IGF-1 increases the expression of fibronectin by Nox4-dependent Akt phosphorylation in renal tubular epithelial cells.

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Running Title: IGF-1 redox signaling in renal epithelial cells

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Abstract

Extracellular matrix accumulation contributes to the progression of chronic kidney disease. Many growth factors including insulin-like growth factor-1 (IGF-1) enhance matrix protein accumulation. Proximal tubular epithelial cells (PTCs) synthesize matrix proteins. NADPH oxidases are major sources of reactive oxygen species (ROS), important signaling molecules that mediate biological responses in a variety of cells and tissue. We investigated the mechanism by which IGF-1 regulates fibronectin accumulation in PTCs and the role of a potential redox-dependent signaling pathway. IGF-1 induces an increase in NADPH-dependent superoxide generation, enhances the release of hydrogen peroxide and increases the expression of Nox4 in PTCs. IGF-1 also stimulates phosphorylation of Akt and inhibition of Akt or its upstream activator PI 3 kinase attenuates IGF-1-induced fibronectin accumulation. Expression of dominant negative Akt also inhibits IGF-1-induced expression of fibronectin, indicating a role for this kinase in fibronectin accumulation. Expression of dominant negative adenovirus Nox4 inhibits IGF-1-induced NADPH oxidase activity, Akt phosphorylation and fibronectin protein expression. Moreover, transfection of siRNA targeting Nox4 decreases Nox4 protein expression and blocks IGF-1-induced Akt phosphorylation and the increase in fibronectin, placing Nox4 and ROS upstream of Akt signaling pathway. To confirm the role of Nox4, PTCs were infected with adenovirus construct expressing wild-type Nox4. Ad-Nox4, but not control Ad-GFP, upregulated Nox4 expression and increased NADPH oxidase activity as well as fibronectin expression. Taken together, these results provide the first evidence for a role of Nox4 in IGF-1-induced Akt phosphorylation and fibronectin expression in tubular epithelial cells.
Introduction

Kidney diseases that eventuate in fibrosis of the tubular/interstitial compartment are characterized by increased expression and release of growth factors and cytokines (1, 2, 3). Glucose or transforming growth factor-beta (TGF-beta) stimulates proximal tubular epithelial cells resulting in an increased production of matrix proteins including collagens and fibronectin (36, 37 43). Insulin-like growth factor 1 (IGF-1) and IGF-1 receptor are upregulated in the kidney in disease states and in particular in diabetes (19, 20, 33, 40). Moreover, the increased levels of IGF-1 in the tubular fluid of animals with proteinuria enhances collagen production in cultured proximal tubular epithelial cells (32). The IGF-1 receptor is a ligand activated tyrosine kinase that signals through PI3K/Akt as well as ERK1/ERK2 MAPK dependent pathways to elicit its cellular responses (26, 46, 34, 42). Proximal tubular epithelial cells express the IGF-1 receptor and IGF-1 activates PI 3 kinase and enhances the phosphorylation of Akt to induce protein synthesis and hypertrophy of proximal tubular epithelial cells (46). Whether IGF-1 enhances fibronectin accumulation and the mechanism by which IGF-1 enhances matrix protein accumulation in proximal tubular cells has not been well defined.

Reactive oxygen species (ROS) have been implicated in growth factor-induced signaling pathways through regulation of the redox state of the target cells (5, 6,10). In the kidney, ROS are primarily produced by NADPH oxidases of the Nox family (11, 45, 25, 9, 6). There is a wide array of evidence that Nox-derived ROS modulate vascular and renal cell function, including extracellular matrix protein synthesis, in response to G-protein coupled as well as ligand activated tyrosine kinase receptors (6, 5, 8, 10,11).
In this study, we explored the effect of IGF-1 on fibronectin accumulation and the potential role of Akt and ROS generated by NADPH oxidases in mediating the effect of IGF-1. We demonstrate that IGF-1 activates the NADPH oxidase-dependent ROS generation and rapidly upregulates the Nox4 isoform, resulting in Akt phosphorylation. Nox4-dependent ROS production and Akt phosphorylation lead to accumulation of the extracellular matrix protein, fibronectin in proximal tubular epithelial cells. Our data also suggest that both transcriptional and translational mechanisms are implicated in the regulation of fibronectin by IGF-1 in renal proximal tubular cells. Thus, alteration of the redox state in pathological conditions in which IGF-1 is implicated (such as diabetic kidney disease) may contribute to matrix accumulation.
Materials and Methods

