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Glucose transporter in human glomerular mesangial cells modulated by transforming growth factor-beta and rhein[1]

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KEY WORDS monosaccharide transport proteins; transforming growth factor beta; glomerular mesangium; rhein

ABSTRACT

AIM: To identify GLUT1 in human mesangial cells and its regulation by TGF-β_1 and rhein. **METHODS**: Identification of GLUT1 was performed in human mesangial cells by analyzing its mRNA expression, protein product, and functional assay. The effects of TGF-β_1 and rhein on glucose uptake and GLUT1 mRNA expression in mesangial cells were examined by [^{3}H]-2-deoxy-*D*-glucose uptake and Northern blotting analysis. **RESULTS**: It was found that human mesangial cells do express functional GLUT1. The 2-DOG uptake of mesangial cells was markedly increased by TGF-β_1 stimulation with simultaneous elevated expression of GLUT1 mRNA. Increased glucose uptake and GLUT1 mRNA expression in mesangial cells induced by TGF-β_1 were markedly attenuated by rhein in a dose-dependent manner. **CONCLUSION**: We found that functional GLUT1 did present in human mesangial cells. TGF-β_1 stimulated the glucose uptake in mesangial cells through upregulation of GLUT1 expression, this effect of TGF-β_1 could be antagonized by rhein.

INTRODUCTION

Diabetes mellitus is a disease of metabolic dysregulation, most notably abnormal glucose metabolism, accompanied by characteristic long-term complications. Recent evidence from randomized controlled clinical trials of both type 1[1] and type 2[2] diabetes have confirmed the early finding in animal studies that strict control of hyperglycemia may delay the onset and slow down the progression of diabetic nephropathy. Accordingly, study of the cell glucose uptake, the first step of glucose metabolism, is critical to understand the pathophysiology of diabetic nephropathy. The uptake of glucose from the surrounding medium into the cells in mammals is a facilitated diffusion mediated by specific glucose transporter protein. Recently, it has been reported that GLUT1 is the main GLUT isoform expressed in mesangial cells from rat and its function plays an important role in the intracellular glucose metabolism and extracellular matrix (ECM) formation of mesangial cells[3-7]. Our previous work demonstrated that the gene polymorphism of GLUT1 was associated with the development of nephropathy in type 2 diabetes mellitus. The frequency of XbaI(-) allele in GLUT1 gene was significantly higher in type 2 diabetes mellitus patients with diabetic nephropathy as compared to either normal subjects or patients without diabetic nephropathy[8]. It is well-known that glomerular mesangial cells play an important part in the causation of diabetic nephropathy. However, the expression of GLUT1 in human mesangial cells and its regulation have not yet been reported. To further evaluate the role of GLUT1 in the pathogenesis of diabetic nephropathy, studies on expression of GLUT1 in human mesangial cells are mandatory.

Transforming growth factor (TGF-β_1) is a key cytokine in the pathogenesis of diabetic nephropathy[9-13]. Recently, it has been reported that TGF-β_1 stimulates glucose uptake by enhancing the expression of GLUT1 in rat mesangial cells, which leads to the acceleration of intracellular metabolic abnormalities in diabetes[14]. Rhein, 4,5 dihydroxyanthraquinone-2-carboxylic acid, is known to inhibit the glycolysis of neoplastic cells by blocking glucose uptake[15,16] and prevents the progression of diabetic nephropathy in animal models[17]. In the present study, we identified the expression of GLUT1 in human

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mesangial cells, and the regulatory effect of TGF-β_1 and rhein on glucose uptake in human mesangial cells were also observed to highlight the role of GLUT1 and its modulation in the pathogenesis of diabetic nephropathy.

MATERIALS AND METHODS

Mesangial cell culture Cultured mesangial cells were prepared as previously described[18]. Isolated glomeruli were cultured in plastic 75 cm^2 flasks containing DMEM (GIBCO BRL, Grand Island, NY, USA) supplemented with 20 % fetal calf serum (FCS.), benzylpenicillin, streptomycin, and glucose 5 mmol/L . Three to four weeks after plating, the cultured mesangial cells became confluent with fusiform or stellate appearance. These cells demonstrated an ability to grow in a medium lacking D-valine. Immunofluorescence staining showed positive results for actin and desmin, and negative for the factor VIII antibody. These characteristics were retained on repeated passages. Experiments were conducted at five to ten passage mesangial cell culture.

