



Original Article

The role of local IL6/JAK2/STAT3 signaling in high glucose–induced podocyte hypertrophy

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Background: Interleukin-6 (IL6) is an important regulator of cellular hypertrophy through the gp130/Janus kinase 2 (JAK2)/signal transducer and activator of transcription 3 (STAT3) pathway. We tested the hypothesis that IL6 and its downstream gp130/JAK2/STAT3 pathway participated in high glucose (HG)–induced podocyte hypertrophy.

Methods: IL6 levels in the media and lysates of podocytes were measured by enzyme-linked immunosorbent assay. Western blots were performed to determine the protein expression levels of gp130/JAK2/STAT3 among podocytes cultured with normal glucose (NG), NG + mannitol, NG + recombinant IL6, HG, and HG + IL6-neutralizing antibodies (IL6NAb). Immunoprecipitation was examined to determine whether gp130 interacted with JAK2 in response to HG or IL6. Podocyte hypertrophy was verified using protein/cell counts and flow cytometry.

Results: IL6 levels were significantly increased in the media and lysates of podocytes cultured in HG compared with the NG groups. The nuclear phospho-STAT3/STAT3 ratio was increased by HG and NG + IL6 and was attenuated in the HG + IL6NAb groups, indicating that nuclear STAT3 was activated following JAK2 and cytosolic STAT3 activation in response to IL6 secreted by HG-stimulated podocytes. Immunoprecipitation showed increased phospho-JAK2 recruitment to gp130 in the HG and NG + IL6 groups, and the addition of IL6NAb in the HG group significantly abrogated these increases. Podocyte hypertrophy was significantly increased in the HG and NG + IL6 compared with the NG condition and was diminished by the addition of IL6NAb to the HG group.

Conclusion: IL6 might play a prominent role in the local activation of JAK2/STAT3 in podocyte hypertrophy under HG conditions. *In vivo* studies examining this pathway are warranted.

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Introduction

Podocyte hypertrophy is an early structural change in diabetic nephropathy [1], although its pathogenic relevance has been a topic of debate. However, recent studies have shown

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that interruption of the signaling pathways related to podocyte hypertrophy ameliorated the long-term advancement of nephropathy in various diabetic animal models [2–5]. This indicates that the hypertrophic signals for podocytes under diabetic conditions may be candidate targets for therapeutic intervention in the early stages of diabetic nephropathy.

It is well established that increased levels of interleukin-6 (IL6) in the plasma or urine correlate with the progression of diabetic nephropathy [6–8]. Moreover, the local expression of IL6 in the kidney is increased in patients with diabetic nephropathy [8,9]. The action of IL6 is mainly mediated through the Janus kinase (JAK)/signal transducers and activators of transcription (STAT) pathway, which plays a pathogenic role in glomerular basement membrane thickening and mesangial expansion during the course of diabetic nephropathy [10–12]. In addition to its impact on extracellular matrix dynamics, several lines of evidence indicate that IL6 mediates cellular hypertrophy through activation of the JAK/STAT pathway in different types of cells [13–15]. Given that activation of the JAK/STAT signaling pathway in the glomeruli is elevated in an early stage of diabetic nephropathy [16], we hypothesized that this pathway may play a critical role in the pathogenesis of podocyte hypertrophy and that IL6 may be a potential upstream regulator of this signaling pathway. However, little is known about the effects of JAK/STAT signaling or its potential upstream regulator, IL6, in podocytes under high-glucose (HG) conditions.

Therefore, to evaluate the role of IL6 in the podocyte hypertrophy contributing to diabetic nephropathy, we examined changes in IL6, JAK2, and STAT3 expression in podocytes cultured under HG conditions and quantified the effect of the signaling pathway on podocyte hypertrophy.

Methods

Podocyte culture

Conditionally immortalized mouse podocytes, which were a gift from Dr Peter Mundel, were cultured as noted in our previous study [17]. The podocytes were grown in media consisting of RPMI 1640 containing fetal bovine serum (10%), penicillin–streptomycin (100 U/mL), and interferon- γ (IFN- γ , 50 U/mL) in collagen-coated flasks at 33°C. Then, the IFN- γ concentration was gradually reduced to 10 U/mL. The cells were trypsinized and subcultured in 100-mm dishes without IFN- γ and differentiated at 37°C for 10 days. Characterization of the podocyte cell line was confirmed by the identification of synaptopodin. After confirming the differentiation of podocytes and following culturing under serum starvation for 24 hours, the medium was replaced with RPMI containing normal glucose (NG, 5.6 mM), NG + 24.4 mM mannitol (MN; NG + MN), or HG (30 mM). The podocytes were activated by recombinant IL6 (rIL6; 10 ng/mL; R&D Systems, Minneapolis, MN, USA), and this effect was reversed with IL6NAbs (1 μ g/mL; R&D Systems). After 48 hours, the cells were harvested with trypsin (0.05%) and ethylenediaminetetraacetic acid (0.25 mM), pelleted at 1,500g for 5 minutes, and resuspended in phosphate-buffered saline (PBS).

