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Tyrosines-740/751 of PDGFRβ contribute to the activation of Akt/Hif1α/TGFβ nexus to drive high glucose-induced glomerular mesangial cell hypertrophy

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Abstract

Glomerular mesangial cell hypertrophy contributes to the complications of diabetic nephropathy. The mechanism by which high glucose induces mesangial cell hypertrophy is poorly understood. Here we explored the role of the platelet-derived growth factor receptor-β (PDGFRβ) tyrosine kinase in driving the high glucose-induced mesangial cell hypertrophy. We show that high glucose stimulates the association of the PDGFRβ with PI 3 kinase leading to tyrosine phosphorylation of the latter. High glucose-induced Akt kinase activation was also dependent upon PDGFRβ and its tyrosine phosphorylation at 740/751 residues. Inhibition of PDGFRβ activity, its downregulation and expression of its phospho-deficient (Y740/751F) mutant inhibited mesangial cell hypertrophy by high glucose. Interestingly, expression of constitutively active Akt reversed this inhibition, indicating a role of Akt kinase downstream of PDGFRβ phosphorylation in this process. The transcription factor Hif1α is a target of Akt kinase. siRNAs against Hif1α inhibited the high glucose-induced mesangial cell hypertrophy. In contrast, increased expression of Hif1α induced hypertrophy similar to high glucose. We found that inhibition of PDGFRβ and expression of PDGFRβ Y740/751F mutant significantly inhibited the high glucose-induced expression of Hif1α. Importantly, expression of Hif1α countered the inhibition of mesangial cell hypertrophy induced by siPDGFRβ or PDGFRβ Y740/751F mutant. Finally, we show that high glucose-stimulated PDGFRβ tyrosine phosphorylation at 740/751 residues and the tyrosine kinase activity of the receptor regulate the transforming growth factor-β (TGFβ) expression by Hif1α. Thus we define the cell surface PDGFRβ as a major link between high glucose and its effectors Hif1α and TGFβ for induction of diabetic mesangial cell hypertrophy.

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Keywords

Diabetic nephropathy; Renal hypertrophy; Receptor tyrosine kinase

1. Introduction

The pathology of diabetic kidney disease consists of changes in the glomeruli with altered hemodynamics and matrix protein expansion [1,2]. The initial structural changes include renal hypertrophy especially glomerular hypertrophy, which leads to hyperfiltration and microalbuminuria. This is followed by fibrosis [3]. In fact, mesangial matrix expansion correlates well with the development of diabetic nephropathy. One third of the glomerular cell types consists of mesangial cells, which are considered as vascular pericytes of the glomerulus. These cells respond to hyperglycemia and contribute to the whole glomerular hypertrophy [4–6]. Hyperglycemia increases the production of hormones and cytokines such as angiotensin II and TGFβ, which contribute to the pathology of mesangial cells [2].

Importantly, the prominent growth factors the mesangial cells respond to are the PDGFs [7]. Mammalian genome codes for four different isotypes of this growth factor: A, B, C and D [8]. By homo or heterodimerization, these proteins form five isoforms. These isoforms bind to two distinct receptors, PDGFRα and PDGFRβ, with variable affinity and specificity. Although PDGF AB and BB bind to both receptors, the PDGF DD interacts with PDGFRββ with high affinity and to a lesser extent to the heterodimeric PDGFRαβ. On the other hand PDGF AA and CC bind to PDGFRα with high affinity; however, CC can interact with PDGFRαβ at a lower affinity [9]. Mesangial cells express all five dimeric PDGFs and their three trimeric receptors [10]. PDGF BB, CC and DD are mitogenic for mesangial cells and play a prominent role in mesangioproliferative glomerulonephritis [7,11,12,13]. On the other hand PDGF AA is not mitogenic for mesangial cells [13]. In fact PDGF BB, which functions predominantly through binding to the PDGFRββ, is the most potent mitogen and activator of various downstream signaling pathways [13,14]. Furthermore, mice deficient in either PDGF-B chain or PDGFRβ lack development of mesangial cells [15]. Although proliferation of glomerular mesangial cells is not a predominant feature of diabetic nephropathy, glomerular expression of PDGF-A is increased in patients with diabetic nephropathy [16]. Augmented urinary excretion of PDGF BB has been reported in the type 2 diabetes patients [17]. Similarly, PDGF B and its receptor beta are expressed in the diabetic mesangial cells [16,18]. Since mesangial cell hypertrophy represents an early event in the pathology of diabetic nephropathy, whether PDGFRβ signal transduction contributes to this phenomenon has not been investigated. In the present study, we show that high glucose initiates the PI 3 kinase/Akt signaling cascade to drive the expression of Hif1α via activation of PDGFRβ. The PDGFRβ autophosphorylation sites Tyr-740 and Tyr-751 are necessary for the expression of Hif1α. Furthermore, we show that tyrosine phosphorylation of these sites in PDGFRβ contributes to the mesangial cell hypertrophy via Hif1α and TGFβ expression.

2. Materials and methods

2.1. Reagents

The following reagents were purchased from Sigma: D-glucose, D-mannitol, protease inhibitor cocktail, phenylmethylsulfonyl fluoride, Na_3VO_4, NP-40, JNJ-10198409 (JNJ) and actin antibody. Tissue culture reagents were obtained from Life Technologies. Antibodies for phospho-p85 (Tyr-458), p85, phospho-PDGFRβ (Tyr-857), phospho-PDGFRβ (Tyr-740), phospho-PDGFRβ (Tyr-751), PDGFRβ, phospho-Akt (Ser-473), phospho-Akt (Thr-308), Akt, phospho-GSK3β (Ser-9) and GSK3β were obtained from Cell Signaling. TGFβ was purchased from R & D. TGFβ antibody was obtained from Abcam. Hif1α antibody, scramble RNA and pooled siRNAs against PDGFRβ were purchased from Santa Cruz. Anti-HA antibody was obtained from Covance. FuGENE transfection reagent and the OPTIMEM transfection medium were purchased from Promega and Life Technology, respectively. MK 2206 was obtained from Selleck Chemicals. ^{35}S-methionine was purchased from PerkinElmer. The PDGFRβ (Y740/751F) mutant plasmid was a gift from Dr. Carl Heldin (Ludwig Institute for Cancer Research, Uppsala University, Sweden). The plasmids expressing HA-tagged Hif1α and HA-tagged Akt K179M were described previously [19].

2.2. Cell culture

Human glomerular mesangial cells were grown in DMEM with 5 mM glucose in the presence of 10% fetal bovine serum as described previously [20,21]. For experiments, cells were grown to confluency and incubated with serum free medium for 24 h. Serum-starved cells were then treated with 25 mM glucose for 24 h or for indicated periods of time. 5 mM glucose plus 20 mM mannitol was used as osmotic control.