Reverse transcription PCR Human GLUT1 primers were chosen to yield an expected product of 313-bp. The sense primer sequence was 5′- CAT GTG CTT CCA GTA TGT GG-3′, and antisense primer was 5′-GTC AGG TTT GGA AGT CTC AT-3′[19]. One aliquot of cells (1×10^5) was washed with PBS, resuspended in 30 μL of PBS containing 2 % Triton, and sonicated for 5 min at 40 ℃. The Triton solution was aliquoted into 4 microcentrifuge tubes to reach a concentration 2.5×10^4 cells in 9 μL. Reverse transcription was performed using a cDNA synthesis kit (Boehringer Mannheim)[20]. The identity of amplified PCR products were verified by size and by restriction enzyme analysis. In control tubes the reverse transcriptase enzyme was omitted to confirm that genomic DNA encoding GLUT1 was not amplified.

Immunofluorescence microscopy Mesangial cells were seeded at a density of 1×10^4 cells to each 0.79 cm^2 well of eight-well chamber slides grown in the medium described above. Mesangial cells were fixed with 2 % paraformaldehyde when cells got subconfluent. All were then washed in PBS, blocked with 10 % goat serum. The chambers were coated with rabbit-GLUT1, followed by fluorescin isothiocyanate (FITC)-conjugated goat anti rabbit IgG, then washed thrice in PBS, and were used for microscopy. Rabbit anti-human GLUT1 antibody was kindly provided by Dr Cuchman SW (Experimental Diabetes, Metabolism, and Nutrition Section, NIDDK, NIH, Bethesda, USA).

GLUT1 analysis by flow cytometry Mesangial cells were detached by vigorous pipetting in the presence of versen (GIBCO BRL, Grand Island, NY, USA). For detection of GLUT1, 1×10^6 cells were washed with 0.1 % PBA (PBS + BSA) and incubated with rabbit anti-GLUT1 antibody for 30 min at 40 ℃. After washing twice with cold 0.1 % PBA, FITC-conjugated goat anti-rabbit IgG F $(ab')_2$ was added for 30 min at 40 ℃ in the dark. After two washes with cold 0.1 % PBA, cells were analyzed on flow cytometry (Coulter EPICS XI). Cells exposed to normal rabbit serum in which the primary antibody was omitted served as a negative control.

Measurement of 2-deoxyglucose (2-DOG) uptake rates and kinetics Uptake rates of glucose were determined using the nonmetabolizable analog 2-deoxy-*D*-[1-^{3}H] glucose (Amersham Life Science, Buckinghamshire, UK) according to the method previously reported[6,7]. Mesangial cells (2×10^5) were seeded in 2.5 cm diameter six-well plates and allowed to grow to a subconfluency. Confluent cells were made quiescent by reducing the concentration of FCS to 1 % for 24 h. The quiescent mesangial cells were incubated in medium containing 1 % FCS with indicated concentrations of TGF-β_1 or/and Rhein at 37 ℃ for the indicated time interval. Time course was performed at 3, 6, 10, and 24 h. After removal of the medium and rinsing with PBS, cultures were incubated in glucose-free PBS for 30 min, and then this buffer solution was replaced with one containing of 37 MBq/L [^{3}H]-2-DOG. The samples were incubated for 10 min. After that, the unincorporated radioisotope was rapidly removed by washing the cell layer with cold PBS, and cells were removed for counting by trpysinization. Rhein was provided by China Pharmaceutical University, Nanjing, China.

Kinetic analysis of glucose uptake was performed on mesangial cells. [^{3}H]-2-DOG was present at a concentration of 37 MBq/L. Unlabeled 2-DOG was added to the extracellular solution in increasing amounts varying from 0.25 to 10 mmol/L to determine the uptake rate of glucose, K_m and V_{max} values. Lineweaver-Burk analysis was performed to calculate the K_m and V_{max} for glucose uptake in the mesangial cells. The concentrations of cellular protein were determined using protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA).