Enzyme-linked immunosorbent assay for IL6

The levels of IL6 were measured in the lysates of podocyte cell lines and the cell culture media using an enzyme-linked immunosorbent assay kit (USCN Life, Wohan, China).

Nuclear cytoplasmic fractionation

To evaluate the expression of nuclear phospho-STAT3, we used nuclear cytoplasmic fractionation in a podocyte cell line. Treated podocytes were harvested and washed in chilled PBS, and the remaining pellets were dried as much as possible. Nuclear cytoplasmic fractionation was conducted using the NEPER nuclear and cytoplasmic extraction reagent kit (Thermo Fisher Scientific, Boston, MA, USA) according to the manufacturer's protocol.

Immunoprecipitation

For immunoprecipitation, the cytoplasmic fractions were incubated with 4 μ g polyclonal anti-gp130 antibody (Millipore Corporation, Bedford, MA, USA) overnight at 4°C and then with protein A/G PLUS-agarose immunoprecipitation reagent (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) for another 3 hours. After 5 washes with TBS-T buffer (Tris-buffered saline, 0.05% Tween 20), the samples were subjected to sodium dodecyl sulfate polyacrylamide gel analysis followed by Western blotting with a 1:1,000 dilution of monoclonal anti-phospho-JAK2 (Tyr1007/1008) antibody (GeneTex, Inc., Irvine, CA, USA) and polyclonal anti-JAK2 antibody (Millipore Corporation).

Western blot analysis

The nuclear and cytoplasmic fractions were aliquoted (60 μ g each) and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis. The proteins were transferred to a polyvinylidene difluoride membrane (Millipore Corporation) using a wet blotting apparatus (Bio-Rad, Inc., Hercules, CA, USA), and the membrane was incubated in blocking buffer [Tris-buffered saline, Tween 20 (0.05%), and bovine serum albumin (5%) or nonfat milk (5%)] for 1 hour at room temperature, followed by overnight incubation at 4°C in a 1:1,000 dilution of polyclonal rabbit anti-phospho-STAT3 (Tyr705; Cell Signaling Technology, Inc., Danvers, MA, USA), polyclonal rabbit anti-phospho-SHP2 (Tyr580; Millipore Corporation), monoclonal mouse anti-STAT3 (Cell Signaling Technology, Inc.), polyclonal rabbit anti-SHP2 (Millipore Corporation), or monoclonal mouse anti- β actin antibody (Sigma-Aldrich, St. Louis, MO, USA). The membrane was then washed 4 times for 10 minutes in TBS-T. Next, the membrane was incubated in 5% nonfat milk blocking buffer containing a 1:3,000 dilution of horseradish peroxidase–linked goat antirabbit and antimouse IgG (Bio-Rad, Inc.). The washes were repeated, and the membrane was developed with chemiluminescence agent (ECL; Amersham Life Science, Inc., Arlington Heights, IL, USA). The band densities were quantified using ImageJ software (NIH Image, Bethesda, MD, USA).

Assessment of hypertrophy in cultured podocytes

Podocyte hypertrophy was confirmed by quantifying the cellular protein/cell counts and using flow cytometry. Small aliquots of podocytes were counted, and the remaining cells were lysed in 0.5 M NaOH. The total protein content was calculated by a modified Lowry method. To measure the cell size directly, the cells were harvested by trypsinization after 48 hours of treatment as described previously, fixed with 75% methanol, and washed and incubated with RNase (100 μ g/mL)

and propidium iodide (10 $\mu\text{g}/\text{mL}$) in PBS for 1 hour at 37°C. The cell size was measured by forward light scatter using a FACScan flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). The data were analyzed with CellQuest Pro software (BD Biosciences).

