



Transforming growth factor- β 1 and phosphatases modulate COX-2 protein expression and TAU phosphorylation in cultured immortalized podocytes

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Abstract

Objective and design The aim of this study is to elucidate TGF- β 1 signaling pathways involved in COX-2 protein induction and modulation of TAU protein phosphorylation in cultured podocytes.

Materials, treatment and methods In vitro cultured immortalized podocytes were stimulated with TGF- β 1 in presence and absence of pharmacologic inhibitors for various signaling pathways and phosphatases. Then, COX-2 protein expression, as well as P38MAPK, AKT and TAU phosphorylation levels were evaluated by western blot analysis.

Results TGF- β 1 induction of COX-2 protein levels was completely blocked by pharmacologic inhibitors of phosphatases, P38 MAPK, or NF- κ B pathways. Time course experiments showed that TGF- β 1 activated p38 MAPK after 5 min of stimulation. Interestingly, podocyte co-incubated with TGF- β 1, high glucose and/or PGE2 showed strong increase in p38 MAPK and AKT phosphorylation as well as COX-2 protein expression levels. Levels of phosphorylated

AKT were further reduced and levels of phosphorylated p38 were increased when PGE2 was added to the culture media. Interestingly, selective phosphatases inhibitors completely abrogated PGE2-induced P38 MAPK and TAU phosphorylation. Also, inhibition of phosphatases reversed TGF- β 1-induced COX-2 protein expression either alone or when incubated with high glucose or PGE2.

Conclusion These data suggest TGF- β 1 mediates its effect in podocyte through novel signaling mechanisms including phosphatases and TAU protein phosphorylation.

Keywords Cyclooxygenase · Diabetic nephropathy · Podocytes · TGF- β 1 · TAU

Introduction

Diabetic nephropathy (DN) is the leading cause of end stage renal disease in diabetic patients [1]. Filtration defects are particularly localized in the glomerulus which is composed of various cell types including glomerular podocytes, mesangial cells and capillary endothelial cells. Glomerular podocytes and capillary endothelial cells are separated by a thick basement membrane which all together forms the glomerular filtration barrier. Clinical features of DN include hyperglycemia and hyperfiltration caused by high glomerular capillary pressure (Pgc) [2]. Studies showed that defective glomerular filtration barrier manifested by proteinuria or excretion of albumin into urine could result from podocytes detachment or death, mesangial expansion and/or increased mesangial extracellular matrix deposition encountered in sclerotic glomeruli [2–4].

Previous studies linked TGF- β 1 to the progression of glomerular injury in various types of kidney diseases but mainly diabetic nephropathy [5, 6]. Diabetic environment can

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modulate glomerular hemodynamics by producing proscletic cytokines such as TGF- β 1 which induces hyperfiltration through dilatation of the afferent arteriole [7]. Accordingly, cultures of mesangial cells, glomerular endothelial cells, or podocytes subjected to mechanical stretching or shear stress can activate various signal transduction pathways or elicit growth responses, cytokine synthesis (e.g., TGF- β 1), and increased production of extracellular matrix proteins [8–10]. These studies proposed that the release of sclerotic growth factors or cytokines by mesangial cells, glomerular endothelial cells, or podocytes following mechanical strain can mediate the harmful effect of mechanical stress that will be translated into structural changes found in diabetic glomeruli. Moreover, TGF- β 1 production in diabetic nephropathy was shown to be mediated through mechanisms involving hyperglycemia and the renin–ANG II system [4]. Upon production and release, TGF- β 1 initiates pleiotropic biological effects on glomerular cells, including stimulation of mesangial matrix expansion and deposition, and promotes podocyte phenotypic changes causing detachment and/or apoptosis [7, 11–13]. TGF- β 1 related physiological and cellular events are mediated through modulation of various downstream intracellular signaling pathways including activation of the P38 MAPK, protein kinase B (PKB/AKT) and SMAD proteins [13, 14]. The activities of the latter signaling pathways can be modulated by specific phosphatases including dual-specificity mitogen-activated protein kinase phosphatases (MKPs) and PTEN [15, 16]. The molecular contribution of each of the above-mentioned phosphatases can be studied through the use of pharmacologic inhibitors targeting specifically their activities. However, the role of phosphatases in podocyte biology is still largely uncovered, except for PTEN which was extensively studied due to the role played by AKT in podocyte survival [17–19]. Additionally, in podocytes TGF- β 1 was shown to modulate key signaling pathways involved in cell adhesion such as integrin-linked kinase (ILK) [20]. TGF- β 1 has an important biologic role in many cell types [21, 22]. It elicits cellular signaling by binding to and activating the TGF- β 1-receptor type II.

Upon binding of TGF- β 1, TGF- β 1-receptor II forms an active heterodimer with its counterpart, the TGF- β 1-receptor type I (TGF β RI) which results in the activation of the kinase activity of TGF β RI. In turn, activated TGF- β -receptor heterodimer phosphorylates SMADs proteins which then translocate to the nucleus to act as transcription factors and induce the expression of target genes [21, 22]. In the kidney, TGF- β 1 has a key effect in the induction of renal fibrosis [23].

Prostaglandins are arachidonic acid-derived metabolites synthesized through the activity of the cyclooxygenase enzymatic cascade [24]. Cyclooxygenase enzymes initiate the rate-limiting step in prostaglandin synthesis via the conversion of arachidonic acid liberated from cytoplasmic membrane into prostaglandin H₂, which then converted by

