein synthesis, the effect of SGK1 on fibronectin formation was studied in human mesangial cells (HMC). Fig 4A shows representative Western blots illustrating fibronectin formation by HMCs following exposure to different glucose concentrations and following transfection with wild type and mutant SGK1. Fig. 4B shows the statistical analysis of the experiments (n=3). Exposure to high extracellular glucose concentration (25 mM) increased fibronectin production in HMCs which were not

Fig. 4. Western blot analysis of glucose and SGK1 sensitive fibronectin formation in human mesangial cells (HMCs). Fibronectin and β -actin protein abundance in nontransfected mesangial cells (NT), in mesangial cells transfected with empty vector (Vec) and in mesangial cells transfected either with constitutively active S^{422D} SGK1 (SD) or with inactive, dominant negative K^{127N} SGK1 (KN). The cells were exposed either to 5.5 mM glucose (normal glucose, NG) or to 25 mM glucose (high glucose, HG). A: Original blots, B: Arithmetic means $\pm$ SEM (n = 3). *P<0.05, ***P<0.001 HG vs corresponding NG.

transfected or transfected with empty vector. At low extracellular glucose concentration (5.5 mM), transfection with wild type SGK1 or inactive K^{127N} SGK1 did not change the fibronectin formation in HMCs, transfection with the active mutant SGK1 (S^{422D} SGK1) tended to increase fibronectin formation, an effect, however, not reaching statistical significance. Following exposure of the cells to high extracellular glucose concentration for 8 hrs led to an increase of fibronectin formation in cells overexpressing active mutant S^{422D} SGK1, which was significantly higher than the increase in non-transfected cells or in cells transfected with the empty vector. In contrast, exposure to high extracellular glucose concentration did not significantly increase fibronectin formation in cells transfected with the transdominant inhibitory K^{127N} SGK1 mutant.

Discussion

The present study confirms previous observations showing enhanced SGK1 expression in diabetic nephropathy [15, 21]. The *in vivo* observations are paralleled by *in vitro* experiments revealing stimulation of SGK1 transcription following exposure of mesangial cells to high extracellular glucose concentrations. In theory, the stimulation could have been due to hyperosmolarity, another strong stimulator of SGK1 transcription [17]. However, the stimulation of SGK1 transcription was significantly stronger following exposure to glucose than following exposure to equimolar concentrations of mannitol. In previous experiments SGK1 has been shown to be a transcriptional target of TGF β [15, 17]. The effect of high glucose concentrations on SGK1 transcript levels could be blunted by neutralizing antibodies against TGF β , suggesting that TGF β participates in the stimulation of SGK1 transcription during diabetic nephropathy [15].

The present experiments reveal a distinct time course of SGK1 expression with peak expression levels approximately 4 weeks following the induction of diabetes. Earlier studies on *in situ* hybridization of SGK1 in fibrosing tissue revealed heterogeneous distribution of SGK1 transcript levels demonstrating clusters of cells with high SGK1 transcript levels and areas with little SGK1 abundance [15]. It is tempting to speculate that SGK1 is important for the initiation of matrix protein formation but disappears when functional tissue is largely replaced by connective tissue. Nevertheless, SGK1 transcript levels and protein abundance were still elevated 12 weeks after induction of diabetes. Thus SGK1 may well contribute to the continued progression of the disease.

Most importantly, the present observations strongly suggest that SGK1 plays an active role in the stimulation of matrix protein formation. Notably, overexpression of constitutively active SGK1 alone has apparently little impact on fibronectin formation indicating that under the experimental conditions chosen SGK1 activity alone is not sufficient to stimulate the formation of matrix proteins. However, the additional exposure to high extracellular glucose concentration increases fibronectin formation

significantly beyond the respective values in nontransfected cells exposed to high glucose concentration and in SGK1 transfected cells exposed to normal extracellular glucose concentration. Apparently, the effect of glucose is potentiated by SGK1 and the effect of SGK1 potentiated by glucose. The experiments further show that the upregulation of SGK1 expression by increased extracellular glucose concentration is not sufficient to allow maximal stimulation of fibronectin formation. Thus, SGK1 expression is apparently rate limiting. SGK1 presumably interacts with other signaling elements in the regulation of matrix protein formation. Diabetic nephropathy leads to upregulation of a wide variety of genes [22-26], which orchestrate the fibrosis of renal tissue.

The present observations did not address the mechanisms accounting for SGK1 dependent fibronectin formation. SGK1 has been shown to stimulate a wide variety of channels [27, 28], carriers [15, 29-35], and the Na⁺/K⁺ ATPase [36, 37]. SGK1 may further influence gene expression. Most recently, SGK1 has been shown to increase NF κ B [38], which in turn upregulates connective tissue growth factor CTGF [39]. CTGF is in turn a well known profibrotic factor [40-42], which participates in stimulation of fibrosis in a variety of diseases [43-46] including diabetic nephropathy [47]. Beyond that SGK1 may translocate into the nucleus and thus more directly modify gene expression [12]. Further studies are required to explore whether the influence of SGK1 on matrix protein expression is mediated by its ability to modify transport and/or gene expression.

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