2.3. Cell lysis, immunoblotting and immunoprecipitation

After incubation with high glucose, the mesangial cell monolayer was lysed in RIPA buffer (20 mM Tris-HCl, pH 7.5, 5 mM EDTA, 150 mM NaCl, 1% NP-40, 1 mM Na_3VO_4, 1 mM PMSF and 0.1% protease inhibitor cocktail) at 4 °C for 30 min. The lysed cells were scraped along with the debris into Eppendorf tube. The cell extracts were centrifuged at 10,000 ×*g* for 20 min at 4 °C. The supernatant was collected. After determining the protein concentration, equal amounts of proteins were separated by SDS polyacrylamide gel electrophoresis. The separated proteins were transferred to PVDF membrane. The membrane containing the proteins was immunoblotted with indicated antibodies. The protein bands were developed with HRP-conjugated secondary antibody using ECL reagent as described previously [4,22,23]. For immunoprecipitation, equal amounts of proteins were incubated with the indicated antibody on ice for 30 min. Protein G agarose conjugated beads were then added. The mixture was rotated at 4 °C for overnight. The immune beads were washed with RIPA buffer and resuspended in SDS sample buffer [21]. The denatured proteins were separated by electrophoresis. The separated proteins were then immunoblotted with the indicated antibody as described above.

2.4. Transfection

The mesangial cells were seeded at 80% confluency. Next day, the cells were transfected with the plasmid vectors or siRNAs against PDGFRβ using FuGENE HD according to vendor's protocol as described previously [4,22]. After 24 h of transfection, the cells were starved in serum free medium and treated with high glucose as described above.

2.5. Protein synthesis

After incubation with high glucose, the mesangial cells were incubated with ^{35}S-methionine and protein synthesis was determined as [^{35}S]-methionine incorporation as described previously [4,5,22].

2.6. Measurement of cellular hypertrophy

At the end of the incubation period, the mesangial cells were trypsinized. The cells were counted in a hemocytometer. After counting, the cells were centrifuged at 4000 ×*g* at 4 °C. The cell pellet was washed with PBS and lysed in RIPA buffer as described above. The protein content in the cells was determined. Hypertrophy was expressed as an increase in the ratio of total cellular protein content to the cell number as described previously [4,22].

2.7. Statistics

The mean ± SE of indicated measurements is shown. The significance of the results was determined using the Graph Pad Prism software. Analysis of variance followed by Students-Newman-Keuls analysis was used as described previously [24,25]. A p value of < 0.05 was considered significant.

3. Results

3.1. Tyrosine phosphorylation of PDGFRβ is necessary for high glucose-induced PI 3 kinase phosphorylation

Recent work demonstrated that the regulatory subunit of PI 3 kinase, the p85 protein, is tyrosine phosphorylated when the PI 3 kinase is activated [26]. We examined the tyrosine phosphorylation of p85 by high glucose. Incubation of mesangial cells with high glucose increased the phosphorylation of p85 at Tyr-458 in a time-dependent manner (Fig. 1A). Since PI 3 kinase is activated by growth factor receptors and recent report showed increased expression of PDGFRβ in diabetic renal glomeruli [18], we tested the status of tyrosine phosphorylation of this receptor in mesangial cells. High glucose time-dependently enhanced the autophosphorylation of PDGFRβ at Tyr-857 (Fig. 1B). To determine the requirement of the tyrosine kinase activity of PDGFRβ for phosphorylation of p85 subunit of PI 3 kinase, we used JNJ-10198409 (JNJ), a PDGFRβ inhibitor [27]. JNJ inhibited the tyrosine phosphorylation of p85 concomitant with attenuation of the autophosphorylation of the PDGFRβ (Fig. 1C). Furthermore, the requirement of PDGFRβ for p85 phosphorylation was also confirmed by siRNAs against this tyrosine kinase (Fig. 1D).

PDGFRβ-mediated activation of PI 3 kinase requires its association with the receptor through phosphorylated tyrosine residues at 740 and 751 [28,29]. Therefore, we examined the phosphorylation of these residues in PDGFRβ. High glucose time-dependently increased

the phosphorylation of Tyr-740 and Tyr-751 in the PDGFRβ (Fig. 1E). To determine the association between PI 3 kinase and PDGFRβ, we performed co-immunoprecipitation experiment. PDGFRβ immunoprecipitates were immunoblotted with p85 antibody. The results in Fig. 1F show association of p85 with the PDGFRβ. Importantly, JNJ, which blocked the tyrosine phosphorylation of PDGFRβ at Tyr-740 and 751 (Supplemental Fig. S1), inhibited this association (Fig. 1F). Reciprocal co-immunoprecipitation experiment confirmed this observation (Fig. 1G). Finally, we examined the requirement of phosphorylated PDGFRβ at Tyr-740 and 751 in p85 phosphorylation by using a phosphorylation deficient mutant of the receptor (Y740F/Y751F) [30]. Expression of the mutant PDGFRβ inhibited the phosphorylation of p85 in response to high glucose (Fig. 1H). These data demonstrate that high glucose increases phosphorylation of PI 3 kinase via enhanced phosphorylation of the PDGFRβ at Tyr-740 and Tyr-751.

3.2. High glucose-induced PDGFRβ controls Akt kinase

Activation of PI 3 kinase promotes Akt kinase activity by inducing phosphorylation of this kinase at the catalytic loop Thr-308 and hydrophobic motif site Ser-473 [31,32]. Incubation of mesangial cells with high glucose increased the phosphorylation of Akt at both these sites in a time dependent manner (Supplementary Fig. S2). Since PDGFRβ was involved in activation of PI 3 kinase, we examined the effect of JNJ on Akt phosphorylation. As shown in Fig. 2A, JNJ inhibited the phosphorylation of Akt at Thr-308 and Ser-473 by high glucose. Since Akt phosphorylation increases its kinase activity, high glucose increased the phosphorylation of the Akt substrate GSK3β, which was inhibited by JNJ (Fig. 2B). Similarly, siRNA-mediated downregulation of PDGFRβ blocked the Akt and GSK3β phosphorylation in response to high glucose (Fig. 2C and D). To determine the role of the PI 3 kinase binding site of the receptor in Akt activation, we transfected the phospho-deficient mutant PDGFRβ (Y740/Y751F) into mesangial cells. Expression of the mutant receptor significantly abrogated the phosphorylation of Akt and GSK3β (Fig. 2E and F). These results indicate that high glucose-stimulated PDGFRβ phosphorylation at Tyr-740 and -751 is required for the Akt activity.

3.3. PDGFRβ regulates high glucose-induced mesangial cell hypertrophy

We have previously shown a role of Akt kinase in rat mesangial cell hypertrophy [5]. We used a recently developed Akt kinase inhibitor MK2206 in human mesangial cells. MK2206 inhibited the high glucose-stimulated Akt phosphorylation, resulting in inhibition of protein synthesis and mesangial cell hypertrophy (Supplementary Fig. S3A–C). Since Akt kinase activity is regulated by high glucose-stimulated PDGFRβ (Fig. 2), we examined the role of this receptor tyrosine kinase in mesangial cell hypertrophy. Inhibition of PDGFRβ by JNJ significantly inhibited high glucose-induced protein synthesis and hypertrophy of mesangial cells (Fig. 3A and B). Similarly, downregulation of PDGFRβ markedly attenuated the protein synthesis and hypertrophy of mesangial cells by high glucose (Fig. 3C and D). Additionally, the expression of phospho-deficient mutant of the PDGFRβ (Y740/Y751F) significantly suppressed the high glucose-induced mesangial cell protein synthesis and hypertrophy (Fig. 3E and F). To confirm the role of Akt in this process, we used constitutively active myristoylated Akt. Expression of constitutively active Myr Akt in the presence of high glucose reversed the siPDGFRβ- and PDGFRβ (Y740/Y751F)-mediated

inhibition of protein synthesis and hypertrophy (Fig. 4A–D). Interestingly, Myr Akt alone was sufficient to induce significantly protein synthesis and hypertrophy similar to high glucose (Fig. 4A–D). These results demonstrate that tyrosine phosphorylation of PDGFRβ at 740 and 751 residues that regulate Akt kinase activity by high glucose controls the mesangial cell hypertrophy.