Northern analysis for GLUT1 mRNA expression Mesangial cells were grown in 75 cm^2 flasks and treated with TGF-β_1 or/and rhein as described previously. Total RNA of mesangial cells was extracted using Trizole

method. RNA samples (20 μg each) were electrophoresed on 1 % agarose/formaldehyde gels and transferred onto nylon membranes. Blots were then prehybridized and probed for GLUT1 and β-actin using the respective cDNAs. The human GLUT1 cDNA was kindly provided by Dr Mueckler M (Department of Cell Biology and Physiology, Washington University School of Medicine, St Louis, MO, USA). The [^{32}P] cDNA were labeled by the random hexamer priming method (Promega). Blots were exposed to X-ray film for 48 h at −70 ℃, and autoradiograms were analyzed by optical scanning densitometry. Relative quantities of GLUT1 mRNA in different experimental designs were compared after normalization to mRNA for β-actin.

Statistics All results are shown as $x \pm s$. Groups were compared using two-tailed t-test for non-paired samples. Stastical significance was defined as $P < 0.05$.

RESULTS

Functional GLUT1 is present in human mesangial cells Expression of GLUT1 mRNA was detected by RT-PCR in human mesangial cells. The specificity of PCR product was confirmed by size and restriction enzyme analysis (Fig 1). Immunofluorescence staining was consistent with the result from the RT-PCR, showing the presence of GLUT1 proteins in mesangial cells. In addition, flow cytometric analysis of the fluorescence intensity profile revealed a shift to the right (Fig 2), confirming the presence of GLUT1 protein in mesangial cells.

Mesangial cells were grown in 2.5 cm diameter six-well plates in DMEM containing 20 % FCS. Measurements of glucose uptake were made at varying glucose concentrations, [^{3}H]-2-deoxyglucose (2-DOG) uptake increased linearly with the glucose concentration of incubation medium. The K_m value determined from the Lineweaver Burk plot was 0.36 mmol/L and the V_{max} value was 462 μmol·g^{-1}(protein)·h^{-1}.

Effect of TGF-β$_1$ on kinetics of 2-DOG uptake and GLUT1 mRNA expression in mesangial cells To test the role of TGF-β$_1$ on the glucose uptake of mesangial cells, various amounts of TGF-β$_1$ (0.5, 1, 2, and 4 μg/L) (Sigma, St Louis, MO, USA) were added to the medium for 10 h. The 2-DOG uptake in mesangial cells was markedly increased after stimulating with TGF-β$_1$ in a dose-dependent manner (Tab 1).

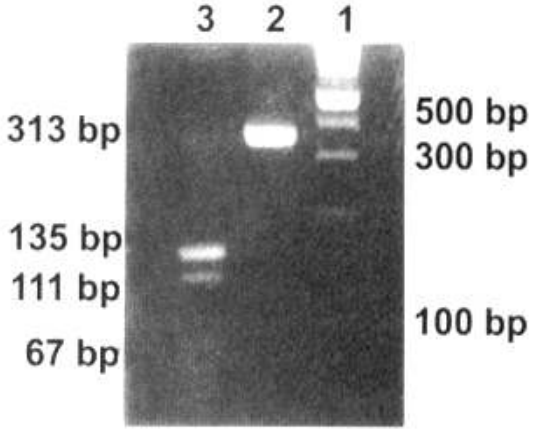

Fig 1. GLUT1 mRNA in human mesangial cell as detected by RT-PCR. Lane 1 is the molecular marker, Lane 2 is the PCR product of GLUT1, and Lane 3 is the PCR product digested by restriction enzyme BstoI, resulting in three fragments of 135 bp, 111 bp, and 67 bp.

