Original Article
Role of caveolae in high glucose and TGF-β₁ induced fibronectin production in rat mesangial cells

Yuantao Liu¹*, Shengxia Lu²*, Yuchao Zhang³, Xiangdong Wang³, Feng Kong⁴, Ye Liu², Li Peng¹, Yuqin Fu²

¹Department of Endocrinology, The Second Hospital of Shandong University, Jinan, Shandong, China; ²Department of Nephrology, The Second Hospital of Shandong University, Jinan, Shandong, China; ³Institute of Cell Biology, Shandong University, Jinan, China; ⁴Central Laboratory, The Second Hospital of Shandong University, Jinan, Shandong, China; ⁵Department of Cardiology, Shandong Electric Power Central Hospital, Jinan, China.
*Equal contributors.

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Abstract: Accumulation of extracellular matrix (ECM) in glomerular mesangium correlates with loss of renal function in diabetic nephropathy. However, the mechanisms underlying are still incompletely known. In the present study, we explored the role of caveolae in ECM production in rat mesangial cells (MCs) stimulated by high glucose or transforming growth factor-β₁ (TGF-β₁), and investigated the possible mechanisms. High glucose (HG) or TGF-β₁ significantly increased collagen-1 and fibronectin expression at both mRNA and protein levels in time-course dependent manners, and simultaneously induced caveolin-1 tyrosine phosphorylation. Disruption of caveolae with Methyl-β-cyclodextrin (β-MCD) prevented HG and TGF-β₁ induced caveolin-1 tyrosine phosphorylation, and attenuated fibronectin but not collagen-1 production. This effect of β-MCD on fibronectin production could be abolished by cholesterol, which restored HG and TGF-β₁ induced caveolin-1 tyrosine phosphorylation, and attenuated fibronectin but not collagen-1 production. In addition, HG and TGF-β₁ induced fibronectin production was attenuated by a caveolin-1 scaffold domain peptide. These findings indicate that mesangial cell caveolae regulate fibronectin production at least partly through caveolin-1 phosphorylation.

Keywords: Caveolae, mesangial cells, high glucose, TGF-β₁, fibronectin

Introduction

Diabetic nephropathy (DN) is a severe microvascular complication of diabetes and the leading cause of end-stage renal disease (ESRD) [1]. A typical morphological alteration in diabetic kidney is the accumulation of extracellular matrix (ECM) in glomeruli that correlates with the loss of renal function [2]. The most common matrix proteins detected include collagen I, III, IV and fibronectin [3]. Under diabetic condition, the synthesis of these proteins is upregulated in mesangial cells (MCs), which ultimately leads to glomerulosclerosis [4].

Hyperglycemia is the primary pathogenic factor for diabetic nephropathy. So far, multiple mechanisms have been proposed for high glucose induced renal injury, including activation of various vasoactive hormone pathways like the renin-angiotensin system and endothelins [5], induction of oxidative stress [6], and production of advanced glycation end products (AGES) [7]. Of note, in vitro studies showed that high glucose could increase the synthesis of certain extracellular matrix (ECM) components in mesangial cells through induction of transforming growth factor-β₁ (TGF-β₁) and TGF-β type II receptor expression [8, 9].