3.4. PDGFRβ-regulated Hif1α controls mesangial cell hypertrophy

A role of Hif1α is reported in renal injury [33]. High glucose increased the expression of Hif1α in mesangial cells in a time-dependent manner (Supplementary Fig. S4). PI 3 kinase has been shown to regulate Hif1α in various cells types [34]. In mesangial cells, MK 2206 and dominant negative Akt (K179M) significantly inhibited the expression of Hif1α in response to high glucose (Fig. 5A and B). Since Akt regulates mesangial cell hypertrophy, we determined the involvement of Hif1α in this process. Downregulation of Hif1α significantly inhibited the high glucose-induced protein synthesis and hypertrophy of mesangial cells (Fig. 5C and D). Conversely, overexpression of Hif1α induced protein synthesis and hypertrophy similar to incubation with high glucose (Fig. 5E and F) As we have shown above that high glucose increases the PI 3 kinase/Akt pathway through PDGFRβ, we investigated the role of this receptor tyrosine kinase in Hif1α expression. Incubation of mesangial cells with the PDGFRβ inhibitor JNJ and siPDGFRβ significantly inhibited the expression of Hif1α by high glucose (Fig. 5G and H). Finally, the mutant PDGFRβ deficient in phosphorylation at 740 and 751 tyrosines suppressed the high glucose-stimulated expression of Hif1α (Fig. 5I).

Since we have shown that PDGFRβ and Hif1α independently regulate the mesangial cell hypertrophy (Figs. 3 and 5), we examined the interaction between this receptor and the transcription factor in this process. As expected siPDGFRβ significantly inhibited the high glucose-induced protein synthesis and hypertrophy. Co-expression of Hif1α with siPDGFRβ prevented this inhibition (Fig. 6A and B). As the PDGFRβ phospho-deficient mutant inhibits the hypertrophy (Fig. 3F), we tested the effect of Hif1α. Overexpression of Hif1α significantly reversed the PDGFRβ mutant-mediated inhibition of protein synthesis and hypertrophy (Fig. 6C and D). These results demonstrate that tyrosine phosphorylation sites 740/751 in PDGFRβ regulates Hif1α to promote mesangial cell hypertrophy in response to high glucose.

3.5. PDGFRβ regulates high glucose-stimulated TGFβ expression by Hif1α

We have previously reported that high glucose-induced mesangial cell hypertrophy is mediated by TGFβ [5]. Since we have shown above a role of PDGFRβ and Hif1α in mesangial cell hypertrophy, we examined the involvement of these proteins in TGFβ expression. Inhibition of PDGFRβ blocked the expression of TGFβ in mesangial cells (Fig. 7A). Similarly, siRNAs against PDGFRβ and the phospho-deficient mutant of PDGFRβ (Y740/751F) significantly inhibited the high glucose-stimulated TGFβ expression (Fig. 7B and C). siRNAs against Hif1α also abrogated the expression of TGFβ in response to high glucose (Fig. 7D). In contrast, overexpression of Hif1α in control cells increased the TGFβ expression similar to high glucose (Fig. 7E). Furthermore, expression of Hif1α reversed the siPDGFRβ- and PDGFRβ mutant (Y740/751F)-mediated inhibition of TGFβ expression

(Fig. 7F and G). These results for the first time demonstrate a role of PDGFRβ-mediated Hif1α in the high glucose-induced TGFβ expression.

4. Discussion

Here we show that PDGFRβ downstream of high glucose increases PI 3 kinase/Akt signal transduction to induce mesangial cell hypertrophy. Specifically, we show that tyrosine phosphorylation at sites 740/751 on the PDGFRβ is necessary for PI 3 kinase/Akt activation leading to high glucose-induced hypertrophy. Furthermore, the PDGFRβ tyrosine phosphorylation at 740/751 regulates the expression of the transcription factor Hif1α. Finally, we provide the first evidence that Hif1α regulates high glucose-induced TGFβ expression. Thus our findings disclose a new mechanism for high glucose-induced mesangial cell hypertrophy.

Chronic hyperglycemia significantly increases the risk of nephropathy in both type 1 and type 2 diabetes [35]. Several factors and multiple cell types in the kidney contribute to the pathology of diabetic nephropathy [36]. The early changes in the kidney during the progression of diabetic nephropathy includes glomerular hypertrophy. Although the signaling mechanism of cell hypertrophy is poorly understood, a role of the PI 3 kinase product PIP_3 has been shown previously to play important role [37]. For example, in *Drosophila*, overexpression of the PI 3 kinase in the eye and wing increased the organ size [38]. Similarly, overexpression of the constitutively active catalytic subunit of the PI 3 kinase, p110, increased the heart size in mouse. Conversely, dominant negative p110 resulted in reduced heart size [39]. In conjunction with these results, we have shown that PI 3 kinase contributes to renal hypertrophy including glomerular mesangial cell hypertrophy in response to high glucose [5,22]. However, the mechanism of PI 3 kinase activation by high glucose has not been investigated in detail. It is established that activation of PI 3 kinase requires upstream tyrosine kinase activity [40]. We considered the involvement of PDGFRβ as the expression of this receptor tyrosine kinase is increased in the glomerular mesangial area of the kidneys of patients and in the rodent model with diabetic nephropathy [18,41]. Also, we have shown that mesangial cells abundantly express PDGFRβ and its activation stimulates PI 3 kinase activity [12,13,42]. In the present study, we show that activation of PI 3 kinase by high glucose correlates with activating phosphorylation of PDGFRβ in mesangial cells (Fig. 1A and B). PI 3 kinase contains an 85 kDa regulatory subunit, which binds to the tyrosine phosphorylated receptors through the SH2 domains [43]. This association facilitates the tyrosine phosphorylation of the PI 3 kinase [26]. We show that inhibition of PDGFRβ blocks the association of PI 3 kinase with the receptor and tyrosine phosphorylation of PI 3 kinase (Fig. 1C, D, F and G). It was previously described that the tyrosine residues 740 and 751 of the PDGFRβ serve as the docking sites for PI 3 kinase [29]. We demonstrate that high glucose uses these residues of PDGFRβ to phosphorylate the PI 3 kinase (Fig. 1H). These results are the first demonstration of the involvement of the PI 3 kinase binding sites of the PDGFRβ in high glucose-induced signal transduction.

