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Activation of PI3K–Akt–GSK3β pathway mediates hepatocyte growth factor inhibition of RANTES expression in renal tubular epithelial cells ☆

Rujun Gong [a,*], Abdalla Rifai [b], Lance D. Dworkin [a]

[a] Division of Renal Diseases, Department of Medicine, Brown University Medical School, Providence, RI 02903, USA
[b] Department of Pathology, Brown University Medical School, Providence, RI 02903, USA

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Abstract

Hepatocyte growth factor (HGF) was recently reported to ameliorate renal inflammation in a rat model of chronic renal failure. HGF exerted its action through suppression of RANTES expression in renal tubules. In the present study, we utilized an in vitro model of human kidney proximal tubule epithelial cells (HKC) to elucidate the mechanisms of RANTES suppression by HGF. HGF significantly suppressed basal and TNF-α-induced mRNA and protein expression of RANTES in a time and dose dependent fashion. HGF elicited PI3K–Akt activation and inhibited GSK3, a downstream transducer of PI3K–Akt, by inhibitory phosphorylation at Ser-9. When the PI3K–Akt pathway was blocked by wortmannin, HGF inhibition of RANTES was abrogated, demonstrating that the PI3K–Akt pathway is necessary for HGF action. In addition, specific inhibition of GSK3 activity by lithium ion suppressed basal and TNF-α-induced RANTES expression, reminiscent of the action of HGF. To further investigate the role of GSK3 in modulating RANTES expression, we examined the effect of forced expression of wild type GSK3β or an uninhibitable mutant GSK3β, in which the regulatory Ser-9 residue is changed to alanine (S9A-GSK3β) in HKC. Overexpression of wild type GSK3β did not alter the inhibitory action of HGF on RANTES. In contrast, expression of S9A-GSK3β abolished HGF inhibition of basal and TNF-α stimulated RANTES expression. These findings suggest that PI3K–Akt activation and subsequent inhibitory phosphorylation of GSK3β are required for HGF-induced suppression of RANTES in HKC.

Keywords: Hepatocyte growth factor; Tumor necrosis factor-α; RANTES; Phosphatidylinositol 3-kinase; Glycogen synthase kinase 3; Renal tubular epithelial cells

Hepatocyte growth factor is a mesenchymal-derived, epithelial-targeted, pleiotropic growth factor that modulates multiple cell processes including mitogenesis, motogenesis, morphogenesis, and anti-apoptosis/survival in a variety of cell types [1–4]. In the kidney, tubular epithelial cells (TEC) are a major target for HGF [5,6]. Evidence from a variety of models, including remnant kidney [7–9], unilateral ureteral obstruction [10,11], and diabetic nephropathy [12], demonstrates that HGF ameliorates chronic renal disease. Several mechanisms have been proposed to account for this beneficial action by HGF including anti-apoptosis, promoting tubular cell proliferation [11], prevention of epithelial-to-mesenchymal transition [10], and increased activity of matrix degradation pathways [7,8]. We recently described a novel aspect of HGF biology that is highly relevant to

☆ *Abbreviations:* BAF, BOC-Asp-CH_2F; DAPI, 4′,6-diamidino-2-phenylindole; ELISA, enzyme-linked immunosorbent assay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GSK, glycogen synthase kinase; HA, hemagglutin; HGF, hepatocyte growth factor; PI3K, phosphatidylinositol 3-kinase; RANTES, regulated on activation, normal T-cell expressed and secreted; RT-PCR, reverse transcriptase-polymerase chain reaction; TEC, tubular epithelial cells; TNF, tumor necrosis factor.

* Corresponding author. Fax: +1 401 444 6849.
E-mail address: rgong@Brown.edu (R. Gong).

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progressive kidney disease [9]. HGF treatment substantially attenuated renal dysfunction and inflammation in the rat remnant kidney model of chronic renal failure. This was associated with reduced tubular production of chemokines, most prominent of which was regulated on activation, normal T-cell expressed, and secreted (RANTES), a pivotal chemokine attracting mononuclear inflammatory cells into the renal parenchyma [9]. Consistently, HGF markedly suppressed the basal and tumor necrosis factor (TNF)-α-induced expression of RANTES in vitro in cultured rat TEC. This study examined the molecular mechanism for this action.