(TGF-β₁) is a critical regulator of ECM that has been implicated in fibrogenesis of various tissues including the kidney [10, 11]. In human and experimental diabetic nephropathy, the expression of transforming growth factor beta is elevated [12]. Disruption of TGF-β₁ signaling significantly attenuated glomerular mesangial matrix expansion in diabetic animal models [13]. All the above studies indicate that both hyperglycemia and TGF-β₁ are involved in ECM accumulation in the mesangium in diabetic nephropathy. Revealing the signaling networks that mediate hyperglycemia and TGF-β₁-induced ECM overproduction may provide novel targets for the prevention and treatment of DN.
Caveolae are flask-shaped, cholesterol-enriched plasma membrane invaginations in a variety of cell types. As caveolae are highly enriched in cholesterol, agents such as β-methyl-cyclodextrin (β-MCD), which bind and sequester membrane cholesterol, can be used to disrupt caveolae to study the function of these microdomains. Caveolin-1 is the principle structural protein of caveolae, and plays an essential role in the formation of caveolae [14]. In addition, as a scaffolding protein, caveolin-1 can interact with multiple signaling molecules, including receptor tyrosine kinases, non-receptor tyrosine kinases and G-proteins. Through these interactions, caveolin-1 sequesters these proteins within caveolae and modulates their activity [15]. In response to various stimuli including oxidative stress and TGF-β1 [16, 17], caveolin-1 can be phosphorylated at tyrosine-14 by the Src family kinases. Although the functional significance of tyrosine phosphorylation on caveolin-1 is unclear, some studies showed that it might have regulatory effects on interactions between caveolin-1 and other proteins [18, 19]. In an idiopathic pulmonary fibrosis mouse model, overexpression of caveolin-1 markedly ameliorated bleomycin (BLM)-induced pulmonary fibrosis [11]. In mesangial cells, caveolae were shown to be required for TGF-β-induced fibronectin production [20].

In the present study, we aimed to further investigate the role of caveolae of mesangial cells in the pathogenesis of diabetic nephropathy, and to explore the possible mechanisms.

Materials and methods

Materials

Rabbit polyclonal anti-caveolin-1 was purchased from BD Transduction Laboratories (Lexington, KY, USA). Anti-phospho-caveolin-1 (Y14) was from Cell Signaling Technology (Danvers, USA). Anti-collagen-1 was from Abcam (MA, USA). Horse radish peroxidase (HRP)-conjugated goat anti-mouse and anti-rabbit secondary antibodies were from ZSGB-Bio (Beijing, China). Recombinant Human TGF-β1 was purchased from Pepro Tech (New Jersey, USA). Methyl-β-cyclodextrin (β-MCD) was from Sigma (St. Louis, MO, USA). All other materials were purchased from standard suppliers.

Caveolin-1 scaffold domain peptide

The caveolin-1 scaffolding domain peptide corresponding to amino acids (CSD peptide, amino acids 82-101, DGWKKASFTTFTVTKYWFYR) with an additional Antennapedia internalization sequence (RQIKIWFQNRRMKWKK) was synthesized by SBS Biotech (Beijing, China). A cav-1 scrambled control peptide (CSC, WGIDKAFFTTSTVTYKF) with an Antennapedia internalization sequence (RQIKIWFQNRRMKWKK) was synthesized for control. The cells were incubated with the peptides for 30 min before treatment with high glucose or TGF-β1.

Cell culture

Rat mesangial cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) containing 5.5 mmol/L glucose, 10% fetal calf serum at 37°C in 5% CO2, and passaged by trypsinization. For experiments, cells were passaged 1:5 by trypsinization and allowed to grow overnight before being made quiescent by serum deprivation for 24 h. The resultant sub-confluent (70-80%) cultures were treated as indicated. To examine the effects of hyperglycemia and TGF-β1 on ECM production, MCs were treated with D-glucose (30 mmol/L) or TGF-β1 (10 ng/ml, Peprotech) for the indicated time periods. Mannitol (24.5 mmol/L) was used for osmotic control. To examine the effects of β-MCD, cells were pretreated with 5 mmol/L β-MCD for 1 h in the presence or absence of cholesterol.

Real time RT-PCR

Total RNA was extracted using TRIZOL Reagent according to the manufacturer’s procedures (Takara Bio, Otsu, Shiga, Japan). The first strand cDNAs served as the template for quantitative PCR performed in eppendorf Real Time PCR System (Eppendorf Mastercycler ep realplex,
Caveolae regulate fibronectin production in mesangial cells

Figure 1. Effects of HG/TGF-β1 on caveolin-1, collagen-1 and fibronectin mRNA expression. MCs cells were treated with HG (30 mmol/L) or TGF-β1 (10 ng/ml) 0, 12, 24 or 48 h. Caveolin-1, collagen-1 and fibronectin mRNA expression was determined by real time RT-PCR (A) HG induced caveolin-1, collagen-1 and fibronectin mRNA expression (n = 3). (B) TGF-β1 induced caveolin-1, collagen-1 and fibronectin mRNA expression (n = 3). *P < 0.05, vs. 0 h.