In the PDGF receptor signaling, one of the downstream targets of PI 3 kinase is the Akt kinase [44]. The PI 3 kinase product PIP_3 upon binding to the PH domain of Akt increases

its initial activating phosphorylation at the Thr-308 residue by the PDK-1 [31,45,46]. Finally, PI 3 kinase-dependent mTORC2 phosphorylates the Ser-473 residue of the Akt [32,47]. In fact, phosphorylation of this residue stabilizes Thr-308 phosphorylation and full activation of Akt [46]. Previously, we have shown increased Akt phosphorylation and activity following exposure of renal cells to high glucose and in the kidneys of mice with diabetes [4,5,22,48]. Our present studies show that high glucose-induced Akt phosphorylation and activation are dependent upon activated PDGFRβ (Fig. 2A–D). In fact, phosphorylation of tyrosines 740/751 of the PDGFRβ is required for high glucose-induced Akt phosphorylation and activation (Fig. 2E and F). Although PI 3 kinase-dependent and independent mechanisms have been reported for full activation of Akt [32,49], our results demonstrating the dependence of the PI 3 kinase on the phosphorylation of PDGFRβ tyrosines 740/751 confirm the lipid kinase dependence of the Akt activation by high glucose in mesangial cells.

As described above that glomerular mesangial cell hypertrophy constitutes a major pathologic feature in the diabetic nephropathy [6]. A role for PI 3 kinase-dependent Akt kinase has been shown in cell size control. For example in *Drosophila*, expression of Akt in the imaginal discs increased the cell size [50]. Similarly, Akt promoted cardiomyocyte and cardiac hypertrophy when activated in vivo [51]. Also, this action of Akt in cardiac hypertrophy was inhibited by the action of PTEN, which dephosphorylates the PI 3 kinase product and inhibits Akt activity [37]. We have previously shown that PI 3 kinase-dependent Akt kinase contributes to glomerular mesangial cell hypertrophy [23,25]. Activation of various receptor tyrosine kinases including PDGFRβ has been shown to participate in the pathogenesis of diabetic nephropathy [52]. However, specific involvement of receptor tyrosine kinase in high glucose-induced renal cell hypertrophy has not been identified. Our results demonstrate that the PDGFRβ, which activates Akt in mesangial cells, regulates high glucose-induced hypertrophy of these cells (Fig. 3A–D). In fact, the tyrosine phosphorylation of PDGFRβ at the residues 740/751 contributes to mesangial cell hypertrophy (Fig. 3E and F). Importantly, our data provide the evidence that Akt kinase downstream of PDGFRβ contributes to high glucose-induced hypertrophy of mesangial cells (Fig. 4).

Oncogenes including receptor tyrosine kinases and loss of tumor suppressor genes have been shown to increase the levels of Hif1α in the absence of hypoxia [53]. Hif1α is regulated by its de novo expression and E3 ubiquitin-mediated degradation. It dimerizes with the constitutive Hif1β to act as a transcription factor to increase expression of genes involved in cell proliferation, apoptosis, angiogenesis, inflammation, pH homeostasis and metabolic switch to glycolysis [33,54]. Homozygous deletion of Hif1α in mice is embryonically lethal although tissue-specific knockout has shown its role in hematopoiesis, osteogenesis, chondrogenesis, innate immunity, adipogenesis and T cell development [55]. During renal development, Hif1α is highly expressed in the glomerular cells; however in the adult kidney it is expressed in the most cells [33,56]. Renal fibrosis is an end point in chronic kidney disease. Interestingly, pro- as well as anti-fibrotic role of Hif1α has been reported in the literature [33]. For example, induction of Hif1α by cobalt chloride reduced the proteinuria and tubulointerstitial damage in rat model of diabetes [57]. On the other hand, administration of a Hif inhibitor attenuated the mesangial matrix expansion, albuminuria and

Nox4 expression in a mouse model of type 1 diabetes [58]. Several reports demonstrate a potent fibrotic role for increased Hif1α in the kidney [59,60]. In fact increased glomerular expression of Hif1α has been shown in the kidneys of patients with moderate to severe diabetic nephropathy [59]. One mechanism by which the abundance of the Hif1α can be maintained at least in certain cancers has recently been worked out. For example, deficiency in the TCA cycle enzymes succinate dehydrogenase and fumarate hydratase in pheochromocytomas and leiomyomata can increase the accumulation of succinate and fumarate to induce a pseudohypoxic state that stabilizes the Hif1α [53]. Recently, using Akita mouse model of diabetic nephropathy, Sharma and coworkers have shown that Nox4-derived hydrogen peroxide inhibits the expression of fumarate hydratase to increase the levels of fumarate in the kidney and in the urine; increased fumarate caused enhanced expression of Hif1α [61]. In agreement with these studies, in vitro we show that high glucose increases the expression of Hif1α in mesangial cells (Supplementary Fig. S4). In the present study, we demonstrate the involvement of PDGFRβ and specifically its 740/751 tyrosine phosphorylation-mediated Akt kinase in high glucose-induced expression of Hif1α (Fig. 5). Furthermore, we provide the first evidence that high glucose-induced Hif1α downstream of PDGFRβ regulates the mesangial cell hypertrophy (Figs. 5 and 6).

A role of TGFβ has been conclusively established in the development of complications of diabetic nephropathy with early glomerular hypertrophy, including mesangial cell hypertrophy [1,6,62]. Administration of anti-TGFβ antibody to streptozotocin-induced type 1 diabetic mice attenuated the glomerular hypertrophy [63]. Similarly, anti-TGFβ antibody prevented the pathologic features of nephropathy in the db/db mouse model of type 2 diabetes [64]. Further evidence for the involvement of TGFβ signaling in glomerular hypertrophy came from the studies where TGFβ receptor II heterozygous mice with streptozotocin-induced diabetes exhibited significantly reduced glomerular hypertrophy [65]. Also, renal cells isolated from TGFβ knock out mice showed impaired response to high glucose-induced hypertrophy [66]. We have shown previously that in cultured mesangial cells, the hypertrophic effect of high glucose is mediated by TGFβ [5]. However, the mechanism by which high glucose increases the expression of TGFβ to induce hypertrophy is poorly understood. We show that activation of PDGFRβ by high glucose increases the expression of TGFβ and mesangial cell hypertrophy (Figs. 3, 7A and B). Overexpression of constitutively active TGFβ receptor I reversed the siPDGFRβ-mediated inhibition of high glucose-induced mesangial cell hypertrophy (data not shown). These results indicate that TGFβ downstream of PDGFRβ contributes to hypertrophy of mesangial cells in response to high glucose. In fact, the tyrosine phosphorylation of PDGFRβ at 740/751 residues is necessary for the high glucose-dependent expression of TGFβ (Fig. 7C and G). Furthermore, we show that high glucose-stimulated Hif1α increased the expression of TGFβ (7D, 7E and 7F). These results are in congruence with a recent in vivo study where the authors observed the expression of Hif1α and TGFβ in the glomeruli of diabetic mice [61]. Recently, we have shown that TGFβ regulates the expression of Hif1α in renal cells [67]. Intriguingly, we demonstrate for the first time that Hif1α regulates the expression of TGFβ and mesangial cell hypertrophy in response to high glucose. Thus Hif1α can act as both an upstream regulator of TGFβ and a downstream effector. Importantly, our data provide significant insight into a potential signal transduction mechanism which involves PDGFRβ

Y740/751 phosphorylation, Akt activation, expression of Hif1α and TGFβ for diabetic mesangial hypertrophy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cellsig.2017.09.017.