tissue-specific isomerase into definitive prostaglandin [24]. These lipid-derived hormones act locally via autocrine and paracrine manner to regulate various physiologic activities including renal hemodynamic in kidney. Prostaglandin E₂ is the main prostaglandin produced by the kidney glomerulus. Two cyclooxygenase isoforms COX-1 and COX-2 are identified and widely expressed in almost all body tissues. While COX-1 expression pattern is constitutive, COX-2 expression is induced following inflammatory conditions and hence increased by various inflammatory stimuli such as interleukin-1 β [24, 25]. Increased COX-2 protein expression and production of the cyclooxygenase (COX) lipid metabolite—prostaglandin E₂ (PGE₂) are etiologically associated with the progression of nephropathies [26]. Additionally, upregulated glomerular COX-2 levels correlate significantly with albuminuria and the extent of kidney damage [26]. Further clinical and experimental data favor the involvement of (COX) metabolites in the pathogenesis of DN and suggest that at the early glomerular alteration and renal hemodynamic abnormalities found in clinical and experimental diabetic model are prostaglandin E₂-dependent. Additionally, increased COX-2 levels correlate significantly with the extent of renal damage and high levels of both cyclooxygenase isoforms (COX-1 and COX-2) are reported in immunological and nonimmunological nephropathies, such as systemic lupus erythematosus, glomerulosclerosis [27], Heymann nephritis and renal ablation [27–29]. Furthermore, renal injury and albuminuria were significantly reduced with pharmacologic administration of COX inhibitors in a subtotal nephrectomised rodent model of chronic kidney disease [30]. Additionally, overexpression of COX-2 in podocyte predispose to proteinuria, podocyte damage and loss [31]. Moreover, we found that mechanical strain (in vitro mimic of high Pgc) strongly induce COX-2 protein production via p38 MAPK activation [32]. Alternatively, we showed that PGE₂ induced COX-2 protein induction is mediated through a positive feedback loop involving activation of cAMP/AMPK/P38 pathway [33]. Interestingly, we were the first to show that PGE₂ strongly inhibited AKT kinase activation, a key kinase involved in podocyte survival and adhesion [34]. Recently, Cheng et al. showed that overexpression of COX-2 significantly increased albuminuria compared to control in streptozotocin mice model of diabetic nephropathy. Moreover, overexpression of COX-2 induced podocytes injury and through induction of the prorenin receptor and activation of the renin–angiotensin system [35]. Finally, calcineurin partly mediated podocyte apoptosis through COX-2 pathway as inhibition of COX2 or blockade of the Gq-coupled E-series prostaglandins receptor protects podocytes from apoptosis [36].

Podocyte foot processes extending out the cell body play a key role in normal function of the glomerular filtration barrier. Foot process effacement, cell hypertrophy, apoptosis

and detachment from the GBM underlie alterations in podocyte function observed in DN [37, 38]. Foot processes retraction and effacement of the podocyte are the most characteristic alterations resulting in a diffuse cytoplasmic covering sheet along affected areas on the GBM [37, 38]. These harmful modifications are the result of impaired cytoskeletal reorganization by an unknown mechanism yet to be identified. TAU is a microtubule-associated protein involved in neurofibrillary tangles formation (NFT) upon hyperphosphorylation. Around 38 different TAU phosphorylation sites have been identified as a target for various signaling kinases including cAMP/PKA pathway that phosphorylates TAU at serine 214, while p38 MAPK and GSK phosphorylate TAU at serine 396. In our cell model cAMP/PKA, p38 MAPK and GSK-3/AKT signaling pathways play a key role in podocyte health and are strongly modulated by the prostaglandin/COX cascade. As such, activation of the arachidonic acid cascade by TGF- β 1 could in part enhance glomerular damage through cyclooxygenase dependent mechanism where PGE2 released in the ambient environment can induce microtubule reorganization through modulation of TAU protein phosphorylation which further explains the detrimental role of TGF- β 1 at the molecular level.

Materials and methods

Cell culture

Culture of conditionally immortalized murine glomerular epithelial cells (podocytes), kindly provided by Dr. K. Endlich, P. Mundel and Dr. B. Kasinath, were carried out as previously described [19]. For experimental protocols and conditions employed in this study, cells were trypsinized and transferred to the 6 well plates, and cultured for an additional 3 days. Following overnight serum starvation in RPMI-1640 medium supplemented with 0.1% FBS, podocytes were subjected to stimulation with PGE2 (1 μ M) (Cayman Chemical), forskolin (F: 10 μ M) /3-isobutyl-1-methylxanthine (IBMX: 0.5 mM) or transforming growth factor- β 1 (10 ng/ml) (R&D) for 10 min to 6 h, in presence or absence of high glucose/low glucose media (25 mM D-glucose, or 20 mM L-glucose + 5 mM D-glucose) as indicated. Control cells (designated as 'non-stimulated') were cultured under identical conditions but were not exposed to bioactive chemicals. MAPK inhibitors SB 202190, (Tocris; Ellisville, MO, USA), and Bay11-7082 as well as phosphatases inhibitors DUSPi (Dual specificity phosphatase 1/6 inhibitor, Calbiochem), SF1670 (PTEN inhibitor, Tocris), Sal003 (Cell-permeable inhibitor of eIF2 α dephosphorylation, Sigma) and L690,333 (Inositol monophosphatase inhibitor, Tocris)

were employed at 25, 2, 20, 2, 75, and 100 μ M, respectively, in RPMI-1640 + 0.1% FBS, with a 30 min incubation prior to stimulation.

Immunoblotting

Western immunoblot experiment was achieved as previously described. Briefly, immortalized cultured podocytes grown on 6 well plates were serum starved, subjected to the appropriate experimental conditions, and were washed twice with ice-cold PBS. Cells lysed with RIPA buffer and (10 μ L) of the lysate was immediately used to measure the total protein concentration using NanoDrop 2000c Spectrophotometer (Thermo Scientific). The total protein from each sample (50 μ g) was analyzed on 10% SDS-polyacrylamide gel electrophoresis. Dilution (1:1) of the protein samples was performed in 2X Laemmli sample buffer and electrophoresed on 10% resolving gels, and electrotransferred to nitrocellulose membranes (Amersham Pharmacia Biotech; Baie d'Urfé, QC, Canada). Membranes incubated with blocking buffer for 1 h were probed overnight at 4 °C with rabbit polyclonal antibodies as follow: anti-COX-2 (1:1000 dilution, abcam15191), actin (1:1000 dilution, Sigma-Aldrich or abcam), phospho-TAU (serine 214), phospho-TAU (serine 396), phospho-TAU (Tyr 181), total-ERK1/2 (all from Abcam), or a rabbit antibody which recognizes the phosphorylated (activated) form of p38 MAP kinase or the Phosphorylated active form of AKT (ser 473) (Cell Signaling Tec or Abcam) at 1:1000 dilution of anti-active p38 or anti-active AKT. After incubation with an appropriate HRP conjugated secondary antibody (1:2000 to 1:40,000) (GE Healthcare UK, or Abcam), blots were incubated in chemiluminescent substrate (Pierce—Rockford, IL, USA) and exposed to blue light-sensitive film (Kodak). Densitometric analysis of resolved blots were carried out using Chemidoc Alpha Imager software (BioRad).