Figure 2. Effects of HG/TGF-β1 on collagen-1 and Fibronectin protein expression. MCs cells were treated with HG (30 mmol/L) or TGF-β1 (10 ng/ml) 0, 12, 24 or 48 h. Collagen-1 protein expression was determined by Western blotting; fibronectin level was determined by ELISA. (A, B) Representative results of Western Blots for collagen-1 (n = 3), (C) Quantitative analysis of fibronectin expression (n = 3). *P < 0.05, vs. controls.
Caveolae regulate fibronectin production in mesangial cells

were performed in triplicate. The results were expressed as the OD ratio relative to GAPDH. Primers used in the experiments were shown in Table 1.

Western blot analysis

Samples were homogenized in RIPA (Radio-Immunoprecipitation Assay) lysis buffer containing protease and phosphatase inhibitors. Protein concentration was determined by a bicinchoninic acid (BCA) method (Pierce, IL, USA). Proteins (80 μg from each sample) were resolved on SDS-PAGE gels and transferred to a NC membrane (nitrocellulose membrane). Subsequently, the membrane was blocked in 5% non fat milk for 1 hour and incubated with primary antibodies overnight at 4°C with gentle agitation, followed by incubation with the HRP-conjugated anti-rabbit or anti-mouse secondary antibody (1:5000 dilution) for 1 hour at room temperature. After washing with PBS, immune complexes were detected using enhanced chemiluminescence (ECL) method. The relative intensity was determined with the Image J software. Primary antibodies used are as follows: Polyclonal anti-caveolin-1 (1:2000, BD), polyclonal anti-p-caveolin-1 Tyr14 (1:1000, CST), anti-type I Collagen-α1 chain (1:1000, Abeam), anti-β-actin (1:1000, ZSGQ).

Enzyme-linked immunosorbent assay

5,000 mesangial cells/well seeded in a 96-well plate, and treated for the indicated time. The supernatant was collected at each time point. Fibronectin concentration was determined using ELISA kit according to the manufacturers’ instructions. Absorbance values were measured with a microplate reader (Bio-Rad iMark, Richmond, CA, USA) at a wavelength of 450
Caveolae regulate fibronectin production in mesangial cells

Effects of HG and TGF-β1 on fibronectin, collagen-1 protein expression

Next, we determined the effects of HG and TGF-β1 on FN and Col-1 protein expression. MC cells were treated with HG (30 mmol/l) or TGF-β1 (10 ng/ml) for the indicated periods. Col-1 protein level was determined by Western blotting; FN concentration was determined by ELISA. As shown in Figure 2, HG significantly increased Col-1 protein expression at 24 h and FN protein expression at 12 h; TGF-β1 significantly increased both Col-1 and FN protein expression as early as 12 h (Figure 2).

Effects of HG and TGF-β1 on caveolin-1 tyrosine phosphorylation

To explore a possible role of caveolin-1 in ECM production, we determined the effects of HG and TGF-β1 on caveolin-1 expression and tyrosine phosphorylation. MC cells were treated with HG (30 mmol/l) or TGF-β1 (10 ng/ml) for the indicated periods. Caveolin-1 expression and phosphorylation were evaluated by Western blotting. As shown in Figure 3, HG treatment significantly induced cav-1 tyrosine phosphorylation.

Statistical analysis

All data were presented as mean ± SEM, and analyzed with one-way analysis of variance (ANOVA) followed by Newman-Keuls test. All analyses were performed using SPSS 17.0. A value of P < 0.05 was considered statistically significant.