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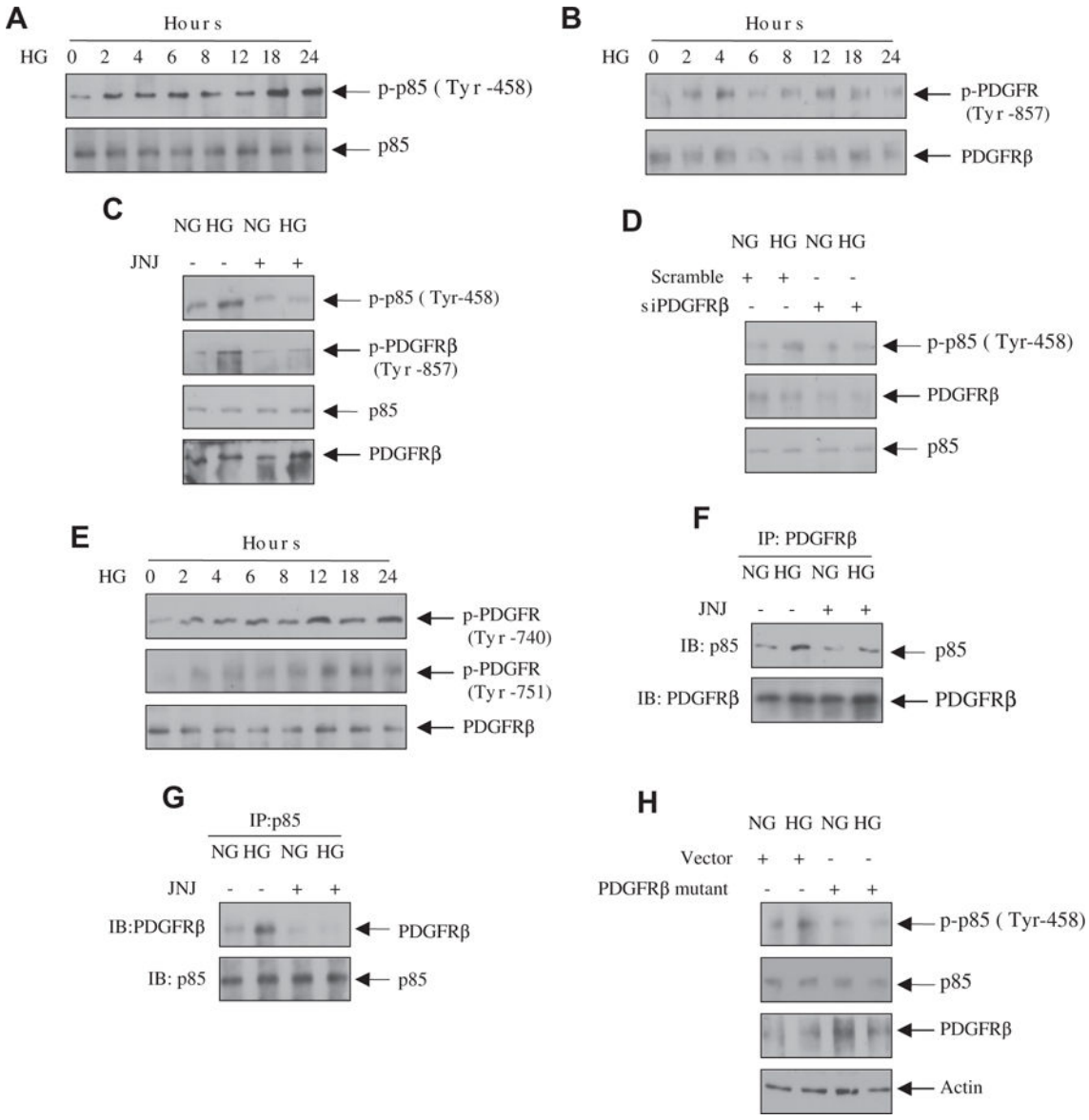

Fig. 1.

High glucose increases the association of PI 3 kinase with the PDGFRβ leading to its phosphorylation. (A, B and E) Mesangial cells were incubated with high glucose (HG, 25 mM glucose) for the indicated time periods. As control (0 h), 20 mM mannitol plus 5 mM glucose was used as described in the Materials and methods section. Equal amounts of cell lysates were immunoblotted with phospho-p85 (Tyr-458), p85, phospho-PDGFRβ (Tyr-857), phospho-PDGFRβ (Tyr-740), phospho-PDGFRβ (Tyr-751) and PDGFRβ antibodies as indicated. (C, F and G) Mesangial cells were treated with 0.1 μM JNJ prior to incubation with high glucose (HG) and normal glucose (NG) for 24 h. In panel C, the cell lysates were immunoblotted with the indicated antibodies. In panels F and G, the cell lysates were immunoprecipitated with PDGFRβ (panel F) or with p85 PI 3 kinase subunit (panel G) antibodies. The immunoprecipitates were immunoblotted with p85 (panel F) or PDGFRβ (panel G) antibodies, respectively. (D and H) Mesangial cells were transfected with scramble

siRNA or siRNAs against PDGFRβ (panel D) or with vector or PDGFRβ (Y740/Y751F) mutant as described in the Materials and methods section. The transfected cells were incubated with high glucose (HG) or normal glucose (NG) as described above. The cell lysates were immunoblotted with the indicated antibodies.

