Allergologia et immunopathologia
Sociedad Española de Inmunología Clínica, Alergología y Asma Pediátrica
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ORIGINAL ARTICLE

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Knockdown of GPRC5B alleviates the high glucose-induced inflammation and extracellular matrix deposition of podocyte through inhibiting NF-κB pathway

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Received 22 December 2021; Accepted 11 January 2022
Available online 1 March 2022

KEYWORDS:
diabetes;
diabetic nephropathy (DN);
high glocuse (HG);
GPRC5B;
ECM deposition;
NF-κB pathway

Abstract
Background: Diabetes is a serious disease that could greatly increase the risk of cardiovascular complications, whereas the underlying pathology of DN is still unknown. GPRC5B is a member of the RAIG subfamily of type 3 (family C) GPCR, and its role in DN is still unclear.
Objective: To unveil the role of GPRC5B in diabetic nephropathy (DN) progression and investigate the potential signaling pathway.
Materials and methods: Podocytes were stimulated with high glucose and expression of GPRC5B was analyzed by qPCR and western blot. Then the level of GPRC5B was depleted by siRNA transfection and inflammatory cytokine level was monitored by ELISA assay. The ECM depostion and the activation of NF-κB pathway were detected by Immunoblot.
Results: We investigated the possible role of GPRC5B in the pathology of diabetic nephropathy. We found GPRC5B was highly expressed in high glocuse (HG) induced podocytes. The depletion of GPRC5B inhibited HG induced cell inflammation. In addition, the ablation of GPRC5B suppressed the HG induced ECM deposition. We further found GPRC5B could alleviate the inflammation and extracellular matrix deposition of HG-induced podocytes through NF-κB pathway.
Conclusion: We therefore thought GPRC5B could serve as a promising target for the treatment of diabetic nephropathy. G-protein-coupled receptors.

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https://doi.org/10.15586/aei.v50i2.566

Introduction

Diabetes is a serious disease that could greatly increase the risk of cardiovascular complications, such as coronary artery disease, myocardial infarction, hypertension, and dyslipidemia. Cardiovascular injury mainly targets two important organs, namely the eyes and kidneys.[1] This makes diabetic nephropathy (DN) as one of the most common complications of diabetes. The main typical renal histological changes in DN are caused by changes in the extracellular matrix (ECM).[2] ECM accumulation in DN leads to expansion in glomerular membrane, tubulointerstitial fibrosis, and irreversible deterioration of renal function.[3] However, so far, the underlying pathology of DN is still unknown.

As is well known, nuclear factor *kappa B* (NF-κB) signaling pathway regulates the expression of various genes involved in inflammatory response. Recent studies have demonstrated that high glucose (HG) could induce phosphorylation and ubiquitination of inhibitor protein IkappaBalpha (IκBα), thereby promoting the activation of NF-κB.[4] NF-κB plays a central regulatory role in DN renal inflammation and fibrosis.[5]. In addition, inhibiting NF-κB pathway could reduce HG-induced mesangial cell inflammation, oxidative stress, and ECM deposition.[6]

G protein-coupled receptors (GPCR) are characterized by seven transmembrane domains and constitute an important class of evolutionarily conserved receptor proteins. These are considered as the most popular drug targets because of their key role in cell signal transduction.[7] GPRC5B, also known as retinoic acid inducible gene 2 (*RAIG2*), is a member of the RAIG subfamily of type 3 (family C) GPCR. In the pancreas, GPRC5B is considered as a negative regulator of insulin secretion.[8] It is significantly up-regulated in common human glomerulopathy (including DN, immunoglobulin A (IgA) nephropathy, and lupus nephritis) and can activate NF-κB pathway.[7] In addition, overexpression of GPRC5B in the fibrotic heart, lung, and liver can enhance the production of collagen in myofibroblasts, thus directly promoting fibrosis in tissues.[9] However, the specific role of GPRC5B in DN is unclear.