Cell Culture-- Rat proximal tubular epithelial cells (PTCs) were maintained in Dulbecco's modified Eagle's medium containing 5 mM glucose (DMEM) plus 10% fetal bovine serum in a 5% CO₂ atmosphere at 37°C. In some experiments, the data were confirmed in primary cultures of rat tubular epithelial cells and in immortalized mouse (MCTs) and human proximal tubular epithelial cell line (HK2). Cells were used between passages 6 and 15. Before addition of agents, the medium was changed to DMEM plus 0.1% FBS and cultured for 24 to 48 h. IGF-1 was used at a concentration of 250 ng/ml. Note that this concentration corresponds to circulating levels of IGF-1 (21) as described previously (26).

qRT-PCR analysis-- Quantitative Real Time PCR analysis was performed as previously described (17, 18). PTCs were grown to 90-95% confluency in 60mm dishes and were serum starved for 24 h. Cells were then treated with IGF-1 (250 ng/ml) for 12, 24 and 48 h respectively. After washing cells twice with PBS, RNA was extracted using RNAzole bee method. Real time PCR gene expression analysis (RT²qPCR primer assay kit, SA Biosciences Frederick, MD) was performed for fibronectin mRNA using ^\textsuperscript{^ΔΔ}Ct method. Fibronectin mRNA expression was quantified using a Realplex mastercycler (Eppendorf, Westbury, NY) with SYBR green dye and RT²qPCR primers (SA Biosciences Frederick, MD). Fibronectin mRNA was normalized to GAPDH mRNA levels.

Fibronectin promoter activity-- A reporter plasmid consisting of the fibronectin promoter adjacent to a firefly luciferase reporter gene was used to determine the transcriptional activity of the fibronectin promoter in PTCs. Cells were grown in 12 well plates to 60%-70%
confluence. Plasmids were then transfected using GeneJuice™ (Novagen, WI). Prior to transfection, cells were washed twice with PBS and media replaced with 1 ml of OPTI-MEM I (Invitrogen). Pre-complex of the DNA with GeneJuice™ in Opti-MEM was mixed and incubated at room temperature for 15 min. GeneJuice™ was added to the complex of DNA and Plus reagent™ and incubated for 15 min at room temperature. DNA with GeneJuice™ complexes was added to each well and incubated at 37 °C with 5% CO2. After incubation for 6 h, media was replaced to complete media. After 24 h of transfection, cells were incubated in serum free media for 24 h followed by treatment with IGF-1 for 12, 24 and 48 h time points. Cells were harvested, washed twice with PBS and lysed in 0.1 ml of lysis buffer. Luciferase activity was determined using the Luciferase Reporter Assay System by a luminometer according to the manufacturer's instructions (Promega, Madison, WI) and normalized by protein content.

**NADPH Oxidase Assay**—NADPH oxidase activity was measured by the lucigenin-enhanced chemiluminescence method as described previously (24, 25). Briefly, cultured cells were homogenized in lysis buffer (20 mM KH₂PO₄, pH 7.0, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, and 0.5 µg/ml leupeptin) using a Dounce homogenizer (100 strokes on ice). To start the assay, 100-µl aliquots of homogenates were added to 900 µl of 50 mM phosphate buffer, pH 7.0, containing 1 mM EGTA, 150 mM sucrose, 5 µM lucigenin, and 100 µM NADPH. Photon emission in terms of relative light units was measured in a luminometer every 30 s for 5 min. There was no measurable activity in the absence of NADPH. Superoxide anion production was expressed as relative chemiluminescence (light) units (RLU)/mg protein. Protein content was measured using the Bio-Rad protein assay reagent. Mean +/- S.E. was calculated for each set of samples.
**Hydrogen peroxide detection**—Extracellular hydrogen peroxide was detected using the Amplex Red Assay Kit as previously described (28, 9) and following the manufacturer's instructions.

**Western Blotting Analysis**—For immunoblotting, proteins were separated using SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with 5% low fat milk in TBS-Tween and then incubated with a rabbit polyclonal Nox4 antibody (25, 7, 9, 10), a rabbit polyclonal anti-fibronectin antibody (catalog number F3648; Sigma) (1:1000), or a mouse monoclonal anti-GAPDH (1:2500) (Sigma). Phospho-antibodies (Akt Ser473, 4E-BP1 Thr37/46 and S6K Thr389) and their unphosphorylated counterparts were obtained from Cell Signaling Technologies and used at a dilution of 1:1000. The appropriate horseradish peroxidase-conjugated secondary antibodies were added, and bands were visualized by enhanced chemiluminescence. Densitometric analysis was performed using NIH Image J software.