Statistics

Statistics analysis was performed using prism software. A *P* value of less than 0.05 was considered to be statistically significant. Differences were evaluated by one-way analysis of variance (ANOVA). Significant main effect differences were tested using Bonferroni's post hoc test for multiple comparison. Differences were considered significant at (**P* < 0.05). All experiments were carried out in triplicate.

Results

TGF- β 1 induced COX-2 expression in cultured immortalized podocytes

Various stimuli have been shown to induce COX-2 protein translation including mechanical strain and PGE2, such induction has been associated in vivo with increased kidney damage. In the present study, we asked whether TGF- β 1 may be able to induce COX-2 protein in podocytes. We, therefore, carried out time course experiments in which cultured podocytes were incubated with 10 ng/ml of TGF- β 1. As shown in Fig. 1, western blot analysis indicates that TGF- β 1 upregulates COX-2 protein in a time-dependent manner. TGF- β 1 rapidly induced COX-2 protein, reaching significant levels after 2 h of stimulation and a steady state level after 4 h, which remained elevated for up to 16 h of stimulation.

TGF- β 1 induced COX-2 expression is mediated through activation of the p38 MAPK and/or NF- κ B pathway

We previously showed that p38 MAPK is expressed and activated following stimulation of podocytes with either mechanical strain or PGE2. Moreover, we demonstrated that PGE2/mechanical strain-dependent activation of p38 MAPK strongly induced COX-2 protein translation, we went to further study the ability of TGF- β 1 to activate p38 MAPK pathway, as shown in Fig. 2a western blot experiments indicated that TGF- β 1 increased phosphorylated activated form of p38 MAPK after 5 min of stimulation for up to 10 min. Inhibition of p38 MAPK with SB 202190 or NF- κ B with Bay 11-7082 completely abrogated TGF- β 1 mediated

induction of COX-2 protein translation (Fig. 2b). These data demonstrate that TGF- β 1 induced COX-2 protein translation is mediated through activation of either p38 MAPK or NF- κ B pathway.

High glucose media strongly potentiates TGF- β 1 induced upregulation of COX-2 protein levels

Hyperglycemic conditions can result in microvascular complications that can cause defective filtration barrier, previous reports showed that COX-2 protein is induced in diabetic glomeruli. In addition, diabetic conditions promote the release of proslertotic TGF- β 1 factor. Interestingly, little is known about the interplay between hyperglycemic media and TGF- β 1 at the molecular level. As shown in Fig. 3a, PGE2 potentiated TGF- β 1 mediated induction of COX-2 protein levels. Also, our data showed that hyperglycemic conditions alone (mimicked by high glucose) significantly activated p38 MAPK (Fig. 3c), but was unable to upregulate COX-2 protein levels (Fig. 3b). Moreover, COX-2 protein levels were strongly induced when TGF- β 1 was added to the hyperglycemic media but significantly higher than levels induced by TGF- β 1 alone (Fig. 3b). These data suggest that the upregulated COX-2 protein levels found in diabetic glomeruli might be due in part through a concerted activation of the p38 MAPK by TGF- β 1 and hyperglycemic conditions.

High glucose modulates p38 MAPK and AKT activation

Hyperglycemic conditions can significantly modulate signal transduction elicited during normal physiological conditions. As such, production of TGF- β 1 and prostaglandins

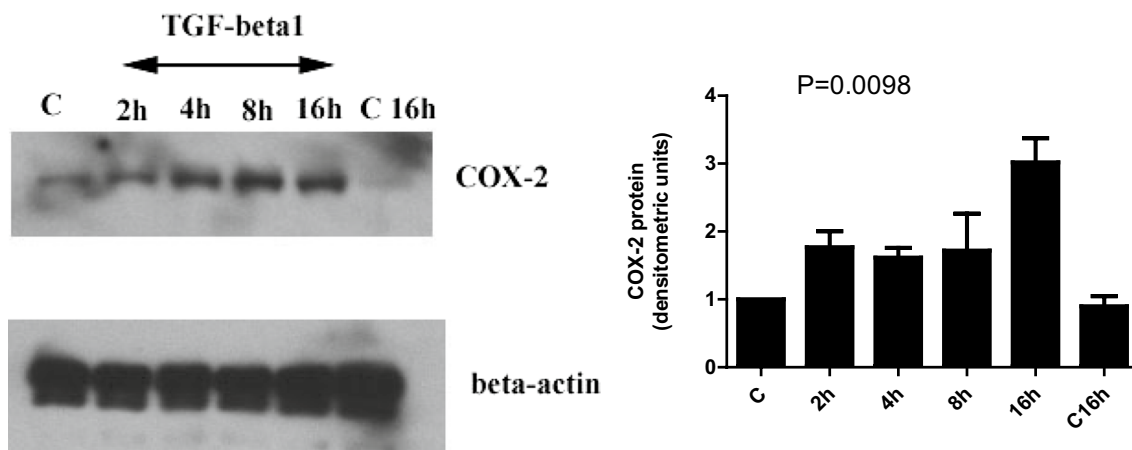


Fig. 1 TGF- β 1 induced COX-2 expression in cultured immortalized podocytes. Immortalized podocytes were incubated with vehicle (PBS) as control (C) or 10 ng/ml of TGF- β 1 for 2, 4, 8 and 16 h, as indicated and lysed with Laemmli buffer. Protein extracts were

resolved by SDS-PAGE and immunoblotted with a COX-2 antibody. Expression levels were normalized to β -actin protein content as assessed by densitometric analysis ($n=3$). * $P<0.01$ vs. vehicle control