In this study, the role of GPRC5B in the pathology of DN was investigated. The collected data revealed that the depletion of GPRC5B could alleviate inflammation and ECM deposition of podocyte upon HG treatment through inhibiting NF-κB pathway. Therefore, GPRC5B could serve as a promising target for the treatment of DN.

Materials and Methods

Cell culture

Mouse podocytes were obtained from the Cell Resource Center of Peking Union Medical College (Beijing, China) and maintained in RPMI1640 medium supplemented with 10% fetal bovine serum (FBS; Gibco, Thermo Fisher, Billings, MT, US), 5-mM glucose, 1% penicillin–streptomycin, and 10-U/mL recombinant mouse IFN-γ (Sigma-Aldrich, USA) in a humidified culture hood with 5% CO_2 at 37°C. For HG stimulation, podocytes were exposed to 30-mM concentration of HG for 36 h and subjected to further detection.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted with RNA extraction kit (Beyotime Biotechnology, Shanghai, China). RNA was reverse-transcribed into Complementary DNA (cDNA) (Thermo Fisher, Waltham, MA, US). qRT-PCR analysis was performed with SYBR Green Supermix (Bio-Rad, Hercules, CA, US) with the Bio-Rad CFX96 system. Following primers were used in the detection:

Primer	Sequences 5′-3′
GPRC5B forward	GGATGAACATAACGCAGCTCTC
GPRC5B reverse	GTCGGCTGATACACATTGCTTC
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) forward	CTCTGCTCCTCCTGTTCGAC
GAPDH reverse	ACCAAATCCGTTGACTCCGA

The following thermocycling conditions were used: Initial denaturation at 95°C for 3 min; followed by 30 cycles of denaturation at 95°C for 30 sec, annealing at 58°C for 30 sec and extension at 72°C for 30 sec. The 2-$\Delta\Delta$Cq method was used to quantify the results.

Western blotting

Cell lysates were collected post-lysis with radioimmunoprecipitation assay (RIPA) buffer. After centrifugation, protein concentration was determined by bicinchoninic acid (BCA) protein assay kit (Beyotime Biotechnology, Shanghai, China). Proteins were then separated with 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes. These membranes were incubated with 5% *bovine serum albumin* (BSA) followed by primary antibodies targeting GPRC5B (1:1000; Novus Biologicals, Southpark Way, Littleton, CO, US), Collagen I(1:1000; Abcam, Cambridge, UK), Collagen IV (1:1000, Abcam), Fibronectin (1:1000, Abcam), *phospho-p65* (p-p65; 1:1000, Abcam), transcription factor p65 (1:1000, Abcam), p-IκBα (1:1000, Abcam), IκBα (1:1000, Abcam), and GAPDH (1:10,000; Abcam). Membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies at a dilution of 1:1000 for 2 h after being washed with tris buffered saline with tween (TBST) for 15 min. The signals were detected with enhanced chemiluminescence (ECL) detection kit.[10]

Cell transfection

The podocytes were transfected with GPRC5B small interfering RNAs (siRNAs) or negative control siRNA using Lipofectamine RNAi MAX (Thermo Fisher Scientific, Waltham, MA, US) according to the manufacturer's guidance. The siRNA targeting GPRC5B was *s82192* (Life

Technologies, Grand Island, NY, US). After transfection, cells were stimulated with HG for 36 h.

Enzyme-linked-immunosorbent serologic assay (ELISA)

The concentrations of Interleukin 1β (IL-1β), tumor necrosis factor-α (TNF-α), and IL-6 in cell lysates were measured with ELISA kit following the guidance of protocols. Briefly, samples were added into wells, and biotin-conjugated primary antibodies were plated into wells before the addition of avidin-conjugated HRP. Enzyme substrate was then added for chromogenic reaction. The absorbance was measured with a microplate reader (R&D Systems, Minneapolis, MN, US).