**siRNA knockdown**—For the RNA interference experiments, a SMART-pool consisting of four short or small interfering RNA (siRNA) duplexes specific for rat Nox4 was obtained from Dharmacon. The SMARTpool of siRNAs was introduced into the cells by transfection using Oligofectamine as described (8, 9, 18). Scrambled (nontargeting) siRNAs served as controls to validate the specificity of the siRNAs. Scrambled control and siRNA for Nox4 were used at a concentration of 400 nM.

**Adenovirus infection**—Cells were grown to 80% confluency and infected with an adenovirus expressing either dominant negative Akt or GFP vector as control, full length active wild type Nox4 (Ad-Nox4) or truncated dominant negative Nox4 (Ad-Nox4ΔFAD/ΔNAD) as
described previously (7, 8, 9, 18, 35). Expression was determined by Western blotting and was maximal at 48 h post-infection. Ad-Nox4 and Ad-Nox4ΔFAD/ΔNAD were generous gift from Dr. B. Goldstein (Merck).

Statistical analyses—Quantitative data are presented as the mean ± standard error of the mean for at least three experiments. Statistical analysis was based on Student's t test for comparison of two groups and one-way analysis of variance for multiple group comparisons. A P value less than 0.05 was used to indicate statistical significance.
**Results:**

*IGF-1 enhances Nox4-dependent ROS generation.* To determine the effect of IGF-1 on ROS generation, we measured NADPH-dependent superoxide generation (NADPH oxidase activity). Treatment of proximal tubular epithelial cells (PTCs) with IGF-1 results in enhanced superoxide generation compared to cells treated with buffer alone (Fig. 1A). Preincubation of the cells with diphenyleneiodonium (DPI), a compound which inhibits flavin-containing oxidases, abrogated IGF-1-induced superoxide generation (Fig. 1A). Dismutation of superoxide (O$_2^•$), spontaneously or enzymatically, by superoxide dismutase (SOD) produces hydrogen peroxide (H$_2$O$_2$). Using Amplex Red as a detector of H$_2$O$_2$, we find that the production of H$_2$O$_2$ was also enhanced in the presence of IGF-1 (Fig. 1B). Taken together, these data suggest that IGF-1-induced ROS generation is most likely mediated by NADPH oxidases. Given that the Nox catalytic subunit Nox4 is highly expressed in the kidney, we examined the effects of IGF-1 on Nox4 protein expression. Cells were incubated with IGF-1 and Nox4 expression was determined by Western blotting. IGF-1 increases Nox4 protein expression in a time dependent manner as early as 5 minutes of treatment and is sustained for at least 30 minutes (Fig. 1C), indicating a role for Nox4 in IGF-1 induced ROS generation. To confirm the role of Nox4 in IGF-1-mediated ROS generation, an adenovirus dominant-negative (DN) form of Nox4, which lacks the FAD and heme binding domains (Ad-Nox4 ΔFAD/ΔNAD) was utilized. Infection of the cells with Ad-DN-Nox4, but not Ad-GFP control, effectively blocked IGF-1-induced NADPH oxidase activity (Figure 1D). Together, these findings indicate that IGF-1 upregulates Nox4 protein expression and that Nox4 mediates IGF-1-induced ROS generation in PTCs.
IGF-1 increases fibronectin expression through a PI3K- and Akt-dependent mechanism.

We and others have previously shown that IGF-1 activates PI3 kinase and enhances Akt phosphorylation in proximal tubular epithelial cells and that this pathway mediates protein synthesis and cell hypertrophy (46). However, the effect of IGF-1 on fibronectin (FN) expression and the role of ROS in mediating the enhancing effect of IGF-1 on matrix protein expression have not been investigated. To identify putative Nox4-dependent redox-sensitive signaling pathways activated by IGF-1, PTCs were treated with IGF-1. As shown in Figure 2A, IGF-1 induced phosphorylation of Akt as early as 5 minutes, an effect that was sustained for up to 60 minutes. There was no change in total Akt protein expression. Similar findings were observed in mouse proximal tubular epithelial cells (data not shown).