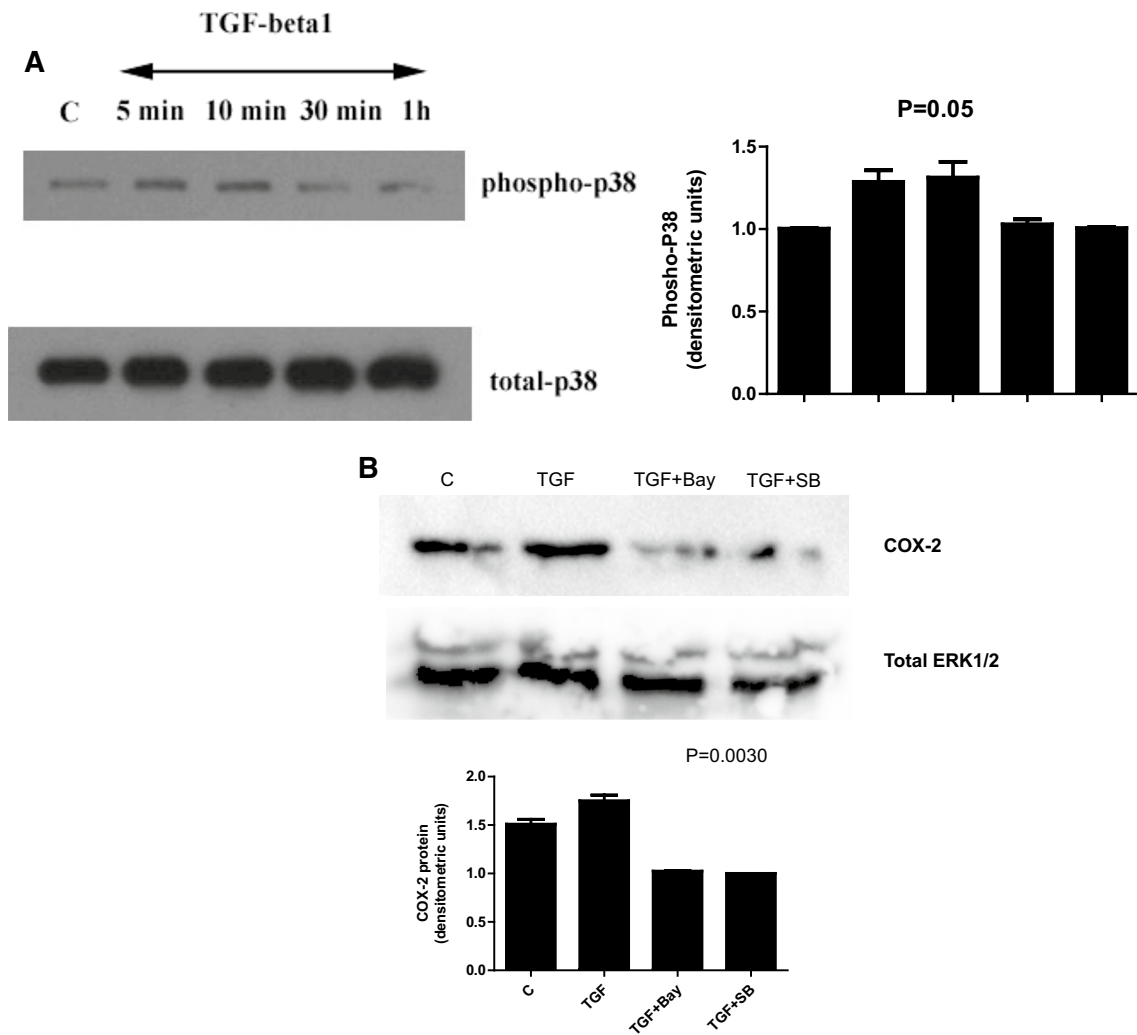


Fig. 2 TGF- β 1 induced COX-2 protein expression through P38MAPK in cultured immortalized podocytes. In **a**, Time and dose-dependent p38 MAPK phosphorylation by TGF- β 1. Immortalized podocytes were stimulated with 10 ng/ml of TGF- β 1 over a range of time points as indicated. In **b**, inhibition of P38 inhibits TGF- β 1-induced COX-2 protein expression. Cells preincubated with SB or

Bay for 30 min were stimulated with 10 ng/ml of TGF- β 1 for 4 h as indicated. Cells were lysed with Laemmli buffer and analyzed by western blot using either anti-COX-2 antibody or phospho-p38 antibody and normalized for total p38 or total-ERK1/2 protein content using anti-p38 or anti-total ERK1/2 antibodies and analyzed by densitometry ($n=3$). * $P < 0.05$ vs. vehicle control

in addition to increase in glucose serum concentration can profoundly alter signaling pathways that are normally inhibited or activated in podocytes. To further assess the effect of hyperglycemia on key signaling pathways in podocytes, we initiated time course experiments in which podocytes were exposed to hyperglycemic conditions mimicked by high glucose media. Our data showed that high glucose strongly activated both p38 MAPK and AKT survival pathways with a maximum activation reached after 10 min of incubating podocytes with high glucose. Levels of the phosphorylated activation of kinases declined fast thereafter Fig. 3c.

Phosphatases inhibitors modulated COX-2 protein expression, as well as P38MAPK and AKT phosphorylation levels in podocytes incubated with TGF- β 1, high glucose and/or PGE2

To further assess the effect of phosphatases on the induction of COX-2 protein expression by either TGF- β 1 or PGE2, podocytes were separately coincubated with TGF- β 1 or PGE2 in presence or absence of phosphatases inhibitors including DUSPi, SF1670 (PTEN inhibitor), Sal003 (Cell-permeable inhibitor of eIF2 α dephosphorylation) and L690,333 (Inositol monophosphatase inhibitor). Interestingly, phosphatases inhibitors DUSPi and Sal003 abrogated