Statistical Analysis

Data analysis was statistically analyzed by one-way ANOVA. Graphs were obtained using the GraphPad Prism 6.0 software (San Diego, CA, US). Data were expressed as mean ± SD, and $P < 0.05$ was considered as a significant difference.

Results

GPRC5B was increased in HG-treated podocytes

At first, the effect of HG on the expression of GPRC5B in podocytes was evaluated. It was noticed that the mRNA level of GPRC5B was elevated in HG (30-mM glucose)-stimulated podocytes (Figure 1a). Similarly, the protein level of GPRC5B was also accumulated in HG-treated podocytes (Figures 1b and b'). These results suggested that GPRC5B could play an important role in DN.

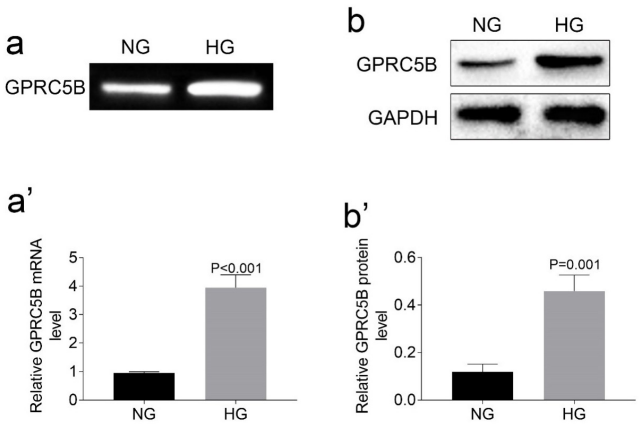

Figure 1 GPRC5B was increased in HG-treated podocytes. (a) The mRNA level of GPRC5B in podocytes was stimulated with HG. (b) The protein level of GPRC5B was enhanced in podocytes treated with HG. Data were represented as mean ± SD.

Knockdown of GPRC5B inhibited inflammation in podocytes stimulated with HG

In order to explore the potential role of GPRC5B in podocytes, the expression of GPRC5B was modulated by transfection of GPRC5B siRNA into podocytes. The expression of GPRC5B was significantly decreased after siRNA transfection (Figures 2a and a'). Cellular inflammation was assessed by detecting the expression level of IL-6, IL-1b, and TNF-a. HG induced a significant increase in the expression of these cytokines. Knockdown of GPRC5B blocked the induction of these cytokines (Figures 2b, b', and b''). Thus, HG treatment- induced inflammation in podocyte was abolished by GPRC5B knockdown.

GPRC5B ablation suppressed the HG-induced ECM deposition

Previous studies believed that multiple proteins were involved in progression of DN, particularly inflammatory cytokines and ECM deposition. Therefore, the ECM deposition via detecting the expressions of collagen I, collagen IV, and fibronectin was evaluated. As expected, HG increased the expressions of collagen I, collagen IV, and fibronectin. However, knockdown of GPRC5B significantly inhibited the HG-induced elevation of collagen I, collagen IV, and fibronectin (Figure 3). Therefore, GPRC5B was involved in HG-mediated ECM deposition.

Knockdown of GPRC5B could alleviate the inflammation and ECM deposition in podocyte on HG treatment via inhibiting NF-κB pathway

In order to analyze further the underlying mechanism of GPRC5B regulating inflammation and ECM deposition in