To determine if IGF-1 induces fibronectin accumulation through a PI3K/Akt-dependent mechanism, we used pharmacologic inhibitors of PI3K or Akt. Treatment of PTCs with IGF-1 increased fibronectin (FN) accumulation, which was reduced when cells were pre-incubated with the PI3K inhibitor LY294002 or the Akt inhibitor Akt-X (Figure 2B). The role of Akt in IGF-1-mediated FN expression was further examined by infecting the cells with an adenovirus vector expressing a dominant-negative mutant of Akt (Ad DN-Akt). GFP adenovirus was used as a control. Infection with Ad DN-Akt prevented IGF-1 induced FN expression compared to infection with Ad GFP alone (Figure 2C), further implicating Akt phosphorylation in IGF-1-induced FN expression.

Nox4 regulates IGF-1 induced Akt phosphorylation in proximal tubular cells.
Because IGF-1 increases Nox4 protein expression and enhances NADPH-dependent ROS generation concomitant with Akt phosphorylation, we investigated the involvement of Nox4 in IGF-1-induced Akt phosphorylation. PTCs were infected with dominant-negative (DN) Nox4 (Ad-Nox4 ΔFAD/ΔNAD) or Ad-GFP control. Following stimulation with IGF-1, phosphorylation of Akt was examined by Western blot analysis. Infection of the cells with Ad-DN-Nox4 blocked IGF-1-stimulated phosphorylation of Akt compared to GFP control (Figure 3A). To further define the role for Nox4 in IGF-1 induced Akt phosphorylation, we employed a Nox4 knockdown strategy. Cells transfected with siRNA Nox4 (siNox4) but not scrambled control (Scr), exhibited reduced phosphorylation of Akt in the presence of IGF-1 (Figure 3B). Knockdown of Nox4 protein expression was confirmed by Western blot analysis.

To confirm our hypothesis that Nox4-derived ROS are involved in the phosphorylation and activation of Akt, we used an adenoviral vector, which expresses full length and active wild type Nox4 (Ad-Nox4). Infection of PTCs with Ad Nox4 increased the expression of Nox4 protein and enhanced NADPH-dependent superoxide generation compared to Adenovirus GFP-infected or non-infected control cells (Figure 4A, upper and lower panel). To examine the role of Nox4 in Akt phosphorylation, PTCs were infected with increasing concentrations of full length Ad-Nox4. Immunoblotting of the cell lysates shows a dose-dependent increase in Nox4 protein expression, which correlates with the increase in Akt phosphorylation (Figure 4B). Taken together, the data indicate a role for Nox4 protein and ROS generation in the phosphorylation of Akt in PTCs.

Nox4 regulates IGF-1-induced fibronectin expression in proximal tubular cells.
We next examined the direct contribution of Nox4 to FN accumulation in PTCs incubated with IGF-1. RNAi-mediated knockdown of Nox4 (siNox4) but not scrambled control blocked FN expression (Figure 5A). Additionally, over-expression of adenoviral dominant-negative Nox4 (Nox4 $\Delta$FAD/$\Delta$NAD) but not GFP adenovirus control blocked IGF-1 induced FN expression (Figure 5B). Conversely, titration of adenoviral wild type Nox4 (Ad-Nox4) induces FN expression in a dose dependent manner (Figure 5C). Taken together, these data indicate that Nox4 is necessary and sufficient for IGF-1-induced FN accumulation.

*Regulation of fibronectin expression by IGF-1 is mediated through transcriptional and translational mechanisms.*

The effect of IGF-1 on FN mRNA expression was assessed by Quantitative Real Time PCR. We show that, in PTCs, IGF-1 enhances the expression of fibronectin mRNA (Figure 6A), peaking at 24 h post-treatment and returning to basal levels by 48 h. This indicates a potential role for transcription in the regulation of IGF-1-induced fibronectin expression. To confirm this, we transfected PTCs with a plasmid construct containing a minimal fibronectin promoter adjacent to the firefly luciferase gene or with the vector only as a control. We found that IGF-1 causes an increase in luminescence, indicating enhanced FN promoter activity (Figure 6B). It should be noted that increased FN promoter activity is concomitant with the increase in FN mRNA expression.