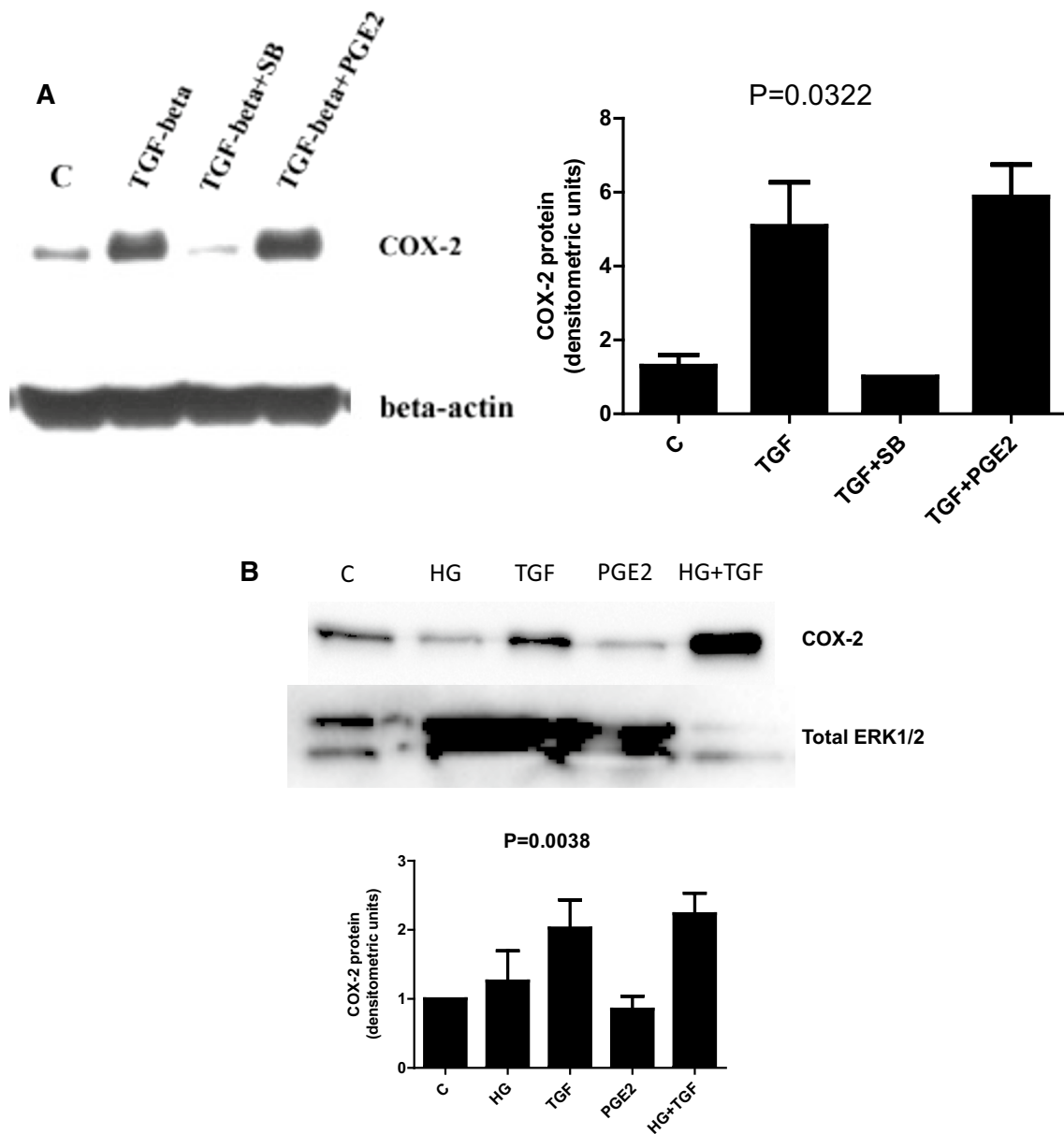


Fig. 3 Synergistic effect of high glucose with either TGF- β 1 or PGE2 mediated COX-2 protein induction. In **a**, immortalized podocytes were incubated for 4 h with either vehicle alone, TGF- β 1 (10 ng/ml), SB 25 (μ M)+TGF- β 1 (10 ng/ml), or PGE2 (1 μ M)+TGF- β 1 (10 ng/ml) as indicated. In **b**, immortalized podocytes were incubated for 4 h with either vehicle alone, high glucose (HG) (25 mM), PGE2 (1 μ M), TGF- β 1 (10 ng/ml), HG (25 mM)+TGF- β 1 (10 ng/ml), or HG (25 mM)+PGE2 (1 μ M) as indicated. Cell lysates were resolved by SDS-PAGE and immunoblot-

ted with a COX-2 antibody. In **c**, immortalized podocytes were incubated with L-Glucose (25 mM) as control, or D-Glucose (25 mM) for 10, 30 min, or 1 or 2 h as indicated. Cells were lysed with Laemmli buffer and analyzed by western blot using either anti-phospho-AKT antibody or anti-phospho-p38 antibody. Expression levels were normalized to beta-actin or total ERK1/2 protein content as indicated in each figure using anti-beta-actin or anti-total-ERK1/2 antibodies and analyzed by densitometry ($n=3$). * $P<0.05$ vs. vehicle control

COX-2 protein translation induced by TGF- β 1 or PGE2 either alone or together in podocytes cultured in normal or high glucose conditions, while the other phosphatases inhibitors were without significant effect in this regard (Fig. 5a). Interestingly, TGF- β 1 induced significant cell death in podocytes pretreated with either DUSPi or Sal003

as marked by complete loss of ERK1/2 protein (Fig. 4a) and cell detachment (data not shown). Furthermore, DUSPi and Sal003 strongly reversed PGE2-induced P38 MAPK phosphorylation, and potentiated the inhibitory effect of PGE2 on AKT phosphorylation, thus levels of phospho-AKT levels were further inhibited in the presence of DUSPi and Sal003.