Upon binding to its cognate receptor c-Met, HGF activates multiple signaling cascades in TEC, including the phosphatidylinositol 3-kinase (PI3K)–Akt, Ras–Mek–Erk, and p38–MAPK pathways [5]. Different pathways mediate the diverse biological actions of HGF in different cell types. For instance, HGF stimulates cell proliferation through the Ras–Mek–Erk pathway [13], while it prevents cell apoptosis via the PI3K–Akt pathway [4]. In this study, we examined the signaling pathway by which HGF modulates tubular expression of RANTES. We found that activation of PI3K–Akt–glycogen synthase kinase (GSK)3β pathway mediates inhibition of RANTES by HGF in TEC.

Materials and methods

Cell culture. Human proximal TEC (HKC-8) (courtesy of Dr. L. Racusen of John Hopkins University, Baltimore, MD) were maintained in Dulbecco's modified Eagle's medium/F12 supplemented with 5% fetal bovine serum. Cells were plated at approximately 70% confluence in the media containing 5% serum for 24 h and then underwent serum starvation for another 24 h. Human recombinant HGF and human recombinant TNF-α (R&D systems, Minneapolis, MN) were added to the culture with fresh serum-free medium at a final concentration of 20 and 2 ng/ml, respectively, or as otherwise indicated. In experiments with GSK3 blockade, the general apoptosis inhibitor BOC-Asp-CH_2F (BAF; Enzyme Systems Products, Dublin, CA) was added to the culture at the final concentration of 50 μM to avoid cell loss due to induction of apoptosis [14]. At different time points, cell number was estimated by hemacytometer. Cell viability was assessed by trypan blue exclusion. Cells and conditioned media were harvested for further investigation.

Transient transfection. The expression vectors encoding the hemagglutin (HA) tagged wild type (WT-GSK3β-HA/pcDNA3) and uninhibitable mutant GSK3β (S9A-GSK3β-HA/pcDNA3) were, respectively, and kindly provided by Dr. Jim Woodgett (University of Toronto, Totonto, Ontario, Canada) [15] and Dr. Gail V.W. Johnson (University of Alabama at Birmingham, Birmingham, AL) [16]. Transient transfection of HKC was carried out by using the Lipofectamine 2000 according to the instructions specified by the manufacturer (Invitrogen, Carlsbad, CA). After transfection with equal amounts of expression plasmid or empty vector pcDNA3 (Invitrogen), HKC were subjected to different treatments as indicated.

Western immunoblot analysis. After treatment, HKC were washed with PBS and lysed with RIPA buffer supplemented with protease inhibitors (1% Nonidet P-40, 0.1% SDS, 100 μg/ml phenylmethylsulfonyl fluoride, 0.5% sodium deoxycholate, 1 mM sodium orthovanadate, 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 5 mM EDTA in PBS). Protein concentration was determined by using a bicinchoninic acid protein assay kit (Sigma). Samples with equal amounts of total protein (50 μg/ml) were fractionated by 7.5–10% SDS–polyacrylamide gels under reducing condition and analyzed by Western immunoblot as described previously [9]. The antibodies against p-Akt, p-GSK3, and hemagglutin were purchased from Cell Signaling Technology (Beverly, MA) and those for Akt, GSK3 were from Santa Cruz Biotechnology (Santa Cruz, CA).

RNA extraction and semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA was extracted from approximately 2×10^6 HKC using TRIzol solution (Invitrogen) and then diluted to 3 μg/μl in RNase free distilled H_2O. The first strand cDNA was prepared using Superscript RT reverse transcriptase (Invitrogen). Aliquots of cDNA were then amplified as described previously [17] using respective primers for different molecules as follows: RANTES, forward 5′-accctgctgctttgcctac-3′, reverse 5′-ggttcacgc cattctcctg-3′; glyceraldehyde-3-phosphate dehydrogenase (GAPDH), forward 5′-atattgttgccatcaatgacccctca-3′, reverse 5′-ttgacaaagtggtcgtt gagggca-3′. GAPDH was used as an invariant normalizing housekeeping gene. Preliminary experiments were carried out to determine the optimal PCR cycle number for the exponential amplification for each molecule. PCR products resolved in 1.5–2% agarose gels were photographed under ultraviolet light.

