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Inhibitor of Bruton's tyrosine kinases, PCI-32765, decreases pro-inflammatory mediators' production in high glucose-induced macrophages

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ABSTRACT

Accumulating evidence has shown that macrophages play a vital role in development and pathogenesis of diabetic nephropathy (DN) by secreting inflammatory cytokines. Although Bruton's tyrosine kinases (Btk) is a biologically important molecule implicated in immune regulation, the role of Btk in high glucose (HG)-stimulated inflammatory response in macrophages and the mechanism involved need further investigation. In our study, we used bone marrow-derived macrophages (BMMs) to investigate the involvement of Btk on HG-induced inflammatory cytokines expression and to explore the underlying mechanisms. We found that high glucose induced phosphorylation of Btk, MAPKs and NF-κB, and the expression of downstream inflammation cytokines monocyte chemo-attractant protein-1 (MCP-1), tumor necrosis factor-alpha (TNF-α) and interleukin-1beta (IL-1β). Btk inhibitor (PCI-32765) not only down-regulated ERK1/2 phosphorylation and NF-κB activation, but also decreased the secretion of MCP-1, TNF-α and IL-1β in HG-treated BMMs. These results indicate that Btk plays an important role in HG-induced inflammatory cytokines expression and that PCI-32765 may be used as an immunoregulatory agent against hyperglycemia-induced inflammatory response in DN.

1. Introduction

Diabetic nephropathy (DN) is one of the microvascular complication of patients with diabetes mellitus [1], leading an important cause of chronic kidney disease and end-stage renal disease [2]. Currently, it has been regarded as a chronic inflammatory disease currently. Accumulated evidence indicated that macrophage, a key component of immune cells, played important roles in development and pathogenesis of DN via secreting the pro-inflammatory cytokines [3–5]. In the hyperglycemia environment, activation of MCP-1 and TNF-α promoted monocyte/macrophage migration and activation [6], subsequently eliciting kidney injury [7]. Furthermore, it has been proven that HG induced inflammatory cytokines and chemokines expression by activating mitogen-activated protein kinases (MAPKs) and nuclear factor kappa B protein (NF-κB) activation in RAW264.7 cell lines [8]. These findings focused on the pathogenic roles of HG in activation of macrophage/monocyte and the progression of DN. However, detailed mechanisms underlying HG-induced inflammation remain to be elucidated.

Bruton's tyrosine kinases (Btk), a non-receptor tyrosine kinase belonging to the Tec kinase family [9], has mainly been studied as a part of adaptive immunity. However, recent study has identified that Btk as emerging key player in innate immunity [10], and secretion of inflammatory cytokines, such as TNF-α, IL-1β [11,13]. Moreover, studies have shown that Btk activity followed the stimulation of several receptors for immune regulation [14–16]. In addition, Btk has been indicated to interact with Toll's like receptors, such as TLR2 and TLR4, and enhanced the transactivation of p65 subunit of NF-κB [16–18]. In conclusion, Btk may play a vital role in inflammatory and immune response.

PCI-32765, also named ibrutinib, is a highly selective small molecule inhibitor of Btk [19,20], which inhibits Btk phosphorylation at Tyr223 and consequently depresses its enzymatic activity [21]. This drug has shown great anti-lymphoma activities in preclinical and clinical trials in recent years [22–24]. In addition, PCI-32765 was administered in murine IC disease models, such as autoimmune arthritis [25]. Here, we studied the effects of PCI-32765 on HG-induced inflammatory cytokines expression via MAPK and NF-κB in BMMs and explored the mechanisms involved of Btk in the development of DN.

2. Materials and methods

2.1. Antibodies and reagents

Glucose and Mannitol were purchased from Sigma (St. Louis, MO,

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USA). Btk inhibitor (PCI-32765) was purchased from (Selleck, USA). The trizol reagent was purchased from Invitrogen (CA, USA), SYBR Green PCR master mix kit and Revert Aid Premium First Strand cDNA Synthesis Kit were obtained from Vazyme (Vazyme Biotech Co, Nanjing, China). FITC stained anti-mouse F4/80, APC stained anti-mouse/human CD11b and isotype controls were gained from Biolegend (Biolegend, San Diego, California, USA). Anti-Btk, anti-phospho-Btk, anti-JNK, anti-phospho-JNK, anti-p38MAPK, anti-phospho-p38MAPK, anti-phospho-ERK, anti-ERK, anti-NF-κBpp65, anti-NF-κBp65, anti-IκB, anti-phospho-IκB, anti-iNOS antibody were gained from Cell Signaling Technology (Beverly, MA, USA); anti-β-actin, anti-rabbit IgG and anti-mouse IgG conjugated to horseradish-peroxidase antibodies were obtained from Wuhan Sanying Biotechnology Inc. (Wuhan, China). Protein Assay Kit was obtained from Beyotime Institute of Biotechnology (Jiangsu, China). CCK-8 kit was purchased from Vazyme (Vazyme Biotech Co, Nanjing, China). ELISA kit for mouse IL-1β was obtained from RayBiotech (RayBiotech, Guangzhou, China), ELISA kit for mouse MCP-1 and mouse TNF-α were bought from Excell Bio (Excell Bio, Shanghai, China).