Statistical analysis

All values are expressed as the mean \pm standard error of the mean. Statistical analysis was performed using the statistical package SPSS for Windows version 21.0 (SPSS, Inc., Chicago, IL, USA). The results were analyzed using the Kruskal–Wallis nonparametric test for multiple comparisons. Significant differences in the test were confirmed by the Mann–Whitney *U* test. A $P < 0.05$ was considered statistically significant.

Results

IL6 levels in the media and lysates of podocytes cultured under HG conditions

To evaluate the production and secretion of IL6 in HG-stimulated podocytes, the levels of IL6 were measured by ELISA in the media and cell lysates of podocytes. The IL6 levels were significantly higher in both the media (NG, 6.31 ± 0.86 pg/mL; HG, 9.12 ± 1.23 pg/mL; $P < 0.05$) and cell lysates (NG, 8.01 ± 0.91 pg/mL; HG, 11.45 ± 1.38 pg/mL; $P < 0.05$) of podocytes cultured in HG conditions (Figs. 1A, B). No effects of osmolarity were observed on the expression of IL6. This result indicated that HG may stimulate the production and secretion of IL6 by cultured podocytes.

Involvement of HG in JAK2 phosphorylation in podocytes via IL6

To investigate whether the phosphorylation of JAK2 is directly mediated by IL6 through gp130, which is the signal-transducing part of the IL6 receptor, we immunoprecipitated proteins with anti-gp130 and immunoblotted with anti-phospho-JAK2. The amount of phospho-JAK2 immunoprecipitated with anti-gp130 antibodies was significantly increased under HG and NG + rIL6 conditions compared with that

observed under the NG condition. However, the phospho-JAK2 levels decreased in podocytes cultured in HG supplemented with IL6NAb (Fig. 2). These data demonstrated that JAK2 was directly activated by IL6 produced by HG-stimulated podocytes.

Effect of HG on JAK2/STAT3 signaling in podocytes

To assess the effect of HG on JAK2 and STAT3 signaling, we examined the protein levels of JAK2, phospho-JAK2, nuclear STAT3, nuclear phospho-STAT3 and cytosolic STAT3, and cytosolic phospho-STAT3 under several conditions, including NG,

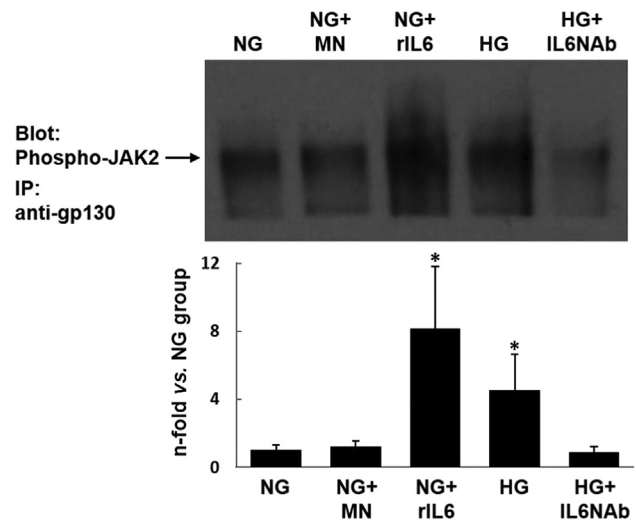


Figure 2. Induction of the phosphorylation of JAK2/STAT3 by HG in podocytes via IL6. Phosphorylation of JAK2 was significantly higher in podocytes cultured in NG conditions with rIL6 and HG conditions compared with NG alone. Increased phosphorylation of JAK2 was reversed under HG conditions by IL6NAb. The data were expressed as fold changes in the phosphorylation of JAK2 relative to podocytes cultured under NG conditions. IP anti-gp130 was immunoblotted with anti-phospho-JAK2 (Tyr1007/1008) antibody.

* $P < 0.05$ versus the NG group.

HG, high glucose; IL6, interleukin-6; IL6NAb, IL6-neutralizing antibody; IP, immunoprecipitated; JAK2, Janus kinase 2; MN, mannitol; NG, normal glucose; rIL6, recombinant IL6; STAT3, signal transducer and activator of transcription 3.