Extracellular matrix protein expression may also be regulated by translational mechanism (36, 43), we sought to determine whether mTOR translational pathway plays a role in IGF-1-induced fibronectin expression. The ribosomal S6 kinase (S6K) and the cap-dependent eukaryotic initiation factor binding protein 4E-BP1 are both downstream
effectors of mTOR signaling. Our data indicate that IGF-1 increases the phosphorylation of mTOR substrates, S6K and 4E-BP1, in a time-dependent manner (Figure 7A). This observation was confirmed in rat PTCs (upper left panel), mouse PTCs (upper right panel) and human PTCs (lower panel). Furthermore, pretreatment of rat PTCs with the specific mTOR inhibitor rapamycin abrogated IGF-1-induced fibronectin expression (Figure 7B). These results suggest that regulation of FN by IGF-1 also requires the activation of mTOR translational pathway.
In this study, we provide strong evidence that in proximal tubular epithelial cells, IGF-1 enhances fibronectin accumulation through ROS generated by the NADPH oxidase isoform Nox4 and enhanced phosphorylation of Akt. The role of Nox4 in the enhanced Akt phosphorylation and fibronectin accumulation was documented by the following observations: First IGF-1 enhances NADPH oxidase-dependent ROS generation and upregulates Nox4. In cells treated with IGF-1, inhibition of Nox4 by dominant negative Nox4 or siRNA targeting Nox4 inhibits NADPH oxidase activity and Akt phosphorylation and decreases fibronectin expression. Moreover, over expression of Nox4 increases Akt phosphorylation and fibronectin accumulation. Collectively, the data indicate that Nox4 is an important mediator of the effect of IGF-1 to enhance Akt phosphorylation and fibronectin accumulation in proximal tubular epithelial cells.

The growth hormone/IGF-1 axis contributes to the pathophysiological manifestations of renal disease (19, 20, 33, 46) and proximal tubular epithelial cells are important target that release matrix proteins such as laminins, collagens and fibronectin (36, 44, 33). Increased filtration of circulating IGF-1 in proteinuric diseases and its upregulation in diabetic nephropathy increases the local concentration of IGF-1 in the proximal tubules and contributes to fibrosis (41). The cellular mechanisms by which IGF-1 exerts its biological activities are not completely defined. IGF-1 signals through multiple signaling pathways to exert specific biological effects including protein synthesis, cell migration and proliferation and matrix accumulation in various cells including renal cells (47, 44, 26, 46, 38). In proximal tubular epithelial cells, IGF-1 induces cell hypertrophy and
protein synthesis at least partially through a PI3K-dependent mechanism (46). Here we
find that IGF-1 induces the accumulation of the extracellular matrix protein fibronectin in a
PI3K/Akt-dependent manner. This is in contrast to the finding in mesangial cells where
IGF-1 enhances fibronectin accumulation in a calcineurin-dependent mechanism and
independent of Akt or ERK (26). This was supported by the finding that inhibition of
calcineurin by the administration of cyclosporine A reduced fibronectin accumulation in the
glomeruli but not in cortical tissue in a rodent model of diabetes (24). In human lens
epithelial cells, for example, IGF-1 counteracts the effect of transforming growth factor-beta
(TGF-beta) on fibronectin expression (14), whereas in vascular smooth muscle cells, IGF-1
increases the expression of fibronectin (47). It is likely that IGF-1 regulates matrix protein
expression in a cell-type specific manner. In proximal tubular epithelial cells, we find that
IGF-1 increases fibronectin expression through phosphorylation of Akt since inhibition of
Akt by pharmacological or genetic means prevents the effect of IGF-1. It should be noted
that the regulation of extracellular matrix by IGF-1 in cells such as glomerular mesangial
cells or vascular smooth muscle cells have been well investigated. Only a few reports
describe the profibrotic action of the growth factor in renal epithelial cells.