Results

Effects of high glucose and TGF-β1 on fibronectin, collagen-1 and caveolin-1 mRNA expression

MC cells were treated with high glucose (HG) (30 mmol/l) or TGF-β1 (10ng/ml) for the indicated periods. At each time point, the mRNA levels of caveolin-1 (Cav-1), fibronectin (FN) and collagen-1 (Col-1) were determined by real time RT-PCR. As shown in Figure 1, both HG and TGF-β1 significantly increased Col-1 and FN mRNA expression as early as 12 h after treatments, and reached peak at 24 h. Cav-1 mRNA expression was not changed.
Caveolae regulate fibronectin production in mesangial cells

Figure 5. Effect of β-MCD on HG/TGF-β1 induced collagen-1 protein expression. MCs were left untreated or pre-treated with β-MCD (5 mmol/l), continued to be present during subsequent treatments) for 1 h, followed by HG (30 mmol/l) or TGF-β1 (10 ng/ml) for 48 h in the absence or presence of cholesterol (15 μg/ml). Collagen-1 protein expression was determined by Western blotting. A. Representative result of Western blots for collagen-1 protein expression induced by HG (n = 3). B. Representative result of Western blots for collagen-1 protein expression induced by TGF-β1 (n = 3). Results of quantitative analyses are presented in the corresponding lower panels. A. *P < 0.05, vs. NG. B. *P < 0.05, vs. controls.

Figure 6. Effect of β-MCD on HG/ TGF-β1 induced fibronectin expression. MCs were left untreated or pre-treated with β-MCD (5 mmol/l) for 1h, and then treated with HG (30 mmol/l) or TGF-β1 (10 ng/ml) in the absence or presence of cholesterol (15 μg/ml) for the indicated periods. The supernatant was collected, and fibronectin concentration was determined by ELISA. A. HG induced fibronectin expression (n = 3). B. TGF-β1-induced fibronectin expression (n = 3). A. *P < 0.01, vs. NG; †P < 0.01, vs. HG; ♦P < 0.01, vs. HG + β-MCD. B. *P < 0.01, vs. CON; †P < 0.01, vs. TGF-β1; ♦P < 0.01, vs. TGF-β1 + β-MCD.
Caveolae regulate fibronectin production in mesangial cells

Figure 7. HG and TGF-β1 induced fibronectin production was attenuated by a caveolin-1 scaffold domain peptide. MCs were pre-treated with caveolin-1 scaffold domain peptide (CSD peptide, 5 uM) or control peptide (CSC peptide, 5 uM) for 30 min, and then treated with HG (30 mmol/l) or TGF-β1 (10 ng/ml) for 48 h. The supernatant was collected, and fibronectin concentration was determined by ELISA. (A) HG induced fibronectin production; (B) TGF-β1 induced fibronectin production. *P < 0.05, vs. control; **P < 0.01, vs. control. #P < 0.05, vs. control; ##P < 0.01, vs. control.

Discussion

In the present study, we demonstrated that both HG and TGF-β1 significantly increased collagen-1 and fibronectin protein and mRNA expression in rat mesangial cells in a time-course dependent manners. Along with these responses, HG and TGF-β1 induced caveolin-1 tyrosine phosphorylation. Disruption of caveolae prevented HG and TGF-β1 induced caveolin-1 tyrosine phosphorylation, and attenuated HG and TGF-β1 induced fibronectin production.
In addition, HG and TGF-β1 induced fibronectin production was attenuated by a caveolin-1 scaffold domain peptide. These findings suggest that mesangial cell caveolae may play a role in fibronectin overproduction under diabetic conditions, which may contribute to the pathogenesis of diabetic nephropathy.

Hyperglycemia is the primary pathogenic factor for diabetic nephropathy. Hyperglycemia may induce renal injury through multiple mechanisms, including activation of renin-angiotensin system and endothelins, induction of oxidative stress, and production of AGEs [5-7]. In the present study, we demonstrated that high glucose directly upregulated collagen-1 and fibronectin expression in rat mesangial cells. In addition to hyperglycemia, many other factors such as TGF-β may also play important roles in the pathogenesis of diabetic nephropathy. In human and experimental diabetic nephropathy, the expression of TGF-β is elevated [12]. TGF-β1 is a well-known mediator in the initiation and progression of fibrosis [21]. In pulmonary fi broblasts, TGF-β1 was shown to induce collagen-1 and fibronectin overexpression [11]. In diabetic animal models, disruption of TGF-β signaling significantly attenuated glomerular mesangial matrix expansion [13]. In the present study, we demonstrated that TGF-β1 significantly increased collagen-1 and fibronectin expression in rat mesangial cells in both mRNA and protein levels.