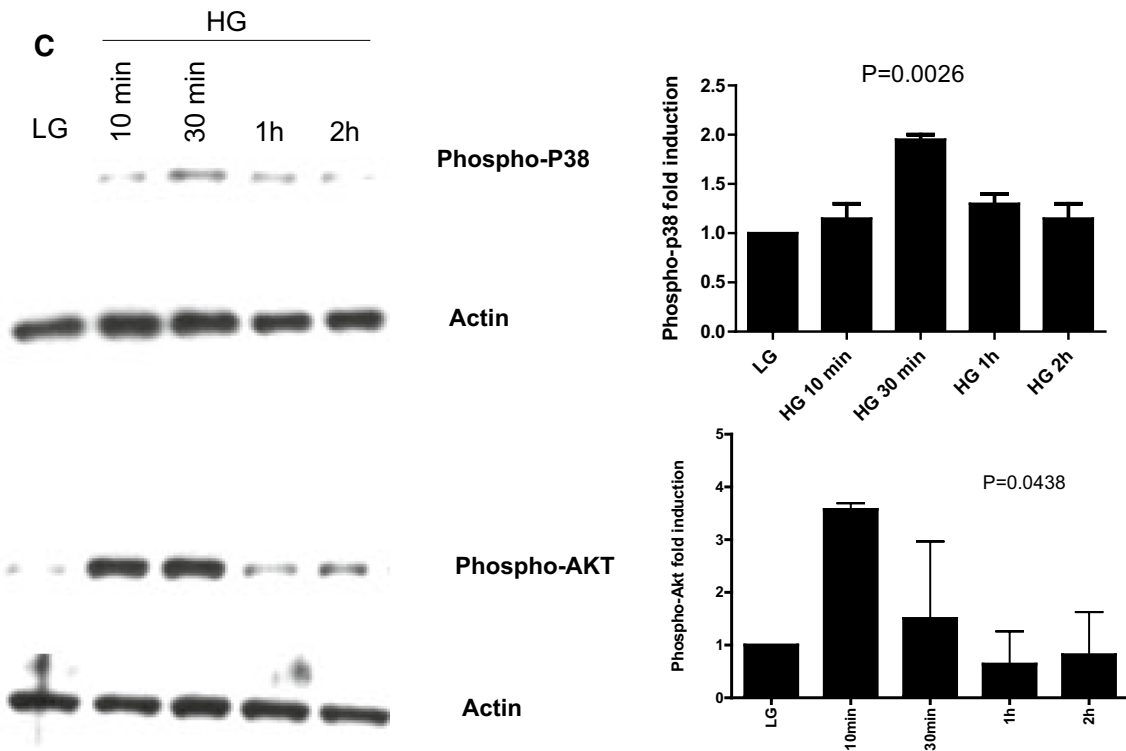


Fig. 3 (continued)

Expectedly, podocyte co-incubated with PGE2 and SF1670 (PTEN inhibitor) showed high phospho-AKT levels when compared to control unstimulated cells (Fig. 5b).

Phosphatases, cAMP and PGE2 modulate TAU protein phosphorylation in podocytes cultured in high glucose media

TAU hyperphosphorylation has been shown to impair microtubules reorganization and hence leads to tangle formation and neuronal apoptosis. As such, we asked whether TAU phosphorylation can be modulated in hyperglycemic conditions when podocytes are exposed to various stimulatory conditions as indicated in Fig. 4b. Interestingly, TAU phosphorylation at serine 214 is significantly modulated by PGE2 and TGF- β 1 in podocytes cultured in high glucose media. We also found that PGE2 or cAMP inducing agents (Forskolin/IBMX) slightly reduced phosphorylated TAU at serine 396, but phospho-TAU (Tyr 181) was not detected (data not shown). Furthermore, podocytes cultured in high glucose conditions and incubated with TGF- β 1 and PGE2 together or in the presence of phosphatases inhibitors showed significant differences in the profile of phosphorylated TAU levels. Interestingly, DUSPi, SF1670 and sal003 completely abrogated phospho-TAU levels at serine 214 regardless the culture conditions. Also, the highest phospho-TAU levels at serine 214 were found in podocytes incubated with both

TGF- β 1 and high glucose, while adding PGE2 was without effect.

Discussion

The role of cyclooxygenase-2 in the etiology of proteinuria and podocytopathies has started to be established. It is now evident based on previous and current investigations done in our lab and by others that cyclooxygenase pathway play a cardinal role in the pathophysiology of glomerular diseases [31, 35, 39]. In addition, the role of TGF- β 1 has been well established as a major contributor to the sclerotic glomeruli [7, 13]. However, the detailed mechanism of TGF- β 1-induced glomerular damage requires further investigations. In the current study, we confirmed a direct link between TGF- β 1 and COX-2, which will further clarify the role of TGF- β 1 in glomerulopathy. It is well known that COX-2 protein induction can be induced following various inflammatory stimuli [25, 33]. Our result showed that TGF- β 1 was able to strongly induce COX-2 protein translation after 2 h of stimulation which confirms a direct effect on cox-2 gene transcription. To our knowledge, we are the first to report that COX-2 protein is upregulated by TGF- β 1 in podocytes.

Regulation of cox-2 gene expression includes both transcriptional and posttranscriptional mechanisms [40]. While posttranscriptional control of COX-2 mRNA is based upon