Reactive oxygen species, including those derived from NADPH oxidases of the
Nox family, have been implicated in receptor tyrosine kinase signaling including signals
activated by IGF-1 (48, 38, 16, 29). In the kidney, NADPH oxidases of the Nox family and
specifically the isoform Nox4 are major sources of superoxide anion and hydrogen peroxide
(23, 6, 25, 9, 5). Although the role of Nox4 as a major source of ROS in the kidney is
known (25, 9), the present study provides the first evidence of the importance of this
oxidase in the propagation of IGF-1 redox signaling in tubular epithelial cells. Our
observation that IGF-1 elicits an increase in Nox4 protein expression is important since in contrast to other enzymes of the Nox family, Nox4 is constitutively active (39, 6, 5). Therefore, increase in the expression of the catalytic unit itself is translated to increase in ROS generation. The regulation of Nox4 activity through control of its expression levels was previously reported in vascular and renal cells upon stimulation by various agonists such as angiotensin II (8), TGF-beta (10) and high glucose environment (9). These observations are consistent with previous report showing that IGF-1 regulates Nox4 protein expression without change in its mRNA levels in cardiac cells (38). Our data suggest that IGF-1 acutely modulates oxidative stress in PTCs via alteration of Nox4 levels. Our studies demonstrating that inhibition of Nox4 by siRNA and the dominant negative Nox4 adenovirus abrogate both Akt phosphorylation and fibronectin accumulation places this oxidase upstream of Akt phosphorylation. The observation that overexpression of Nox4 enhances fibronectin accumulation indicates that this oxidase is essential for the accumulation of this matrix protein. The present work identifies for the first time Nox4 as a proximal activator of Akt in the redox pathway linking IGF-1 receptor to fibronectin accumulation in renal tubular epithelial cells. Although it is known that Nox4 is present in epithelial cells including kidney epithelium (12, 49, 22), its role has not being well characterized in these cells. To our knowledge, apart from one report (45) no data exist regarding the function of Nox4 and the redox signaling pathways engaged by the oxidase in renal epithelial cells.

The proximal tubular epithelium is an important contributor to interstitial fibrosis, which ultimately leads to kidney failure. Interstitial fibrosis is characterized in part by the accumulation of matrix proteins such as collagens and fibronectin in the renal tubulo-
interstitial compartment. Fibronectin is typically the first protein to appear in an interstitial scar and serves as both a recruiter of fibroblasts and a scaffold for the deposition of other matrix proteins. Our results support the concept that Nox4-dependent oxidative stress is involved in fibrotic processes as it was proposed in lung (31, 12), heart (4) or kidney (25) fibrosis. The present work provides additional rational for the consideration of Nox4 as a primary target for the design of novel therapeutic intervention in fibrotic disease in general and kidney fibrosis in particular.

The mechanism by which IGF-1 acts through Nox4 to enhance FN accumulation remains to be determined. We have evidence suggesting that IGF-1 may regulate fibronectin expression through both translational as well as transcriptional pathways. On one hand, we show that IGF-1 regulates FN mRNA levels and FN promoter activity, indicating that transcriptional mechanisms are implicated in the action of the growth factor. On the other hand, the fact that IGF1 activates the mTOR translational pathway (as evidenced by enhanced 4E-BP1 and S6K phosphorylation) together with the finding that the mTOR inhibitor rapamycin prevents IGF1-induced FN expression demonstrate that translational mechanisms also play a key role in the control of FN accumulation. This is consistent with our observation that PI3K and Akt, two known upstream activators, of mTOR are implicated in the modulation of FN expression in PTCs.

In conclusion, we describe for the first time that Nox4 is upregulated in response to IGF-1 and mediates IGF-1-induced FN expression through Akt-dependent mechanism in proximal tubular epithelial cells. Collectively, the data support the idea that oxidative stress contributes to matrix accumulation in diseases characterized by upregulation of IGF-1 or its receptor such as diabetic kidney disease. Importantly, our observations also suggest that
mTOR inhibition together with antioxidants targeting Nox4 may represent a potential therapy to reduce fibrosis in kidney disease. IGF-1 treatment was reported to ameliorate diabetic neuropathy in animals (50, 51). However, our data showing that IGF-1 contributes to renal cell injury suggest that therapeutic interventions proposing IGF1 administration in diabetes should be taken with caution. This is supported by the fact that, similar to the kidney, IGF-1 has been shown to be deleterious in diabetic retinopathy (14, 30). Therefore, pathways that are commonly implicated in diabetes complications such as Nox4 and mTOR signaling may represent a more appropriate target for the development of therapeutic interventions.
Figure legends