Enzyme-linked immunosorbent assay of RANTES. The contents of RANTES in conditioned media from HKC were determined by specific Quantikine sandwich enzyme-linked immunosorbent assay (ELISA) kits for human RANTES (R&D systems). The results were normalized for cell number in culture.

Fluorescent immunocytochemistry. Indirect immunofluorescence staining was performed using an established procedure [9]. Briefly, cells cultured on coverslips were washed twice with cold PBS and fixed with cold methanol/acetone (1:1) for 10 min at −20 °C. Following three extensive washings with PBS containing 0.5% BSA, the cells were blocked with 20% normal donkey serum in PBS buffer for 30 min at room temperature and then incubated with the specific primary antibody. Finally, cells were double stained with DAPI (4′,6-diamidino-2-phenylindole) to visualize the nuclei. Stained cells were mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, California, USA). Results were interpreted using a fluorescence microscope.

Statistics. For immunoblot analysis and RT-PCR, bands were scanned and the integrated pixel density was determined using a densitometer and the NIH image analysis program. All data are expressed as means ± SD. Statistical analysis of the data from multiple groups was performed by ANOVA followed by Student–Newman–Kuels tests. Data from two groups were compared by Student's *t* test. Linear regression analysis was applied to examine possible relationships between two parameters. A value of $P < 0.05$ was considered significant.

Results

HGF suppresses basal and TNF-α-induced RANTES expression in HKC

As presented in Fig. 1, TNF-α strongly stimulates RANTES mRNA expression. HGF treatment significantly suppressed basal and TNF-α-induced RANTES mRNA expression in a time- and dose-dependent fashion. ELISA of RANTES in the culture supernatant demonstrated that RANTES content significantly increased in response to TNF-α stimulation. HGF blunted the basal and TNF-α-induced production of RANTES at 24, 36, and 48 h.

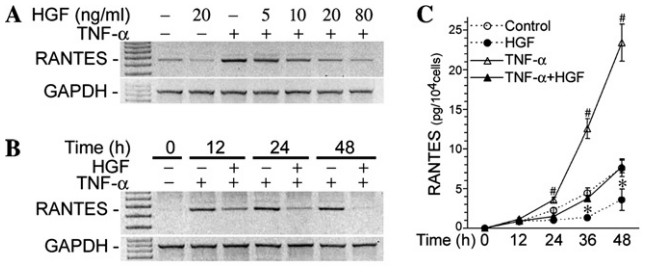

Fig. 1. HGF suppresses basal and TNF-α-induced mRNA and protein expression of RANTES in HKC in a time and dose dependent fashion. (A) HKC were treated with HGF at different concentrations in the presence or absence of TNF-α (2 ng/ml) for 24 h before mRNA was extracted and subjected to semi-quantitative RT-PCR. (B) HKC were treated with HGF (20 ng/ml) in the presence or absence of TNF-α (2 ng/ml) for different intervals before mRNA was extracted and subjected to semi-quantitative RT-PCR. (C) HKC were treated with HGF (20 ng/ml), TNF-α (2 ng/ml) or the combination for different intervals. Conditioned media were harvested and RANTES levels were determined by ELISA. RANTES content was normalized for cell number. $^{\#}P < 0.01$ vs cells treated with both TNF-α and HGF at the same time point; $^{*}P < 0.05$ vs control at the same time point. $n = 3$.