2.2. Primary culture of bone marrow-derived macrophages

Bone marrow-derived macrophages (BMMs) were isolated from male C57 mice as previously described [26]. Briefly, the femur and tibia bones of 6–8-week-old C57 mice were used and flushed out cells into 2% fetal bovine serum (FBS) + cold PBS (about 6 ml/mouse). Bone marrow stem cells were centrifuged at 500*g* for10 min. And then added 3 ml red blood cell lysis buffer to the precipitation and centrifuged at 500*g* for 5 min again. Precipitated cells were suspended in low glucose DMEM containing 10% FBS, 1% penicillin/streptomycin and 15% L-929 cell culture supernatant at 37 °C in an atmosphere containing 5% CO2. The medium was changed on day 3. BMMs were identified with flow cytometry analysis on day 7.

2.3. Cell viability assay

Cells viability was detected using CCK-8 kit according to the manufacturer's instruction. Briefly, 1×10^4 BMMs were plated in 96-well plates. After overnight culturing, the BMMs were incubated with different concentrations of PCI-32765 or DMSO (as control) for 45 min and then were exposed to HG for 24 h. Separately, 10 μl CCK-8 solution was added to each well and cultured for another 4 h. Cell viability was determined by detecting the absorbance of the converted dye at 490 nm using a microplate reader. The average optical density (OD) in the control cells was set as 100% viability, and treatment results expressed as a percentage of control.

2.4. RNA extraction and qRT- PCR

RNA extraction and real-time PCR were operated as what we our previously reported [27]. Concisely total RNA was extracted from the cells using Trizol reagent according to the user's protocol. 1 μg RNA was reverse-transcribed to cDNA using Reverse Transcription System. The cDNA was amplified by real-time PCR instrument with AceQ qPCR SYBR Green Master Mix. The primers to detect mRNA were as follows: GAPDH, as the internal control: Forward primer: 5′- ACCCCAGCAAG GACACTGAGCAAG -3′, Reverse primer: 5′- GGCCCCTCCTGTTATTAT GGGGGT -3′; TNF-a: Forward primer: 5′- CCCTCCTGGCCAACGGC ATG-3′, Reverse primer:5′- TCGGGGCAGCCTTGTCCCTT-3′; MCP-1: Forward primer: 5′-TTGACCCGTAAATCTGAAGCTAAT-3′, Reverse primer: 5′-TCACAGTCCGAGTCACACTAGTTCAC-3′; IL-1β: Forward primer: 5′-GCCTCGTGCTGTCGGACCCATAT-3′, Reverse primer: 5′-TCCTTTGAGGCCCAAGGCCACA -3′. Calculate the relative mRNA levels using $2^{-\Delta\Delta Ct}$ methods with values normalized to a reference gene GAPDH.

2.5. Enzyme-linked immunosorbent assay (ELISA)

Supernatants from macrophages cultures were harvested and removed the supernatant after centrifugation. MCP-1, IL-1β and TNF-a were detected using ELISA kit according to the manufacturer's instructions.

2.6. Confocal microscopy analysis

Cells were plated on cover glasses bottom dishes. Macrophages were pre-incubated with different concentrations of PCI-32765 or not for 45 min and then exposed with HG for another 24 h. Cell samples were fixed by 4% formaldehyde at RT for 30 min. After blocking for 2 h with mixed solution (5% BSA + 0.2% TritonX-100), the macrophages were incubated with anti-iNOS or NF-κBp65 primary antibody at 4 °C overnight. After washing 3 times with PBS, Alexa Fluor 647-conjugated F4/80 primary antibody and Alexa Fluor 488 secondary antibody were added for 2 h at 37 °C in the dark. After washing 3 times with PBS, nuclear were stained by 40,6 0-diamidino-2-phenylindole (DAPI) for 10 min, and the macrophages were visualized under Leica TCS SP5 laser confocal microscope (Leica, Germany).

2.7. Flow cytometry (FCM) analysis

1×10^6 mature BMMs were suspended in PBS and blocked for unspecific binding with anti-CD16/CD32, followed by surface staining of FITC anti-mouse F4/80, APC anti-human/mouse CD11b or isotype controls. The detail steps were as follows: the cells were washed and centrifuged, and then added PBS (500 μl/tube) containing FITC-conjugated anti-mouse F4/80, APC-conjugated anti-mouse CD11b to resuspended macrophages. Then, samples were incubated at room temperature for 30 min in the dark. Macrophages were subsequently centrifuged and washed. Finally, 500 μl PBS was added to the tube to resuspend macrophages and then the macrophages were examined with the Backman FACS Calibur.

2.8. Western blotting analysis

Macrophages were pre-incubated with of PCI-32765 or not for 45 min, and then exposed in HG for the indicated time. The total protein was resolved and transferred to polyvinylidene difluoride (PVDF) membranes. Subsequently, the protein membranes were blocked in 5% nonfat dried milk for 2 h at 37 °C, and then incubated with primary antibodies at 4 °C overnight, and then incubated with HRP conjugated secondary antibodies before washing 3 times with PBST. Then the protein membranes were detected with enhanced chemiluminescence (ECL) reagent and captured protein bands using a chemiluminescence GE 600 system after being washed with TBST for three times. Finally, the results were presented as the relative ratio and quantified by image J.

2.9. Statistical analysis

In this study, all data was showed as the means ± SD. Data was analyzed using a one-way ANOVA in this study. Differences were considered significant if the p value was lower than 0.05.

3. Results

3.1. Isolation of BMMs

F4/80 and CD11b were used as markers for pan-macrophage. As shown in Fig.1A, mature BMDMs were defined as F4/80 + CD11b + cells. High purity of macrophages could be obtained on day 7 when 94.5% of them are presented as F4/80 and CD11b double positive cells.