**Figure 1:** A. IGF-1 (250 ng/ml) induces the NADPH-dependent production of superoxide in primary isolated rat PTCs. This effect is strongly inhibited by DPI (10 nM). NADPH-dependent superoxide production was measured using the lucigenin-based chemiluminescence assay and expressed as relative light units (RLU). B. Exposure of rat PTCs to 250 ng/ml IGF-1 for 1 h enhanced hydrogen peroxide production. Hydrogen peroxide was assessed using Amplex Red method. C. IGF-1 rapidly increases Nox4 protein expression in primary isolated rat PTCs. D. Nox4 is required for IGF-1-induced reactive oxygen species production. Rat PTCs were infected with either an adenovirus expressing dominant negative Nox4, (Ad-Nox4 ΔFAD/ΔNAD) or Ad-GFP as a control. After 48 h, cells were then treated with IGF-1 (250 ng/ml) for 10 min and then assayed for NADPH-dependent superoxide production using the lucigenin-based chemiluminescence assay. Values are the means ± S.E. (n =3), ** p<0.01

**Figure 2:** A. IGF-1 rapidly induces Akt phosphorylation and activation. Akt is phosphorylated on Serine 473 within 5 minutes in response to treatment with 250 ng/ml IGF-1. Western blots were performed on 30 ug of total cellular lysates prepared from primary isolated rat PTCs. B. IGF-1 (250 ng/ml)-induced fibronectin (FN) expression is inhibited by chemical inhibitors of PI3K (LY294002 20 uM) or Akt (Akt inhibitor X, 2.5 uM). C. Dominant negative Akt adenovirus (Ad DN-Akt) abolishes IGF-1 (250 ng/ml)-induced expression of fibronectin in PTCs. Adenovirus encoding GFP (Ad GFP) was used as a control. Histograms represent the intensity of the fibronectin expression bands to the
GAPDH band quantified by densitometry. Values are the means ± S.E. (n = 3), increased ** p<0.01, or reduced compared to IGF-1 induced, # p<0.05.

Figure 3: Nox4 is required for IGF-1-induced Akt phosphorylation. A. PTCs were infected with either an adenovirus expressing dominant negative Nox4 (Ad-Nox4 ΔFAD/ΔNAD) or Ad-GFP as a control. After 48 h, cells were then treated with IGF-1 (250 ng/ml) for 10 min. Dominant negative Nox4 adenovirus abolished IGF-1-induced Akt phosphorylation at Serine 473 as assessed by Western blot analysis. B. siRNA against Nox4 results in a significant reduction of Nox4 protein levels (middle panel), and a concomitant reduction in IGF-1-induced Akt phosphorylation (upper panel). Histogram (lower panels) represents the intensity of the pAkt bands to the Total Akt or GAPDH respectively and expressed as Percent of Control.

Figure 4: Wild type Nox4 overexpression results in increased NADPH-dependent superoxide production and phosphorylation of Akt. Rat PTCs were infected with a full length active wild type Nox4 adenovirus (Ad-Nox4) and after 48 h, the lysates were assayed for Nox4 protein expression (A, top panel) and NADPH-dependent superoxide production (A, bottom panel). A titration of the wild type Nox4 adenovirus (B) shows a dose-dependent increase in Nox4 protein production concomitant with increased phosphorylation of Akt at Serine 473. Ad-GFP was used as a control.
Figure 5: Nox4-derived reactive oxygen species are required for IGF-1-induced Nox4 expression of fibronectin in PTCs. A. siRNA against Nox4 effectively abrogates the expression of fibronectin (FN) at 48 h post-IGF-1 treatment relative to control (scrambled, Scr) siRNA. B. Infection of PTCs with an adenovirus expressing dominant negative Nox4 (Ad-Nox4 ΔFAD/ΔNAD) inhibits IGF-1-induced fibronectin expression. C. Overexpression of full length wild type Nox4 adenovirus (Ad-Nox4) increases fibronectin (FN) expression in a dose-dependent manner. Histogram (lower panels) represents the intensity of the fibronectin expression to GAPDH quantified by densitometry. Values are the means ± S.E. (n =3), increased ** p<0.01, or reduced compared to IGF-1 induced, # p<0.05.

Figure 6: IGF-1 increases Fibronectin mRNA expression and fibronectin promoter activity at similar time points. A. PTC’s were serum deprived for 24 h and then treated with IGF-1 (250ng/ml) for 12, 24 and 48 h respectively. RNA was extracted using RNAzole bee method. RT-PCR was performed for fibronectin mRNA using ΔΔCt method. Fibronectin mRNA expression was quantified with SYBR green dye and RT²qPCR primers. Data represent the relative induction of fibronectin mRNA to GAPDH mRNA levels. Data are the mean and SEM of three experiments. B. The reporter plasmid containing fibronectin promoter that drive the expression of the Luciferase gene was transfected into the PTC’s using GeneJuice™. After 24 h of transfection, cells were incubated with serum free media for 24 h followed by treatment with IGF-1 (250ng/ml)
for indicated time points. Luciferase activity was determined and normalized by protein content. pCDNA3 was used as a control vector. Data are mean and SEM of three experiments. Data demonstrate that IGF-1 significantly induces the fibronectin promoter activity at 12 and 24 h. The results are expressed as the means ± SE. *P < 0.05 and **P < 0.01 compared with uninduced timed control.