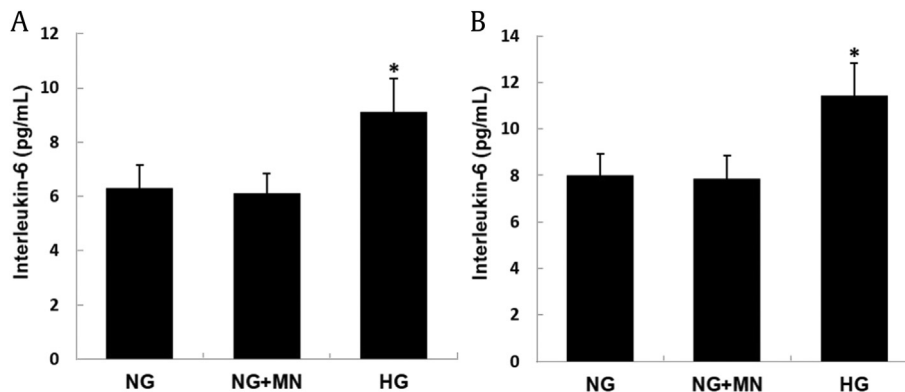


Figure 1. Expression of IL6 in the media (A) and cell lysates (B) of podocytes determined by ELISA. MN was used as an osmotic control. IL6 expression was significantly higher in both the media and lysates from podocytes cultured in HG than in those from podocytes cultured in NG or NG + MN.

* $P < 0.05$ HG versus NG groups.

ELISA, enzyme-linked immunosorbent assay; HG, high glucose; IL6, interleukin-6; MN, mannitol; NG, normal glucose.

NG + MN, NG + rIL6, HG, and HG + rIL6NAb by Western blotting. We confirmed that the expression levels of gp-130 and the ratio of gp-130/ β -actin did not differ among the groups. The ratios of phospho-JAK2/JAK2 and both nuclear and cytosolic phospho-STAT3/STAT3 were increased in the HG and NG + rIL6 groups compared with those in the NG and NG + MN groups. A significant decrease in the ratio of nuclear and cytosolic phospho-STAT3/STAT3 was observed in podocytes cultured in HG supplemented with rIL6NAb (Figs. 3A, B). These results indicate that JAK2 and both cytosolic and nuclear STAT3 were phosphorylated as a result of the IL6 produced by HG-stimulated podocytes.

Effects of HG on podocyte hypertrophy

To examine the effect of IL6 produced by HG-stimulated podocytes on cellular hypertrophy, we quantified the cellular protein/cell counts using a forward light scattering method. Flow cytometry showed that the cell size was significantly increased in podocytes cultured in HG and NG + rIL6 compared with those cultured in NG alone. Podocyte hypertrophy induced by HG was suppressed by the addition of IL6NAb (Fig. 4). The cellular protein/cell count ratio of the podocytes was significantly increased in the HG and NG + rIL6 groups compared with the NG group (NG, 0.75 ± 0.21 ; NG + rIL6, 1.32 ± 0.33 ; HG, 1.12 ± 0.25 ; HG + IL6NAb, 0.85 ± 0.22 ; * $P < 0.05$ vs. NG groups; Fig. 5).

Discussion

Although the role of podocyte hypertrophy in the pathophysiology of diabetic nephropathy is still not clearly defined, it is generally considered a principal factor in the early stage of diabetic nephropathy, and preceding glomerular morphologic changes have been documented in patients with type 2 diabetes [18] as well as in a diabetic animal model [1]. A recent morphometric analysis also suggested that podocyte hypertrophy was related to glomerular enlargement and occurred before podocyte depletion and segmental sclerosis [19]. In addition, podocyte hypertrophy accompanied by changes in

podocyte biology affects the glomerular filtration barrier and results in a further increase in proteinuria [20]. Podocytes are thought to have a limited capacity for cell differentiation, and the proportion recruited to the glomerulus is finite; therefore, cellular hypertrophy leads to the overall loss of podocytes [20,21]. Consequently, preventing podocyte hypertrophy may be one important strategy for treatment in the early stage of diabetic nephropathy.

IL6 is a cytokine regulating an extensive range of biological activities, including inflammatory responses. Much evidence has accumulated to demonstrate an association between serum IL6 and clinical findings such as proteinuria as well as pathologic findings, including glomerular basement membrane widening, in diabetic nephropathy [8,22]. The results from the present study suggest that IL6 might be expressed systemically and locally in glomerular cells stimulated with HG. To date, the role of IL6 in glomerular cells has mainly been investigated in mesangial cells as a growth factor [23,24]. In addition, this proinflammatory cytokine has also been shown to induce various types of cellular hypertrophy [25,26]. However, the role of IL6 in podocyte hypertrophy under HG conditions has not been well studied. Moreover, there have been few studies on the signaling pathways involved in podocyte hypertrophy under HG conditions [2].