Caveolae are small flask-shaped invaginations of the plasma membrane that serve as platforms for multiple signaling processes [14]. Caveolin-1, a 21 to 24-kDa integral membrane protein, is a principal structural component of caveolae [15]. In the kidney of both human and rodents, caveolin-1 is most abundant in the muscle layer of blood vessels, parietal epithelial cells (PECs) and endothelial cells [22]. Renal mesangial cells have similar characteristics as vascular smooth muscle cells, which are rich in caveolin as well. There is evidence showing that platelet-derived growth factor (PDGF) receptor is localized in caveolae membrane in mesangial cells, and caveolin may play a role in the pathogenesis of the mesangial proliferative glomerular diseases through PDGF signaling [22]. So far, the role of mesangial cell caveolae in the pathogenesis of diabetic nephropathy has not been well documented. A recent study showed that caveolin-1 expression is reduced in the endothelial cells of renal blood vessels in streptozocin-induced diabetic rats, which may contribute to the development of diabetic nephropathy through inhibition of endothelial NO synthase (eNOS) and NO production [23].

To explore the potential role of caveolae in mesangial cell in diabetic nephropathy, we examined the effects of β-MCD on TGF-β1 and HG induced ECM production. β-MCD is a cholesterol-sequestering agent, which is the most commonly used agent to disrupt caveolae. Our results showed that β-MCD treatment significantly attenuated TGF-β1 and HG induced fibronectin production, but had no evident effect on collagen-1 expression. These results suggest that mesangial cell caveolae may have a preferential regulatory role in fibronectin production.

Caveolin-1 is the structural protein of caveolae. Previous studied showed that caveolin-1 can physically interact with multiple signaling molecules and modulates their activity [15, 19, 24]. Caveolin-1 was first identified as a major substrate of Src kinase [25]. Micro-sequencing of Src-phosphorylated caveolin-1 revealed that phosphorylation occurs within the extreme N-terminal region of full-length caveolin-1 [26]. In addition to Src, other stimuli including mechanotransduction, oxidative stress, insulin and EFG have been also showed to induce caveolin-1 tyrosine phosphorylation [19, 27-29]. Importantly, tyrosine phosphorylation on caveolin-1 may enhance the interactions between caveolin-1 and other proteins [18, 19]. In the present study, we demonstrated that both TGF-β1 and HG significantly induced caveolin-1 tyrosine phosphorylation, which coincided with an upregulation of fibronectin production. Disruption of caveolae with β-MCD prevented HG and TGF-β1 induced caveolin-1 tyrosine phosphorylation, and attenuated fibronectin production. Furthermore, this effect of β-MCD on fibronectin production could be abolished by cholesterol, which restored HG and TGF-β1 induced caveolin-1 tyrosine phosphorylation. These results suggested that caveolae may play a role in regulation of fibronectin production through caveolin-1 phosphorylation.

A recent study showed that phosphorylated caveolin-1 could interact with and activate RhoA, and RhoA mediated downstream signaling pathways play essential roles in regulation of ECM genes expression [20]. This study high-
Caveolae regulate fibronectin production in mesangial cells

lights the importance of interactions between caveolin-1 and other signaling molecules in regulation of ECM production. In line with this study, we demonstrated that a caveolin-1 scaffold domain peptide attenuated HG and TGF-β1 induced fibronectin production. The caveolin-1 scaffold domain peptide is supposed to compete with endogenous caveolin-1 for interacting with other proteins.

In summary, we demonstrated that the mesangial cell caveolae play a role in regulation of fibronectin production in response to high glucose and TGF-β1 stimulation. The mechanism may involve the phosphorylation of caveolin-1 and its interactions with other signaling molecules.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Yuqin Fu, Department of Nephrology, The Second Hospital of Shandong University, 247 Beiyuan Street, Jinan 250033, Shandong, China. E-mail: sdufyqin@163.com

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