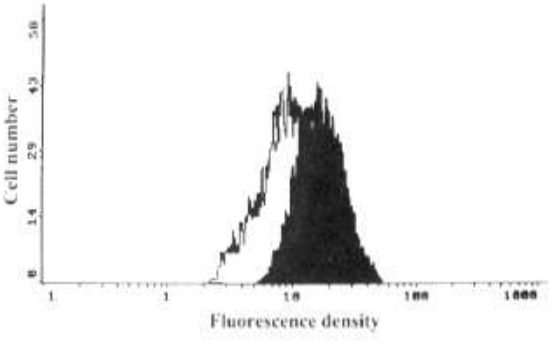

Fig 2. Histograms of anti-GLUT1 antibody staining of mesangial cells (solid peak) compared to matching unstained controls (blank peak) as analyzed by flow cytometry.

Tab 1. TGF-β$_1$ stimulates the glucose uptake in mesangial cells. n = the number of separate well. $^{a}P < 0.01$ *vs* control (TGF-β$_1$ 0 μg/L).

TGF-β$_1$/ μg·L^{-1}	n	Glucose uptake/ $10^{-8} \times$ Bq·g^{-1} protein
0	4	2.83 ± 0.15
0.5	4	7.3 ± 0.8^{a}
1	4	9.0 ± 1.8^{a}
2	4	14.3 ± 1.8^{a}
4	4	15.3 ± 1.7^{a}

TGF-β$_1$ increased 2-DOG uptake in mesangial cells by approximately 4-fold. Analysis of the kinetics of 2-DOG uptake showed that TGF-β$_1$ increased the V_{max} from 848

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mol·g^{-1}(protein)·h^{-1} to 960 mol·g^{-1}(protein)·h^{-1} and decreased K_m from 1.14 mmol/L to 0.39 mmol/L in mesangial cells (Fig 3). These data suggest that TGF-β_1 stimulation not only increases the affinity of transporters to glucose, but also affects the number of glucose transporters in mesangial cells. Furthermore, the effect of cycloheximide was examined to assess the requirement of new protein synthesis in TGF-β_1 stimulated glucose uptake in mesangial cells. The glucose uptake induced by TGF-β_1 stimulation was completely blocked by the addition of cycloheximide (1 mg/L), suggesting that new protein synthesis was needed for the TGF-β_1 stimulated glucose uptake in human mesangial cells. As shown in Fig 4, TGF-β_1 could stimulate the expression of GLUT1 mRNA after a coculture for 8 h in mesangial cells.

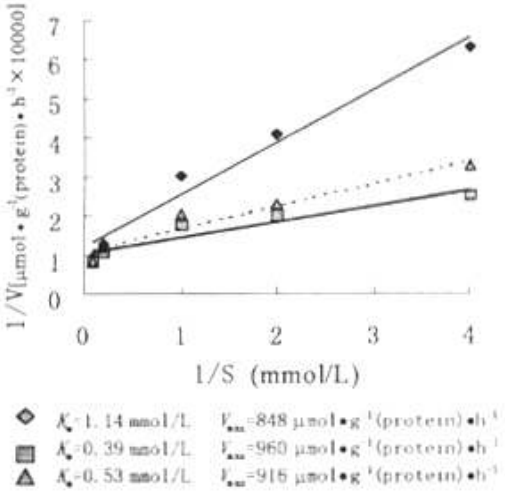

Fig 3. Kinetics analysis of 2-DOG uptake in mesangial cells. ◆ Mesangial cells cultured in control medium. ■ Mesangial cells incubated with TGF-β_1 (2 μg/L) for 10 h. ▲ Mesangial cells incubated with TGF-β_1 (2 μg/L) and rhein (50 mg/L) for 10 h. Results presented are the combined values from 4 separate experiments.

Effect of rhein on 2-DOG uptake and its kinetics and the GLUT1 mRNA expression in mesangial cells The amount of 2-DOG uptake in mesangial cells treated with various doses of rhein (25 mg/L or 50 mg/L) showed no significant change as compared to the untreated control. Kinetics study of 2-DOG uptake revealed that the values of K_m and V_{max} were similar in mesangial cells with or without rhein treatment [K_m 0.31 mmol/L *vs* 0.29 mmol/L, V_{max} 860 μmol·g^{-1}(protein)·h^{-1} *vs* 801 μmol·g^{-1}(protein)·h^{-1}].