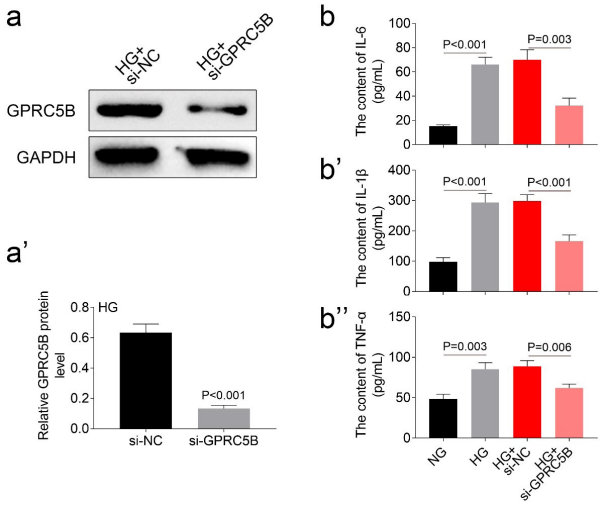

Figure 2 Knockdown of GPRC5B inhibited inflammation in podocytes stimulated with HG. (a) Knockdown of GPRC5B was detected by Western blot test. (b) Expression of inflammatory cytokines was detected in control or GPRC5B-depleted cells treated with HG. Data were represented as mean ± SD.

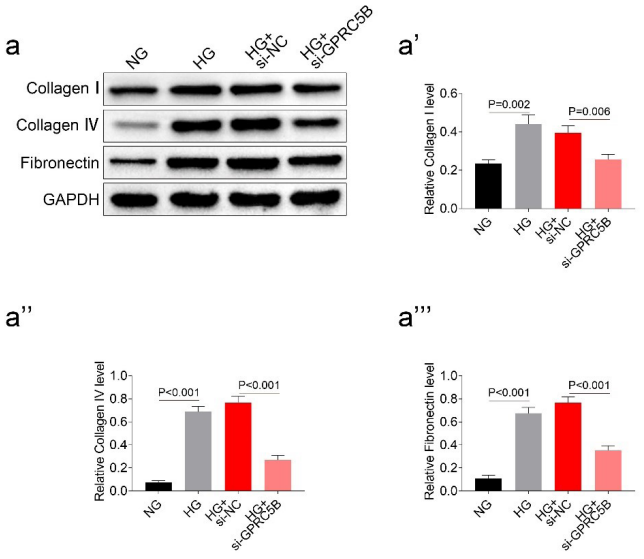

Figure 3 GPRC5B ablation suppressed the HG-induced ECM deposition. The expression of collagen I, collagen IV and fibronectin was detected in control or GPRC5B-depleted cells treated with HG. Data were represented as mean ± SD.

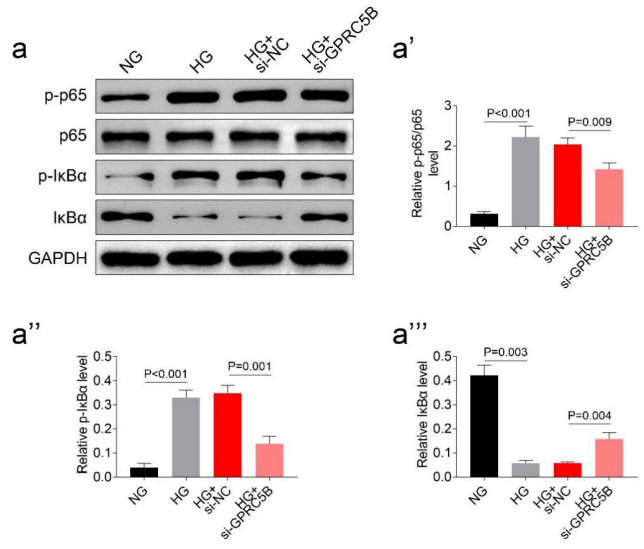

Figure 4 Knockdown of GPRC5B could alleviate the inflammation and ECM deposition of podocyte upon HG treatment via inhibiting NF-κB signaling pathway. The activation of NF-κB pathway was measured in control or GPRC5B-depleted cells treated with HG. Data were represented as mean ± SD.

podocytes treated with HG, the relative expressions of p-p65, p65, p-IκBα, and IκBα were analyzed. HG treatment significantly induced the activation of p-p65 and p-IκBα. Besides, GPRC5B depletion suppressed the activation of p-p65 and p-IκBα, suggesting that knockdown of GPRC5B inhibited the activation of NF-κB signaling pathway and therefore modulated the inflammatory response and ECM deposition of podocytes with HG treatment (Figure 4).