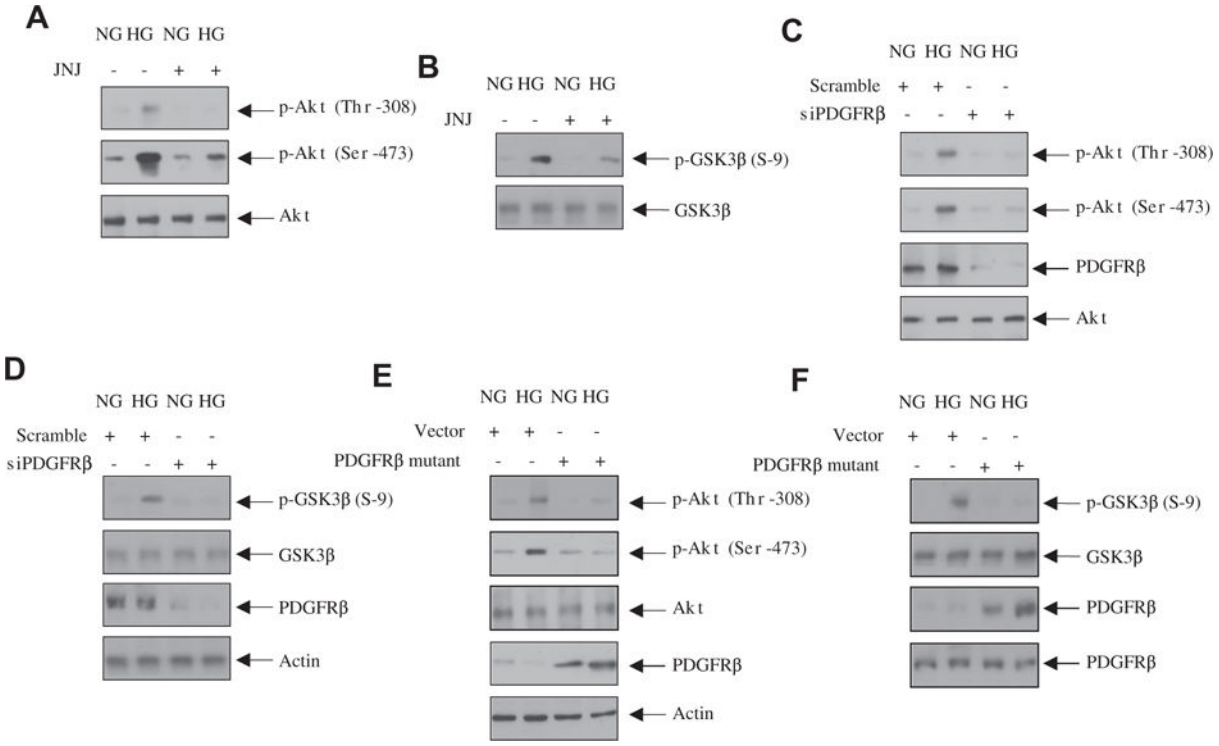

Fig. 2.

PDGFRβ regulates high glucose-stimulated Akt kinase activity. (A and B) Mesangial cells were treated with 0.1 μM JNJ for 1 h prior to incubation with high glucose (HG) or normal glucose (NG) for 24 h. Equal amounts of cell lysates were immunoblotted with the indicated antibodies. (C–F) Mesangial cells were transfected with siPDGFRβ (panels C and D) or with PDGFRβ Y740/751F (E and F) mutant. The cells were then incubated with high glucose (HG) or normal glucose (NG) for 24 h. The cell lysates were immunoblotted with indicated antibodies.

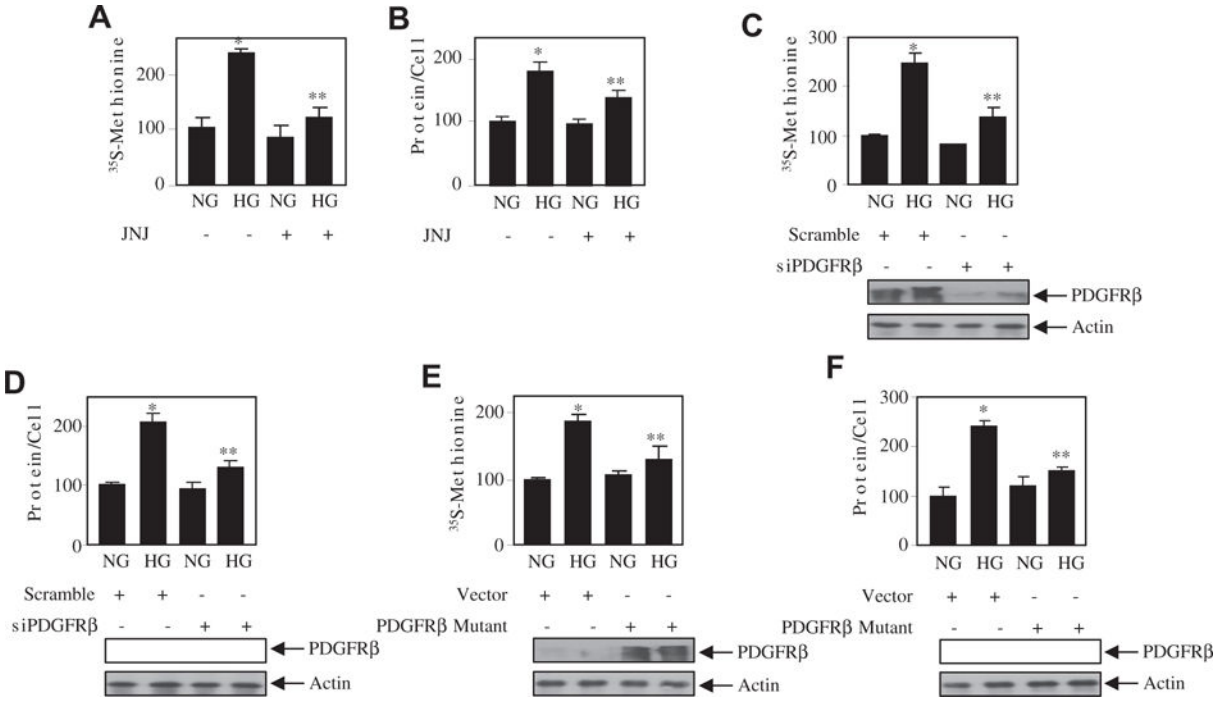

Fig. 3.

PDGFRβ controls mesangial cell hypertrophy by high glucose. (A and B) Mesangial cells were treated 0.1 μM JNJ for an hour prior to incubation with high glucose (HG) for 24 h. Protein synthesis was measured by ^{35}S-methionine incorporation as described in the Materials and methods section (panel A). Mean ± SE of triplicate measurements is shown. *p < 0.01 vs NG; **p < 0.01 vs HG. Hypertrophy was determined as the ratio of total amount of protein to cell number as described. Mean ± SE of 6 measurements is shown. *p < 0.001 vs NG; **p < 0.05 vs HG [22,25]. (C–F) Mesangial cells were transfected with siPDGFRβ or scramble (panels C and D) or PDGFRβ mutant (Y740/751F) or vector (panels E and F). The transfected cells were incubated with normal glucose (NG) or high glucose (HG) for 24 h. The protein synthesis and hypertrophy were determined as described above. Mean ± SE of 3–6 measurements is shown. *p < 0.01 vs HG. For panels B and E, **p < 0.05 vs HG; for panels C, D and F, **p < 0.01 vs HG. The bottom panels show the expression of PDGFRβ and actin.