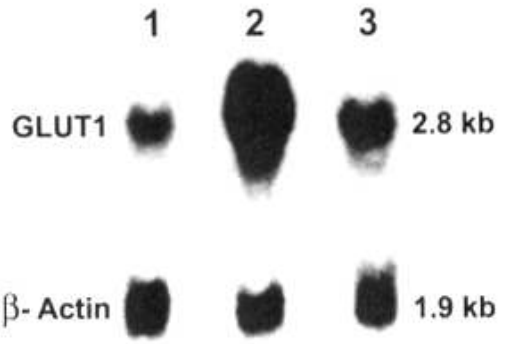

Fig 4. Northern analysis of GLUT1 mRNA expression in cultured mesangial cells. Lane 1 is the mesangial cells cultured in the control medium. Lane 2 is mesangial cells incubated with TGF-β_1 (2 μg/L) for 8 h and lane 3 is mesangial cells incubated with TGF-β_1 (2 μg/L) and rhein (50 mg/L) for 8 h.

Interestingly, the increased 2-DOG uptake in mesangial cells stimulated by TGF-β_1 was remarkably attenuated by the addition of rhein (Tab 2), with an increased K_m and decreased V_{max} values as shown in Fig 3. The enhanced expression of GLUT1 mRNA by TGF-β_1 in mesangial cells was also diminished by rhein (Fig 4).

Tab 2. Rhein inhibits the increased glucose uptake stimulated by TGF-β_1 in mesangial cells. n = the number of separate wells. $^cP<0.01$ *vs* controls. $^eP<0.05$, $^fP<0.01$ *vs* TGF-β_1.

Groups	n	Glucose uptake/ $10^{-6}\times$ Bq·g^{-1} protein
Control	4	2.40±0.03
TGF-β_1	4	11.2±0.4^c
TGF-β_1 + Rhein (20 mg/L)	4	8.2±1.0^e
TGF-β_1 + Rhein (50 mg/L)	4	4.0±0.7^f

DISCUSSION

Immunogold labeling and *in situ* hybridization studies demonstrated that glucose transporters were widely distributed from glomeruli down to the medullary collecting duct in normal kidneys and GLUT1 was the main isoform of glucose transporters in the rat mesangial cells[21,22]. As evidenced by the observation of mRNA expression, protein synthesis, and glucose uptake assay of the present study, we have proved that human glomerular mesangial cells do express GLUT1. This data provides the basis for further investigation of the role of GLUT1 in the pathogenesis of diabetic nephropathy. At the early phase

of STZ-induced diabetic rats, renal medullary GLUT1 mRNA levels were significantly increased[23,24]. Cultured rat mesangial cells transduced with the GLUT1 gene demonstrated a marked increase in glucose uptake and metabolism as well as significant stimulation of ECM synthesis, even when the cells were grown in a normal extracellular glucose concentration[6]. As change of mesangium is the basic lesion for diabetic nephropathy, it is rational to assume that functional alteration of GLUT1 might be implicated in the process of excessive uptake of glucose by the mesangial cell and the production of renal damage in diabetes.

TGF-β_1 plays an important role in the development of diabetic nephropathy. TGF-β_1 promotes tubular epithelial cell hypertrophy and regulates the glomerular production of ECM. TGF-β_1 also blocks the destruction of newly synthesized ECM by upregulating the synthesis of protease inhibitors and down-regulating the synthesis of matrix-degrading proteases[11,25]. Glomeruli isolated from STZ-diabetic rats demonstrated an increased expression of matrix components associated with a progressive increase in TGF-β_1 mRNA and protein levels[12,26]. Furthermore, glomeruli from kidneys in patients with established diabetic nephropathy overexpress TGF-β_1 protein[12,27], and the increased TGF-β_1 production present in diabetic patients even prior to the establishment of the kidney lesion[10]. Recently, TGF-β_1 was reported to enhance glucose uptake and GLUT1 mRNA expression in mesangial cells[14]. This observation indicated that TGF-β_1 might modulate the cell function by altering glucose metabolism. Our data also provided evidence that TGF-β_1 stimulated the glucose uptake in human mesangial cells by upregulating the expression of GLUT1 mRNA expression. The changed patterns of the K_m and V_{max} values in kinetics analysis of 2-DOG uptake indicated an increase in affinity of transporter to glucose and an increase in number of glucose transporters by TGF-β_1 in human mesangial cells. The fact that increased glucose uptake by TGF-β_1 stimulation was abolished by the addition of cycloheximide means requirement of new protein synthesis for the TGF-β_1-induced enhancement of glucose uptake in mesangial cells.