In the present study, we studied the role of IL6 in the local activation of the JAK/STAT pathway and the consequent effects on podocyte hypertrophy. HG induced the production and secretion of IL6 in podocytes and increased the relative ratios of phosphorylation of cytosolic and nuclear STAT3. Moreover, local activation of the JAK2/STAT3 pathway induced podocyte hypertrophy, and this effect was abrogated by the addition of IL6NAb to the media of HG-stimulated podocytes.

Although IL6 is a well-known upstream activator of the JAK2/STAT3 pathway, it can also activate other pathways, such as the mitogen-activated protein kinase-dependent and phosphatidylinositol 3-kinase-dependent pathways [27]. In addition, several IL6 family cytokines share the common signal transducer gp130 and further activate various downstream pathways [28]. In this study, we performed immunoprecipitation with anti-gp130 and blotted with anti-phospho-JAK2

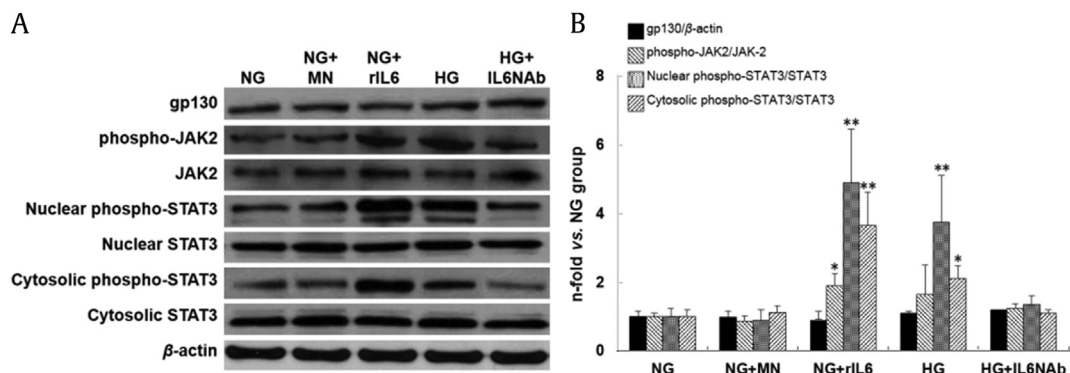


Figure 3. Effect of HG on the JAK2/STAT3 signaling pathway. (A) A representative Western blot of JAK2, pJAK2, nuclear STAT3, nuclear phospho-STAT3, cytosolic STAT3, and cytosolic phospho-STAT3 is shown. (B) The ratios of nuclear phospho-STAT3/STAT3 and cytosolic phospho-STAT3/STAT3 were significantly higher in the NG + rIL6 and HG conditions compared with the NG controls. The addition of IL6NAb to HG significantly diminished these increases in nuclear and cytosolic phospho-STAT3 activation. The ratio of pJAK2/JAK2 was significantly higher under NG + rIL6 conditions compared with NG. * $P < 0.05$ versus NG groups. ** $P < 0.01$ versus NG groups. HG, high glucose; IL6NAb, IL6-neutralizing antibody; JAK2, Janus kinase 2; MN, mannitol; NG, normal glucose; pJAK2, phospho-JAK2; rIL6, recombinant IL6; STAT3, signal transducer and activator of transcription 3.