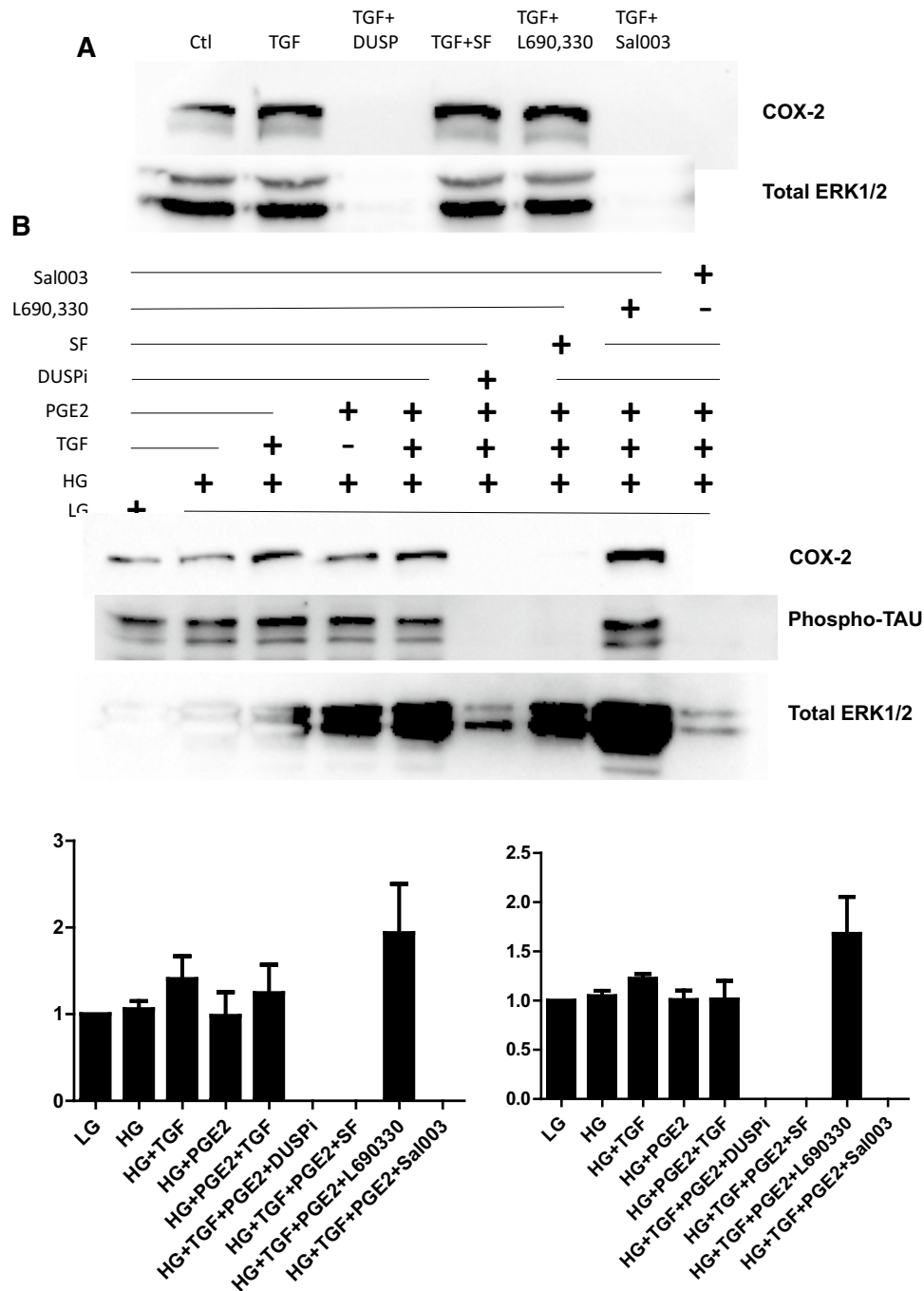
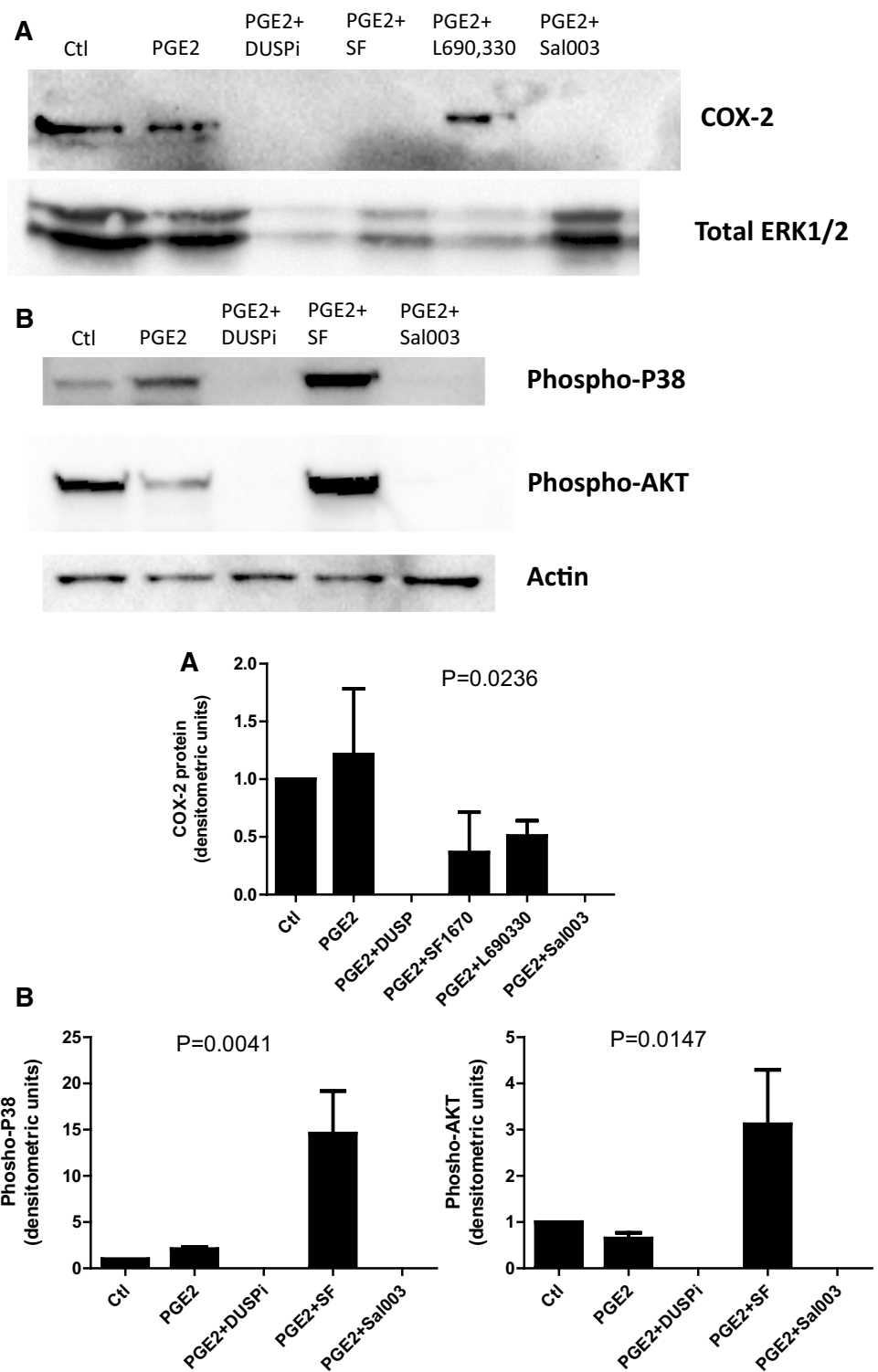


Fig. 4 Phosphatases modulated phospho-TAU (Ser 214) and COX-2 protein expression induced by TGF-β1 and PGE2 in normal or high glucose-treated immortalized podocyte. In **a**, immortalized podocytes were treated for 5 h with vehicle as control, or with TGF-β1 (10 ng/ml), or with TGF-β1 (10 ng/ml)+DUSPi (20 μM), or with TGF-β1 (10 ng/ml)+SF1670 (2 μM), or with TGF-β1 (10 ng/ml)+L690,330 (100 μM), or with TGF-β1 (10 ng/ml)+Sal003 (75 μM) and expression levels were normalized to total-ERK1/2 protein content using anti-total ERK1/2 antibody (*n*=3). In **b**, immortalized podocytes were treated for 5 h with L-glucose (25 mM) alone as control, or with high glucose (HG) (25 mM) alone, or with HG (25 mM)+TGF-β1 (10 ng/ml), or with HG (25 mM)+PGE2 (1 μM),

or with HG (25 mM)+TGF-β1 (10 ng/ml)+PGE2 (1 μM), or with HG (25 mM)+TGF-β1 (10 ng/ml)+PGE2 (1 μM)+DUSPi (20 μM), or with HG (25 mM)+TGF-β1 (10 ng/ml)+PGE2 (1 μM)+SF1670 (2 μM), or with HG (25 mM)+TGF-β1 (10 ng/ml)+PGE2 (1 μM)+L690, 333 (100 μM), or with HG (25 mM)+TGF-β1 (10 ng/ml)+PGE2 (1 μM)+Sal003 (75 μM). Cells were first lysed with RIPA buffer and 50 μg of each sample were diluted 1:1 in sample buffer 2X then resolved onto 10% SDS-PAGE using anti-COX-2 or anti-phospho-TAU (Ser 214) antibodies as indicated. Expression levels were normalized to total-ERK1/2 protein content using anti-total ERK1/2 antibody and analyzed by densitometry (*n*=3). **P*<0.05 vs. vehicle control