HGF inhibition of RANTES expression is dependent on PI3K–Akt activation

PI3K–Akt is a major signaling pathway triggered by HGF in kidney TEC [5,18]. After HGF treatment, the PI3K–Akt pathway was immediately activated in HKC in the presence or absence of TNF-α, as shown by the elevated phosphorylation of Akt (Fig. 2). TNF-α alone, however, had little effect on Akt activation in HKC. Wortmannin, a specific chemical inhibitor of PI3K, abolished HGF-induced Akt activation, implying that PI3K activation is essential for HGF-induced Akt phosphorylation (Fig. 3). Whether the PI3K–Akt signaling pathway is responsible for RANTES inhibition by HGF in HKC was examined next. We found that the suppressive effect of HGF on RANTES was blocked at both the message and protein levels by the selective PI3K inhibitor,

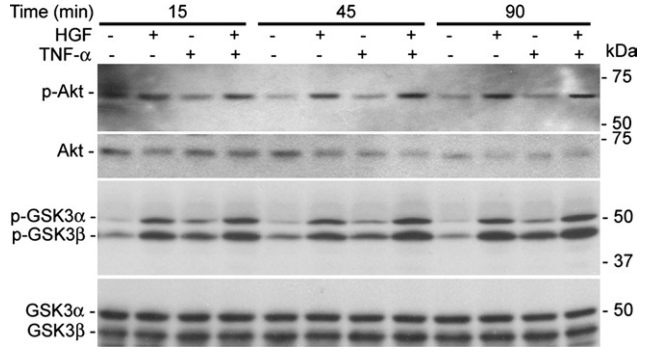

Fig. 2. HGF treatment elicits Akt activation and inhibitory phosphorylation of GSK3 in HKC. HKC were treated with HGF (20 ng/ml), TNF-α (2 ng/ml), or the combination for different intervals. Total cell lysates were subjected to Western immunoblot for different molecules.

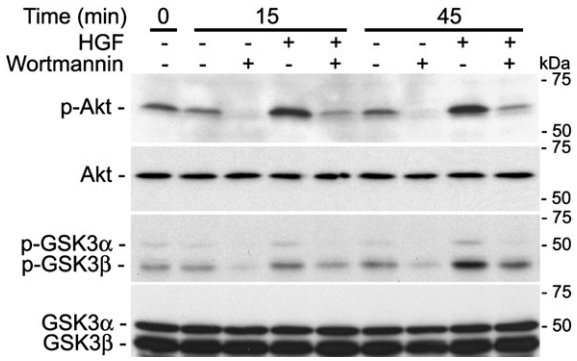

Fig. 3. Specific blockade of the PI3K–Akt pathway by wortmannin abrogates HGF-induced inhibitory phosphorylation of GSK3. HKC were treated with HGF (20 ng/ml) for different intervals after pretreatment with wortmannin (50 nM) for 30 min. Total cell lysates were subjected to Western immunoblot for different proteins.

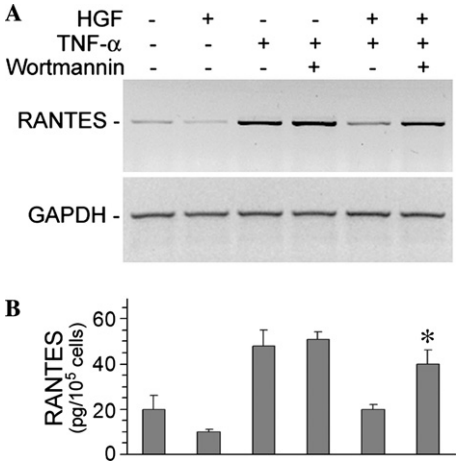

Fig. 4. Specific blockade of the PI3K–Akt pathway by wortmannin abolishes HGF inhibition of RANTES expression in HKC. HKC were treated with HGF (20 ng/ml), TNF-α (2 ng/ml), or the combination for 24 h after wortmannin (50 nM) pretreatment for 24 h. (A) mRNA was extracted and subjected to semi-quantitative RT-PCR. (B) Conditioned media were assayed for RANTES by ELISA. RANTES content was normalized for cell number. $^{*}P < 0.05$ vs cells treated with TNF-α and HGF. $n = 3$.

wortmannin (Fig. 4), implying that PI3K–Akt pathway is required for HGF's inhibition of RANTES. In contrast, two other chemical inhibitors, U0126 and SB203580, respectively, specific for Ras–MEK–ERK and p38–MAPK pathways, failed to block the inhibitory effect of HGF (data not shown), suggesting that the ERK and the p38–MAPK pathways are not involved in HGF's inhibition of RANTES expression in HKC.