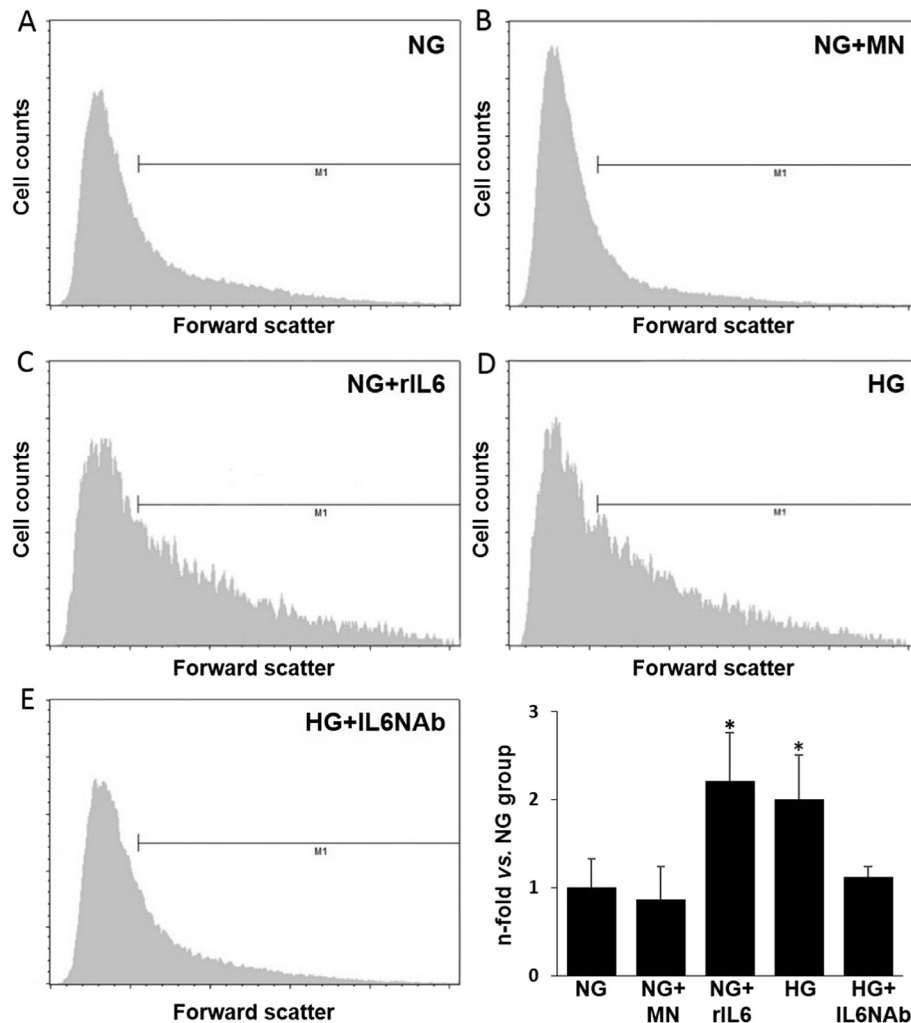


Figure 4. Assessment of podocyte hypertrophy by flow cytometry. A representative histogram of podocyte size as confirmed by forward light scatter (A–E). The size of the podocytes was increased under NG+rIL6 (C) and HG (D) conditions compared to that observed with NG (A) or NG+MN (B) media. IL6NAb (E) reduced the increase in the cell size of HG-cultured podocytes.

* $P < 0.05$ versus the NG group.

HG, high glucose; IL6NAb, IL6-neutralizing antibody; MN, mannitol; NG, normal glucose; rIL6, recombinant IL6.

antibody to verify whether JAK2 could be activated by HG stimulation through direct interaction with gp130. Podocytes treated with HG showed increased amount of phosphorylated JAK2 in anti-gp130 immunoprecipitates. Increased activation of JAK2 induced by HG stimulation was also shown in NG conditions with the addition of rIL6 and was attenuated at a similar level as in NG-cultured podocytes with addition of IL6NAb. Based on these findings, we have observed that the IL6/gp130/JAK2 interaction was upregulated in HG-stimulated podocytes.

Transforming growth factor- β and angiotensin II are also known as upstream activators of the JAK/STAT pathway, and these factors induce mesangial cell proliferation. To the best of our knowledge, no known studies to date have investigated the role of the JAK2/STAT3 pathway in podocyte hypertrophy under HG stimulation. The addition of IL6NAb almost completely inhibited the activation of the JAK2/STAT3 pathway in HG-stimulated podocytes. Therefore, we carefully suggest that a local activation of the JAK2/STAT3 pathway is the main signaling mechanism underlying podocyte hypertrophy.

The JAK/STAT pathway has an important role in several renal diseases, where it acts on cell proliferation and apoptosis through cytokine and growth hormone signaling [29]. The JAK2/STAT3 signaling pathway, induced by angiotensin II, affects mesangial matrix protein production and consequently induces glomerular hypertrophy. STAT3 activation requires phosphorylation induced by JAK2, and phosphorylated STAT3 dimers translocate to the nucleus to induce the expression of STAT regulatory genes that are essential for cell proliferation, differentiation, and survival. Our study showed that HG increased the activation of both cytosolic and nuclear STAT3 in podocytes, and this effect was diminished by the addition of IL6NAb. Therefore, HG increased the activation of nuclear STAT3 by affecting upstream cytosolic STAT3 and JAK2 through IL6. The phosphorylation of JAK2 was evaluated by immunoprecipitation with anti-gp130 and was found to be induced via the IL6 signal transduction component gp130, which is expressed in podocytes and is known to mediate signals mainly via JAK/STAT [30].