Discussion

In this study, an *in vitro* cell model of DN was developed. Our data confirmed that GPCR, GPRC5B, had the potential to regulate the inflammation and ECM deposition of podocytes. DN is one of the most important complications of diabetes.[11] The treatment of DN is often more difficult than other kidney diseases if it develops to end-stage renal disease because of its complex metabolic disorders. Hence, timely prevention and treatment for inhibiting the progression of DN is of great significance.[12] Nephrotic syndrome in DN is often more pronounced in edema than in general primary glomerular disease, and is often accompanied with severe hypertension.[13] In the pathogenesis of DN, accumulation of ECM can lead to dilation of the glomerular membrane, renal tubulointerstitial fibrosis, and irreversible deterioration of renal function.[14] In order to improve the prognosis of patients, it is necessary to study the pathogenesis of DN and identify key regulatory proteins.

In this study, the podocytes were treated with HG to simulate DN *in vitro*. The qRT-PCR and Immunoblot assays revealed that the expression of GPRC5B was high in podocytes with HG treatment. Results of Immunoblot and ELISA assays suggested that knockdown of GPRC5B suppressed HG-induced inflammation and ECM deposition in podocytes. As a GPCR, GPRC5B had multiple cellular and physiological functions.[15,16] For example, GPRC5B contributed to the production of collagen in myofibroblasts.[17] GPRC5B could also mediate smooth muscle contractility and differentiation by suppressing the prostacyclin receptor pathway.[17] In addition, GPRC5B could modulate inflammatory response as well as fibrotic pathways in cardiac fibroblasts and mice hearts.[18] GPRC5B also activated the obesity-associated inflammatory signaling in adipocytes. Similarly, it was also observed that GPRC5B exhibited pro-inflammatory effect on podocytes.[19]

It was noticed that GPRC5B modulated the HG-induced inflammatory response and ECM deposition in podocytes through mediating the NF-κB signaling pathway. Similarly, a previous study confirmed that GPRC5B modulated the inflammatory response in glomerular diseases by regulating NF-κB signaling pathway, which played an important role in regulating inflammatory progression.[20] NF-κB plays a central regulatory role in renal inflammation and fibrosis in DN, and the inhibition of NF-κB pathway could reduce HG-induced inflammation, oxidative stress, and ECM deposition in mesangial cells.[21] Several studies confirmed that multiple proteins affected progression of DN, particularly inflammatory response and ECM deposition via this pathway. For example, yes-associated protein 1 (YAP1) could promote HG-induced inflammation and ECM deposition in glomerular mesangial cells by activating NF-κB pathway.[22] Sirtuin 4 (SIRT4) suppressed NF-κB pathway to alleviate podocyte pyroptosis in DN. These studies, including the findings of the present study, confirmed that NF-κB pathway could serve as a promising target for treating DN.

In conclusion, it was established that the expression of GPRC5B was lifted in HG-treated podocytes. Depletion of GPRC5B inhibited HG-induced inflammation and ECM deposition in podocytes. It was further established that GPRC5B could alleviate the inflammation and ECM deposition

of HG-treated podocytes via targeting NF-κB pathway. Therefore, GPRC5B could serve as a promising target for treating DN.

Competing interests

The authors stated that there were no conflicts of interest to disclose.

Contribution of authors

Yiying Tao and Youwen Lin designed and carried out the study. Ling An, Yijuan Tao, and Yulin Li supervised data collection, and analyzed and interpreted the data. Jianfang Han, Wenbo Hu, and Wenhua Liu reviewed the draft of manuscript and prepared the same for publication. All authors read and approved the final manuscript.

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