HGF induces PI3K–Akt dependent inhibitory phosphorylation of GSK3β in HKC

GSK3 is an important downstream transducer of the PI3K–Akt signaling pathway [19,20]. GSK3 is

inactivated in response to PI3K signaling, as a result of Akt-mediated phosphorylation of an N-terminal serine, Ser-9 in GSK3β and Ser-21 in GSK3α [21]. In HKC, HGF treatment immediately elicited inhibitory phosphorylation of GSK3α and GSK3β (Fig. 2), as probed by a specific antibody against phosphorylated GSK3α (S21) and GSK3β(S9). This effect persisted for at least 90 min in the presence or absence of TNF-α, while TNF-α alone had only a minor effect. In addition, HGF-induced inhibitory phosphorylation of GSK3 was partially abolished by wortmannin (Fig. 3), implying that activation of PI3K–Akt pathway is required for this action.

Inhibition of GSK3 mimics HGF suppression of RANTES in HKC

Preliminary experiments (not shown) and previous reports show that blockade of GSK3 can cause significant cellular apoptosis [22], typically in the presence of TNF-α. To avoid cell loss due to induction of apoptosis, the general apoptosis inhibitor BAF was added to the culture. At the optimal concentration not associated with significant apoptosis, lithium (20 mM), a selective inhibitor for GSK3 [23], markedly attenuated basal and TNF-α-induced message and protein expression of RANTES in HKC (Fig. 5), reminiscent of the action of HGF. As an osmolality control, potassium had no effect.

Ectopic expression of uninhibitable mutant GSK3β abolishes HGF inhibition of RANTES in HKC

Previous studies have shown that GSK3β is essential for TNF-α or IL-1β-induced inflammatory responses [22]. To further examine the role of inhibitory phosphorylation of GSK3β in HGF inhibition of RANTES, we studied the effect of forced expression of GSK3β on RANTES in HKC. Vectors encoding the wild type GSK3β or an uninhibitable mutant GSK3β, in which the regulatory Ser-9 residue was changed to alanine (S9A-GSK), were transfected to HKC. As a control, pcDNA3 was used in transfection. To evaluate the levels of expression, whole cell lysates were analyzed by immunoblotting for HA or HA-GSK3β (Fig. 6). The con-

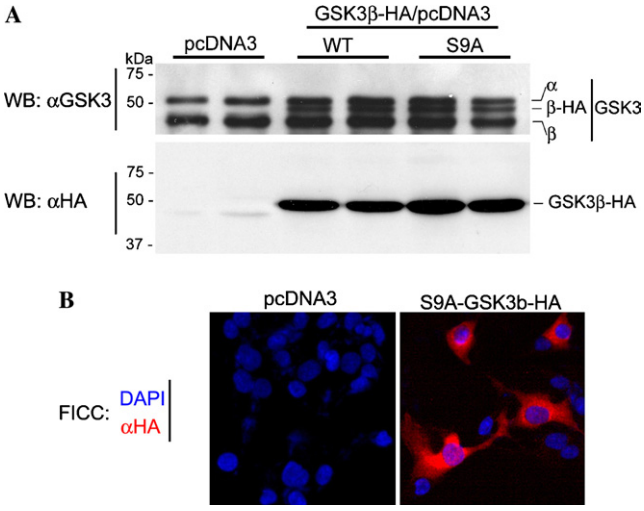

Fig. 6. Forced expression of vectors encoding wild type and mutant GSK3β in HKC. HKC were transfected with the empty vector pcDNA3 or vectors encoding t20e HA-tagged wild type (WT-GSK3β-HA/pcDNA3) or the uninhibitable mutant (S9A-GSK3β-HA/pcDNA3). (A) 24 hours after transfection, cell lysates were subjected to Western immunoblot (WB) for GSK3 or HA. (B) Fluorescent immunocytochemistry (FICC) staining shows expression of HA tag in the transfected cells.