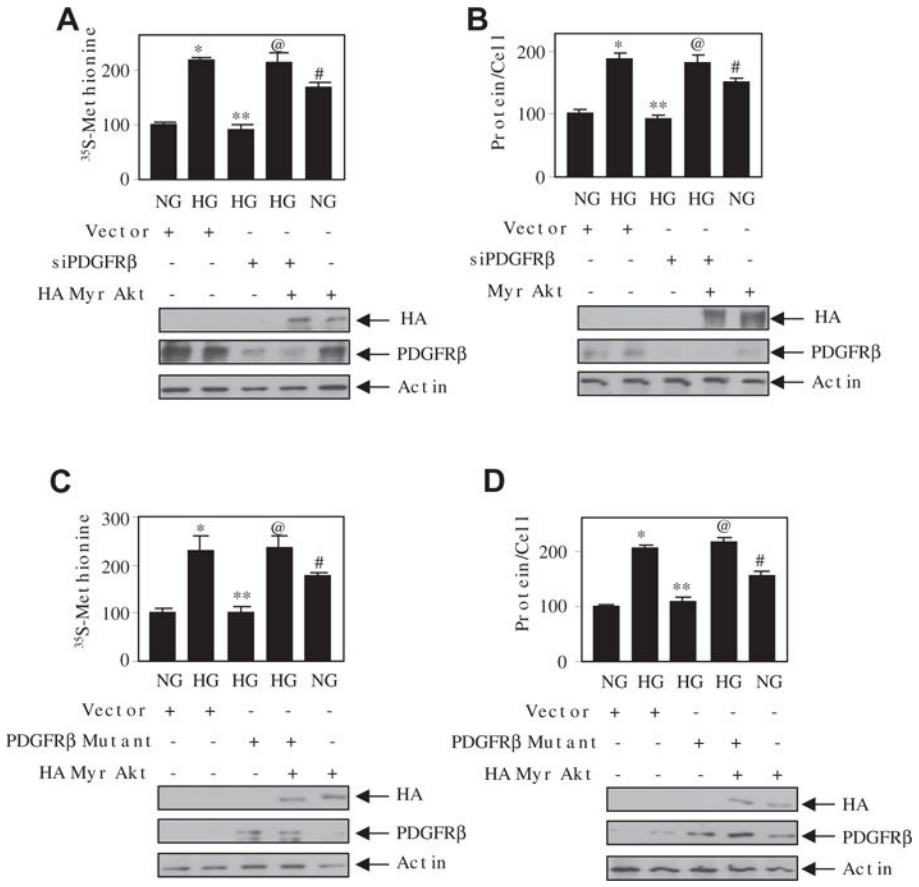

Fig. 4.

Akt kinase regulates high glucose-induced PDGFRβ-mediated hypertrophy of mesangial cells. Mesangial cells were transfected with siPDGFRβ and Myr Akt (panels A and B) or PDGFRβ mutant (Y740/751F) and HA Myr Akt (panels C and D) as indicated. The transfected cells were incubated with normal glucose (NG) or high glucose (HG). The protein synthesis and hypertrophy were determined as described in the Materials and methods section [22,25]. Mean ± SE of 4 measurements is shown. *$p < 0.01$ vs NG; **$p < 0.01$ vs HG alone; $^{@}p < 0.01$ vs HG + siPDGFRβ or PDGFRβ mutant; $^{\#}p < 0.01$ or 0.05 vs NG. The bottom panels show the expression of HA Myr Akt, PDGFRβ and actin.

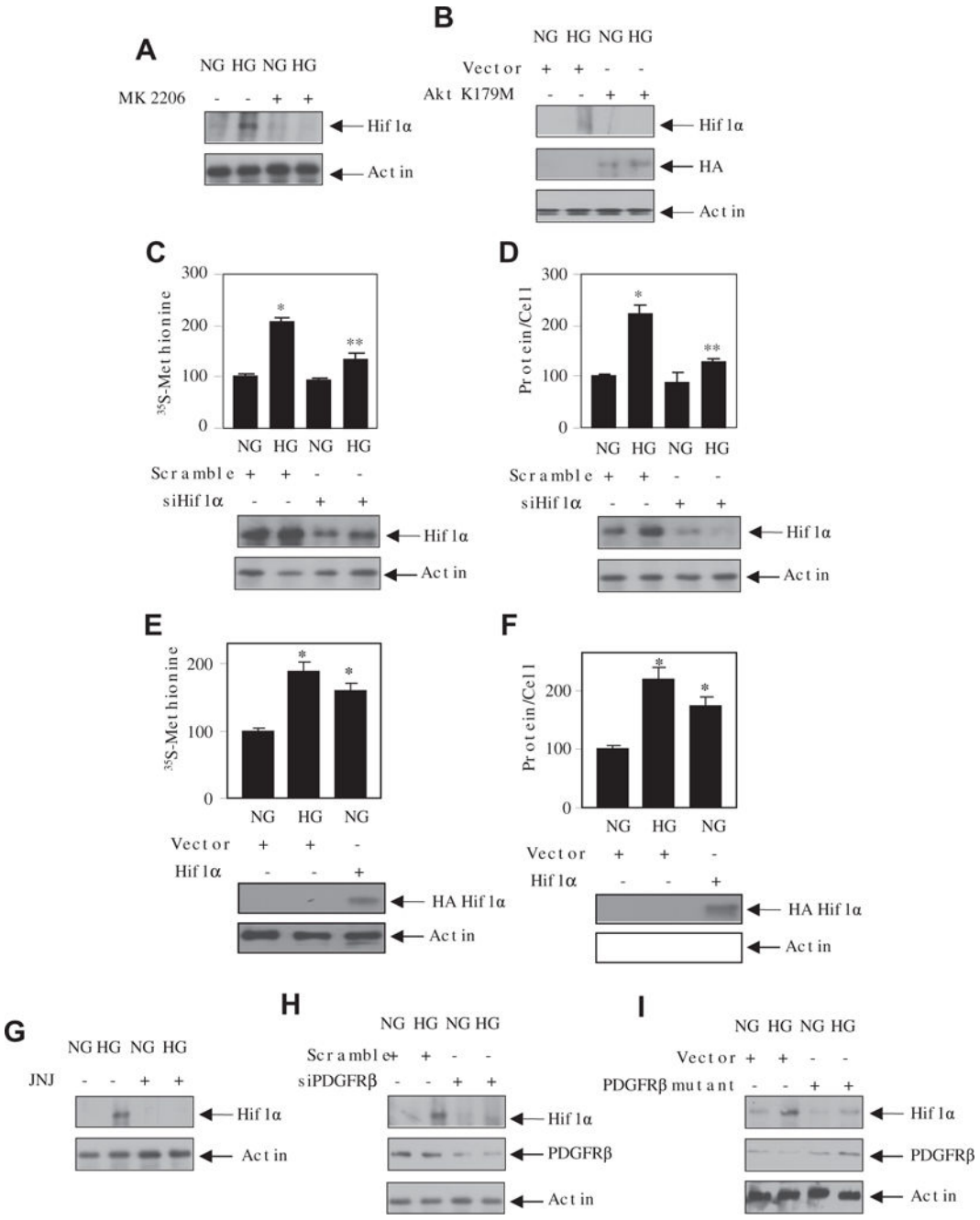

Fig. 5.

Akt-dependent Hif1α regulates mesangial cell hypertrophy. (A and G) Mesangial cells were treated with 0.1 μM JNJ (panel A) or 1 μM MK 2206 (panel G) for 1 h followed by incubation with normal glucose (NG) or high glucose (HG) for 24 h. The cell lysates were immunoblotted with Hif1α and actin antibodies. (B, H and I) Mesangial cells were transfected with dominant negative HA Akt K179M (panel B) or siPDGFRβ (panel H) or PDGFRβ mutant (Y740/751F) (panel I). The transfected cells were incubated with normal glucose or high glucose. The cell lysates were immunoblotted Hif1α, HA, PDGFRβ or actin antibodies as indicated. (C–F) Mesangial cells were transfected with siHif1α or scramble

(panels C and D) or HA Hif1α expression vector (panels E and F). The protein synthesis and hypertrophy were determined as described in the Materials and methods section [22,25]. Mean ± SE of 3–6 measurements is shown. *p < 0.01 or 0.001 vs NG; **p < 0.01 vs HG. The bottom panels show the expression of Hif1α.