**Figure 7:** IGF-1 activates the mTOR pathway leading to fibronectin expression. A. The mTOR substrates S6 kinase (S6K) and 4E-BP1 are phosphorylated within 5 minutes in response to treatment with 250 ng/ml IGF-1. 30 ug of RIPA lysates were separated by SDS-PAGE and probed as described in Methods in rat, mouse and human proximal tubular epithelial cells. B. IGF-1-induced fibronectin expression is inhibited by a selective inhibitor of mTOR (rapamycin 25 nM) in rat PTCs. Histogram (lower panel) represent the intensity of the fibronectin expression to GAPDH quantified by densitometry. Values are the means ± S.E. (n =3), **p<0.01.
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Disclosures:

No potential conflicts of interest relevant to this article were reported.
References


Figure 1

A. NADPH-dependent superoxide generation (RLU) over time (min.)

B. H₂O₂ production (Amplex Red fluorescence at 563 nm)

C. Western blot analysis of Nox4 and GAPDH over time (min.): IGF-I, 0, 5, 10, 15, 30 min.

D. NADPH-dependent superoxide generation (RLU) for different conditions: Ad-GFP, Ad-GFP + IGF, Ad-Nox4, Ad-Nox4 ΔFAD/NAD, Ad-Nox4 ΔFAD/NAD + IGF.
**Figure 2**

(A) Western blot analysis of Akt phosphorylation (P-Akt) and total Akt (Akt) in response to IGF-I treatment with or without LY294002. GAPDH is used as a loading control.

(B) Western blot analysis of FN expression in the presence of IGF-I, LY294002, and Akt-X. GAPDH serves as a loading control.

(C) Quantification of fibronectin expression levels. Ad GFP, Ad GFP ΔAkt, Ad ΔAkt, and Ad ΔAkt + are compared in the presence of IGF-I and LY294002. The graph shows fibronectin expression as a percentage of control, with ** indicating statistically significant differences (p < 0.01) and # indicating trends towards significance (p < 0.05).
Figure 3

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Akt phosphorylation (Percent of Control)

- Ad-GFP
- Ad-GFP +IGF
- Ad-Nox4 ΔFAD/ΔNAD
- Ad-Nox4 ΔFAD/ΔNAD +IGF

- Ctrl
- Ctrl +IGF
- siNox4
- siNox4 +IGF
Figure 4

A

B

Graph showing the effect of Ad-GFP and Ad-Nox4 on Nox4 activity. The graph indicates a significant increase in Nox4 activity with Ad-Nox4 compared to Ctrl and Ad-GFP.

Bar graph comparing the NADPH-dependent superoxide generation in Ctrl, Ad-GFP, and Ad-Nox4 conditions. The graph shows a significant increase in superoxide generation with Ad-Nox4.

Western blot analysis showing the expression levels of Nox4, P-Akt, and GAPDH. The blots indicate increased expression of Nox4 and P-Akt with Ad-Nox4 compared to Ctrl and Ad-GFP.
Figure 5

A

B

C
Figure 6

A

Fibronectin mRNA expression (percent of control)

- - 12 h  +

- - 24 h  +

- - 48 h  +

B

Fibronectin promoter activity (percent of control)

- - 12 h  +

- - 24 h  +

- - 48 h  +
**Figure 7**

**A**

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Rat (RPTC)

Mouse (MCT)

**B**

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Rapamycin

IGF-1

Fibronectin

GAPDH

**Rat (RPTC) vs Mouse (MCT)**

**Human (HK2)**

**Bar Graph**

**Fibronectin expression (percent of control)**

IGF-1

- - + + Rapa

**Bar Graph**

**Figure 7**

**A**

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**Rat (RPTC)**

**Mouse (MCT)**

**Human (HK2)**

**Bar Graph**

**Fibronectin expression (percent of control)**

IGF-1

- - + + Rapa

**Bar Graph**