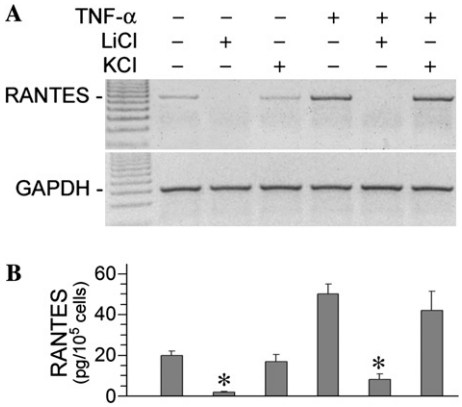

Fig. 5. Inhibition of GSK3 by lithium suppresses basal and TNF-α induced RANTES expression. HKC were treated with LiCl (20 mM) or KCl (20 mM) in the presence or absence of TNF-α (2 ng/ml) for 24 h. To avoid substantial cell apoptosis, the apoptosis inhibitor BAF (50 μM) was added to all cultures. (A) mRNA was extracted and subjected to semi-quantitative RT-PCR. (B) Conditioned media were assayed for RANTES by ELISA. RANTES content was normalized for cell number. $^*P < 0.01$ vs cells treated without lithium. $n = 3$.

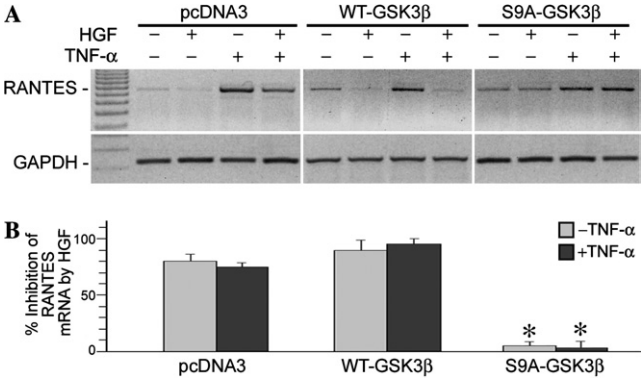

Fig. 7. Ectopic expression of S9A-GSK3β abolishes HGF-induced inhibition of RANTES expression in HKC. After transfection with different vectors as described in the text (Fig. 6), HKC were treated with HGF (20 ng/ml), TNF-α (2 ng/ml) or both for 24 h. (A) mRNA was extracted and subjected to semi-quantitative RT-PCR. (B) Relative inhibition of RANTES mRNA by HGF was estimated by densitometric analysis of the RT-PCR bands. $^*P < 0.01$ vs cells transfected with pcDNA3 or WT-GSK3β. $n = 3$.

structs were abundantly expressed 24 h after transfection. Immunofluorescent detection using an antibody against the HA epitope revealed that ~70% of the cells expressed the HA-tagged constructs. Shown in Fig. 7, HGF inhibition of basal and TNF-α-induced expression of RANTES was evident in HKC transfected with pcDNA3 or WT-GSK3β. In contrast, ectopic expression of S9A-GSK3β abolished the HGF suppressive action on RANTES expression. Collectively, these findings suggest that inhibitory phosphorylation of GSK3β at Ser-9 is required for HGF inhibition of RANTES in HKC.

Discussion

Although considerable evidence exists demonstrating that HGF is a potent renoprotective agent with therapeutic potential for human renal disease [24], relatively little is known about the mechanisms of this beneficial action. Renal inflammation, a frequent pathological finding in almost all types of chronic renal diseases, is a key secondary process perpetuating renal injury [25–27]. Recently, we reported that that HGF ameliorates renal inflammatory infiltration by suppressing tubular cell expression of chemokines. However, the mechanism by which HGF blunts tubular chemokine expression is unknown. In the present study, we report that HGF blocks tubular expression of RANTES via activation of the PI3K–Akt–GSK3β pathway.