Rhubarb (*Rheum officinale*), a Chinese herbal medicine, has been found to be of value in preventing the development of diabetic nephropathy in STZ dibetic rats[17]. Isolated perfusion kidney studies also demonstrated that the oxygen consumption of the renal tissue of the rats treated with rhubarb was significantly lower as compared to the controls[28]. In addition, rhubarb has shown a blunting effect on the hypertrophy of either the remnant or diabetic kidney[28]. All these data indicated that the protective effect of rhubarb on the development of diabetic nephropathy might be achieved by reducing the renal metabolism in diabetes. Rhein, 4,5 dihydroxyanthraquinone-2-carboxylic acid, is one of the most important ingredient components of rhubarb. The effect of rhein in reducing glucose uptake has been reported in tumor cells[15,29]. In the present study, we found that the increased glucose uptake and GLUT1 mRNA expression by TGF-β_1 in mesangial cells could be markedly attenuated by the addition of rhein. The mechanism by which TGF-β_1 stimulates the glucose uptake in mesangial cells remains uncertain. The result of Northern blot and kinetics of 2-DOG uptake analysis indicated that TGF-β_1 was able to stimulate the mRNA expression of GLUT1 and increases the affinity of glucose transporters to glucose as well as the number of glucose transporters. In addition, the observation that coincubation of cycloheximide with TGF-β_1 could completely block TGF-β_1 induced stimulation of 2-DOG uptake indicated that a newly synthesized protein was required for this process. The inhibitory effect of rhein on protein synthesis in tumor cells has also been reported[29,30]. Thus, the inhibitory effect of rhein on protein synthesis may attenuate the process of TGF-β_1 stimulated glucose uptake. In conclusion, functional GLUT1 do present in human mesangial cells. Rhein plays a role in antagonizing the effect of TGF-β_1 to increase glucose uptake of mesangial cells through GLUT1. These findings have placed us closer in identifying therapeutic approaches for aborting GLUT1 overexpression in diabetic nephropathy.

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转化生长因子β及大黄酸对肾小球系膜细胞葡萄糖转运蛋白功能的影响[1]

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关键词 单糖转运蛋白类;转化生长因子β;肾小球系膜;大黄酸

目的:对人肾小球系膜细胞葡萄糖转运蛋白-1(GLUT1)进行鉴定.研究转化生长因子(TGF-β_1)对GLUT1表达和功能的影响及大黄酸的干预作用.**方法**:分别用RT-PCR、免疫荧光染色、流式细胞仪、Northern杂交和[^{3}H]-2脱氧葡萄糖摄入率对系膜细胞GLUT1 mRNA表达、蛋白质分布和功能进行了研究.观察不同浓度TGF-β_1在加或不加大黄酸的情况下对系膜细胞葡萄糖摄入以及GLUT1 mRNA表达的影响.**结果**:研究发现人类肾小球系膜细胞上存在功能性的GLUT1.TGF-β_1能上调系膜细胞GLUT1 mRNA的表达和增加系膜细胞葡萄糖摄入率.大黄酸对正常糖浓度培养条件下系膜细胞葡萄糖摄入无影响,但能明显抑制TGF-β_1增加系膜细胞GLUT1 mRNA表达和葡萄糖摄入的作用.**结论**:TGF-β_1增加人肾小球系膜细胞GLUT1 mRNA的表达和细胞葡萄糖的摄入,该作用能够明显地被大黄酸所拮抗.

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