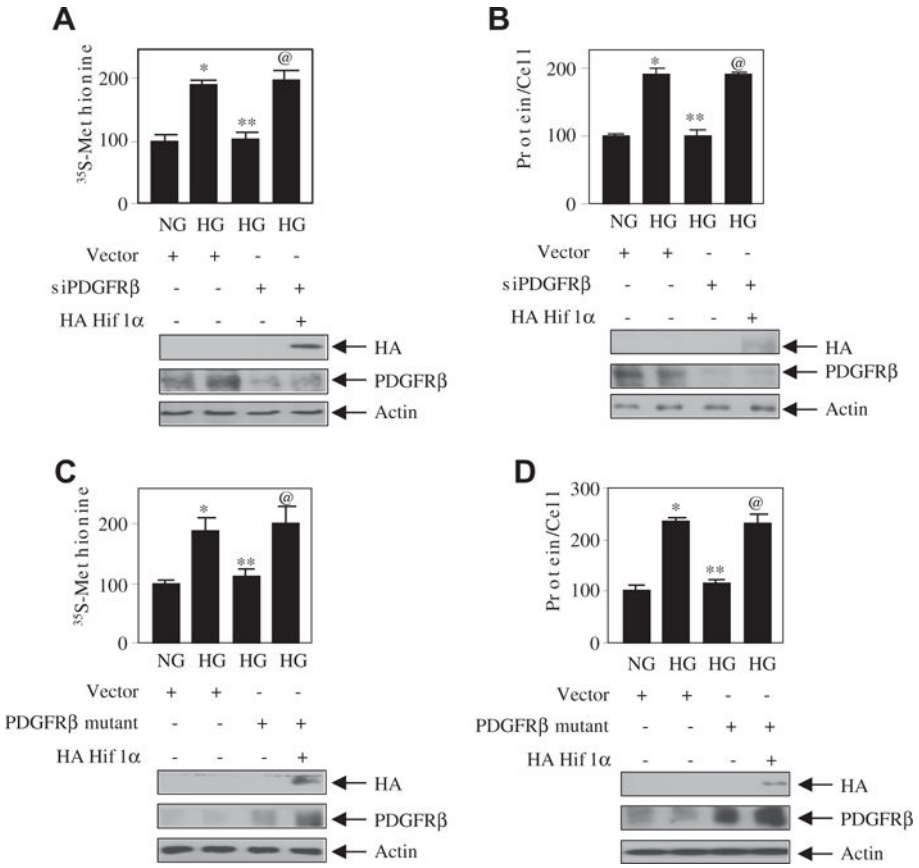

Fig. 6.

Hif1α downstream of PDGFRβ controls high glucose-induced mesangial cell hypertrophy. Mesangial cells were transfected with siPDGFRβ or PDGFRβ mutant (Y740/751F) along with HA Hif1α. The transfected cells were incubated with normal glucose or high glucose. The protein synthesis and hypertrophy were determined as described in the Materials and methods section [22,25]. The bottom panels show the expression of HA Hif1α, PDGFRβ and actin. Mean ± SE of 3–6 measurement is shown. *p < 0. 01, 0.05 or 0.001 vs NG; **p < 0. 01, 0.05 or 0.001 vs HG; $^{@}$p < 0.05, 0.01 or 0.001.

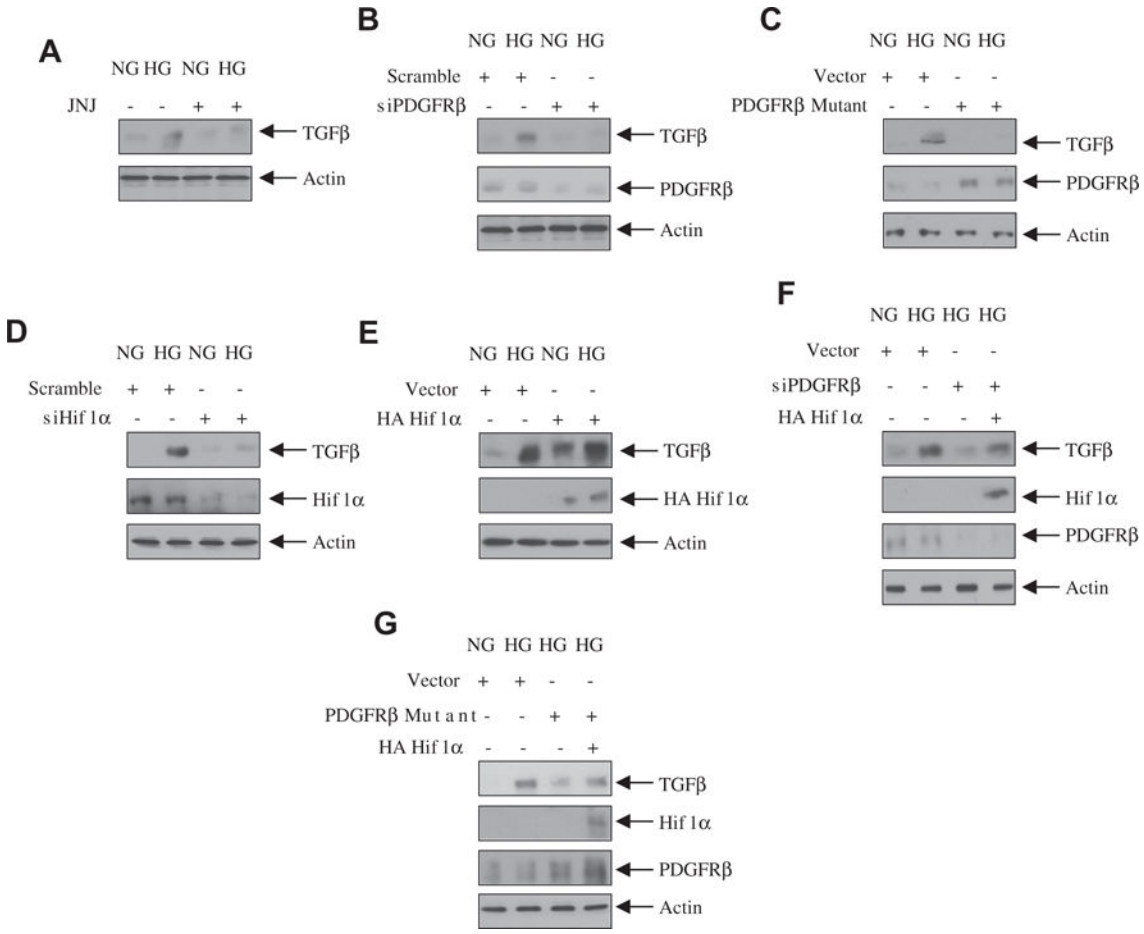

Fig. 7.

PDGFRβ-stimulated Hif1α regulates high glucose-induced TGFβ expression. (A) Mesangial cells were treated with 0.1 μM JNJ for 1 h prior to incubation with high glucose (HG) or normal glucose (NG) for 24 h. The cell lysates were immunoblotted with TGFβ or actin antibodies. (B–G) Mesangial cells were transfected with siPDGFRβ (panel B) or PDGFRβ mutant (Y7f40/751F) (panel C) or siHIf1α (panel D) or HA Hif1α (panel E) or siPDGFRβ plus HA Hif1α (panel F) or PDGFRβ mutant (Y740/751F) plus HA Hif1α (panel G). The cell lysates were immunoblotted with TGFβ, PDGFRβ, HA and actin antibodies as indicated.