PI3K–Akt is a major signaling cascade triggered by c-Met receptor tyrosine kinase after binding to its cognate ligand HGF [28]. However, the exact role of PI3K–Akt in modulation of RANTES expression is controversial and may vary with cell type. For example, specific inhibition of PI3K by LY294002 inhibits lysophosphatidylcholine-induced RANTES expression in human microvascular endothelial cell [29]. Similarly, in lung epithelial cells, PI3K/Akt mediates double-stranded RNA and influenza A virus stimulated RANTES expression [30]. In contrast, in human hepatoma cells [31], blockade of PI3K/Akt by wortmannin stimulated basal and augmented TNF-α provoked expression of RANTES, consistent with an inhibitory effect of PI3K/Akt on RANTES. These conflicting results might result from transduction of the PI3K/Akt signal to distinct downstream transducers in different cell types. Of note, PI3K has multiple isoforms [32], including α, β, and γ, and different isoforms were found to mediate distinct biological responses in different cells [33,34]. In our study, specific PI3K blockade blunted Akt activation and abolished the HGF-induced suppression of RANTES, suggesting that activation of PI3K–Akt in tubular epithelial cells is responsible for the inhibitory effect of HGF on RANTES expression.

One important downstream substrate of PI3K–Akt involved in modulation of TNF-α-induced responses is GSK3 [22], a ubiquitously expressed serine-threonine kinase encoded by two isoforms, GSK3α and GSK3β. Initially identified as a glycogen synthase kinase [36], GSK3 is now known to be a key regulator of a large number of cellular processes and has been implicated in diverse diseases including diabetes, Alzheimer's disease, stroke, and inflammatory conditions [37]. GSK3 is a unique signal transducer in that it is constitutively active in cells under normal conditions. GSK3 is inactivated in response to PI3K signaling, as a result of Akt-mediated inhibitory phosphorylation [35], however, whether GSK3 is involved in RANTES regulation has not been documented previously. We found that selective inhibition of GSK3 by lithium suppressed basal and TNF-α-induced RANTES expression in renal TEC, similar to the action of HGF. Furthermore, ectopic expression of a mutant construct encoding an uninhibitable GSK3β abolished HGF-induced suppression of RANTES. This suggests that HGF inhibits RANTES via inhibitory phosphorylation of GSK3β at serine 9.

How GSK3β inactivation mediates RANTES inhibition is unclear. Recent data suggest that GSK3β is an essential element for NF-κB activation [22,38,39]. Genetic disruption of GSK3β abrogates NF-κB activation and NF-κB-mediated inflammatory responses to TNF-α or IL-1β [22]. In another study, Schwabe et al. [40] reported that active GSK3β directly phosphorylated and activated NF-κB p65. More recently, GSK3β was reported to regulate NF-κB1/p105 stability [41], which is highly relevant to modulation of NF-κB activity. Of note, sequence analysis reveals the presence of several putative κB elements in the promoter region of the RANTES [42]. Consistently, our previous studies demonstrated that NF-κB is responsible for TNF-α stimulated RANTES expression in TEC and HGF blunts this expression via NF-κB inhibition [9]. Therefore, it is attractive to speculate that HGF-induced inhibition of RANTES in TEC might be attributable to suppression NF-κB activity via GSK3β inactivation. In support of this hypothesis, Deng et al. [43] reported that inhibition of GSK3β abrogates NF-κB activation by TNF-α in human colon cancer cells. Whether GSK3β inactivation via inhibitory phosphorylation at Ser-9 mediates HGF's inhibition of NF-κB activity in renal TEC merits further investigation.

In summary, our data suggest that the PI3K–Akt signaling pathway mediates HGF-induced inhibition of RANTES expression in renal TEC, via inhibitory phosphorylation of GSK3β at Ser-9. This novel mechanism may account for HGF's anti-inflammatory action in chronically diseased kidneys.

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