Fig. 5 Phosphatases inhibitors modulated p38MAPK and AKT activity induced by PGE2. In **a**, immortalized podocytes were treated for 5 h with vehicle as control, or PGE2 (1 μ M), or with PGE2 (1 μ M)+DUSPi (20 μ M), or with PGE2 (1 μ M)+SF1670 (2 μ M), or with PGE2 (1 μ M)+L690,330 (100 μ M), or with PGE2 (1 μ M)+Sal003 (75 μ M). Cells were first lysed with RIPA buffer and 50 μ g of each sample were diluted 1:1 in sample buffer 2X then resolved onto 10% SDS-PAGE using anti-COX-2 antibody. Expression levels were normalized to total ERK1/2 protein content using anti-total ERK1/2 antibody and analyzed by densitometry ($n=3$). * $P<0.05$ vs. vehicle control. In **b**, immortalized podocytes were treated for 20 min with vehicle as control, or PGE2 (1 μ M), or with PGE2 (1 μ M)+DUSPi (20 μ M), or with PGE2 (1 μ M)+SF1670 (2 μ M), or with PGE2 (1 μ M)+Sal003 (75 μ M). Cells were first lysed with RIPA buffer and 50 μ g of each sample were diluted 1:1 in sample buffer 2x then resolved onto 10% SDS-PAGE using anti-phospho-p38 or anti-phospho-AKT antibodies as indicated. Expression levels were normalized to actin protein content using anti-actin antibody and analyzed by densitometry ($n=3$). * $P<0.05$ vs. vehicle control



mRNA stability, transcriptional stimulation requires the activation of specific enhancer sites found in *cox-2* gene promoter including NF- κ B [41]. Moreover, both mechanisms are highly modulated by p38 MAPK [42]. Our data in podocytes favor both transcriptional and mRNA stability mechanisms in the regulation of *cox-2* mRNA and

protein translation which are found to be mostly mediated via the activation of p38 MAPK. Accordingly, strong and fast upregulation of COX-2 protein expression after 2 h of stimulation coincide with rapid activation of p38 MAPK after 10 min of stimulation. These observations demonstrate that direct transcriptional effects are involved in COX-2

protein upregulation. However, posttranslational control requires longer duration to accumulate significant levels of mRNA and consequently COX-2 protein, which explains long-lasting increased levels of COX-2 protein in podocytes for up to 16 h. Accordingly, we previously showed that p38 activation promote mRNA stability of *cox-2* mRNA for up to 16 h [25]. We further delineated the role of NF- κ B in *cox-2* gene expression. Interestingly inhibition of NF- κ B pathway showed detectable inhibitory effect on COX-2 protein expression induced by TGF- β 1. Knowing that COX-2 promoter contains NF- κ B site, our result showed that TGF- β 1 can signal in part via the activation of NF- κ B pathway to induce COX-2 protein expression.

Although, podocyte incubated with high glucose increased phosphorylated activated levels of p38 MAPK, COX-2 protein levels were barely detectable. These results can be explained by the fact that high glucose was found to block AMPK a key kinase involved in COX-2 protein upregulation in podocytes [43]. Interestingly, cocubation of podocytes with either PGE2 or TGF- β 1 showed significantly high levels of COX-2 protein. These findings raise the possibility that the induction of glomerular sensitive genes such as *cox-2* requires the presence of additional harmful/sclerotic factors such as TGF- β 1 in addition to hyperglycemia. As such, glomerular damage caused by hyperglycemic conditions can be strongly exacerbated upon production of factors that mainly upregulate COX-2 protein including TGF- β 1. The fact that podocyte incubated in high glucose media and stimulated with TGF- β 1 showed higher levels of COX-2 protein compared to TGF- β 1 or high glucose conditions alone highlight the important role of COX pathway in glomerular biology. In addition to glomerulosclerosis caused by TGF- β 1 signaling, the physiologic relevance of these finding can be highlighted by the fact that PGE2 strongly abrogated phosphorylated activated levels of AKT a key survival pathway in podocytes. The effect of PGE2 was entirely mediated via the EP4 receptor subtype as sulprostone (EP1/3 agonist) and SC19223 (EP1 agonist) were without detectable effect (data not shown). The current findings cumulate and confirm our previous describing that PGE2 induced COX-2 protein synthesis through a mechanism involving EP4/cAMP/AMPK/P38 [33]. The novelty of our findings is that TGF- β 1 along with high glucose can strongly induce COX-2 protein synthesis will, therefore, leads to a sustained PGE2 production in the vicinity of podocytes. The finding that TAU proteins can be phosphorylated in podocytes is of considerable importance and can largely explain podocyte foot process retraction/effacement in disease states. The fact that TAU phosphorylation can be modulated by PGE2 and phosphatases further suggests that protein kinase phosphatases and the arachidonic acid cascade are additional important molecular players involved in podocyte microtubule reorganization. It remains to be identified the impact of

such modulation on glomerular filtration barrier function in both health and disease states. Finally, we suggest that TGF- β 1 induced following hyperglycemic conditions will induce COX-2 protein translation which will be further exacerbated by ambient high glucose. Subsequently, released PGE2 in the ambient environment of podocytes contribute to microtubule reorganization and perhaps foot process effacement. The above-mentioned signaling events will eventually lead to concomitant production of PGE2 through a sustained positive feedback loop which causes AKT inhibition and TAU protein hyperphosphorylation leading to podocyte injury. To our knowledge, this is the first study that documents the ability of TGF- β 1 to induce cyclooxygenase protein translation in podocyte, and modulation of TAU phosphorylation by PGE2 a mechanism that could mediate in part the harmful effect of TGF- β 1 in sclerotic glomeruli.

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Compliance with ethical standards

Conflict of interest No competing financial interests exist.

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