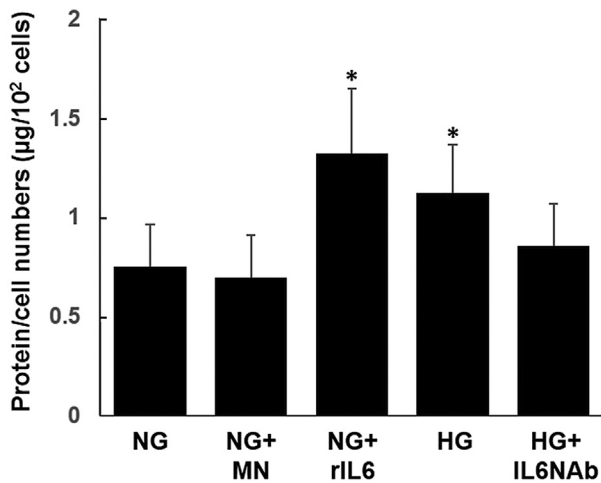


Figure 5. Assessment of podocyte hypertrophy by cellular protein/cell counts of podocytes. The ratio of total cellular protein/the total number of cells was significantly higher in podocytes exposed to NG + rIL6 and HG, and this effect was inhibited when IL6NABs were added to cells cultured under HG.

* $P < 0.05$ versus the NG group.

HG, high glucose; IL6NAB, IL6-neutralizing antibody; MN, mannitol; NG, normal glucose; rIL6, recombinant IL6.

This study has a limitation in that we examined nuclear and cytosolic STAT3 protein expression level at only 1 time point. We did not evaluate the results over time to confirm that the source of nuclear STAT3 was cytosolic STAT3. Cytosolic STAT3 levels could be increased by positive regulation of nuclear STAT3 transportation. However, nuclear STAT3 levels are commonly increased by cytosolic STAT3 translocation; therefore, the increased nuclear levels of activated STAT3 may still have resulted from import of cytosolic STAT3.

Moreover, in a previous study, podocytes treated with HG underwent cell cycle arrest and consequent apoptosis through IL-6-associated changes in the expression of the cell cycle inhibitory proteins p21Cip and p27Kip in podocytes [31]. Local activation of the IL-6/JAK2/STAT3 pathway in podocytes could activate p21Cip and p27Kip expression. Further studies are needed to investigate whether podocyte hypertrophy could be induced by cell cycle arrest through this mechanism. A previous study showed that diabetic mice with a 25% reduction of STAT3 activity showed increased IL6 messenger RNA expression in the glomeruli compared with those with a 75% reduction in STAT3 activity [11]. We suggested that the IL6 produced by STAT3 activity could further stimulate STAT3-related pathways, resulting in an aggravating effect on inflammatory responses in diabetic nephropathy [11]. Another study evaluated kidney biopsy samples from diabetic nephropathy patients using microarray gene analysis and found that factors participating in the JAK/STAT pathway were increased in the early stage of diabetic nephropathy in the glomerular compartment compared with those of control patients [16]. Collectively, these findings and our present results strongly suggest that the IL6/JAK2/STAT3 signaling pathway may be involved in podocyte hypertrophy in the early stage of diabetic nephropathy.

In conclusion, we demonstrated that HG-stimulated podocytes produced and secreted IL6, which activated the JAK2/STAT3 pathway via autocrine or paracrine mechanisms and participated in the process of cellular hypertrophy *in vitro*. This

effect was attenuated by the addition of IL6NABs to podocytes cultured under HG conditions, directly demonstrating that IL6 was the cause of HG-induced podocyte hypertrophy. Further *in vivo* studies are needed to validate this *in vitro* study, specifically those focusing on the role of IL6 in JAK2/STAT3 signaling that leads to podocyte hypertrophy. Further investigation of these mechanisms is expected to elucidate therapeutic targets for diabetic nephropathy. To establish the pathogenesis of diabetes-induced podocyte hypertrophy, it is essential to examine this pathway in an *in vivo* animal model.

Conflicts of interest

All authors have no conflicts of interest to declare.

Acknowledgments

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