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EGF Receptor Inhibition Alleviates Hyperuricemic Nephropathy

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Abstract

Hyperuricemia is an independent risk factor for CKD and contributes to kidney fibrosis. In this study, we investigated the effect of EGF receptor (EGFR) inhibition on the development of hyperuricemic nephropathy (HN) and the mechanisms involved. In a rat model of HN induced by feeding a mixture of adenine and potassium oxonate, increased EGFR phosphorylation and severe glomerular sclerosis and renal interstitial fibrosis were evident, accompanied by renal dysfunction and increased urine microalbumin excretion. Administration of gefitinib, a highly selective EGFR inhibitor, prevented renal dysfunction, reduced

urine microalbumin, and inhibited activation of renal interstitial fibroblasts and expression of extracellular proteins. Gefitinib treatment also inhibited hyperuricemia-induced activation of the TGF-β1 and NF-κB signaling pathways and expression of multiple profibrogenic cytokines/chemokines in the kidney. Furthermore, gefitinib treatment suppressed xanthine oxidase activity, which mediates uric acid production, and preserved expression of organic anion transporters 1 and 3, which promotes uric acid excretion in the kidney of hyperuricemic rats. Thus, blocking EGFR can attenuate development of HN *via* suppression of TGF-β1 signaling and inflammation and promotion of the molecular processes that reduce uric acid accumulation in the body.

Keywords: Cell signaling, chronic kidney disease, interstitial fibrosis, fibroblast, TGF-beta

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Serum uric acid is enhanced in patients with CKD regardless of whether it is primary or secondary. Hyperuricemia-related diseases were historically viewed with limited interest.[1] However, increasing evidence has indicated that the increased level of uric acid is tightly associated with the development and progression of CKD as well as many other diseases, such as hypertension, cardiovascular diseases, and diabetes.[2–7] For example, a recent meta-analysis of a prospective cohort study showed a 12% rise in mortality for every 1-mg/dl rise in serum uric acid in persons with coronary heart disease.[8] Other pilot investigations indicate that lowering plasma uric acid concentrations slows and delays the development of CKD.[9–15] Thus, uric acid is likely an important mediator and risk marker in CKD.

Uric acid is the final metabolic product of purine metabolism in humans and is excreted in urine. Serum uric acid levels are controlled by the balance of uric acid synthesis and renal excretion.[16] Increased cell turnover (*e.g.,* hemolysis, tumor growth and necrosis) leads to increased extracellular levels of adenosine, inosine, and guanosine.[1] These nucleobases are further degraded to hypoxanthine and xanthine, which are the substrates for the enzyme xanthine oxidase (XOD), a key enzyme in the formation of uric acid.[16] Uric acid is then secreted into the proximal tubular lumen by two processes: It is first translocated across the basolateral membrane from blood to proximal tubular cells by organic anion transporters, OAT1 (*SLC22A6*) and OAT3 (*SLC22A8*), and then secreted to the tubular lumen. When these transporters lose their function, excessive uric acid is accumulated in the body, leading to hyperuricemic nephropathy (HN). Therefore,

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OAT1 and OAT3 may play a pivotal role in uric acid transport and metabolism.[17–19]

HN is characterized by glomerular hypertension, arteriolosclerosis, and tubulointerstitial fibrosis. Traditionally, hyperuricemia has been assumed to induce renal disease through deposition of uric acid crystals in the collecting duct of the nephron in a manner similar to gouty arthropathy.[20,21] Recently, multiple mechanisms leading to HN have been reported. These include endothelial dysfunction, renal angiotensin system activation, oxidative stress, and tubular epithelial cell transition.[22–26] In addition, uric acid can also trigger vascular smooth muscle cell proliferation[27] and inflammation by activating transcription factor, such as NF-κB and inducing production of chemokines/chemokines-like TNF-α, IL-1β, monocyte chemoattractant protein-1 (MCP-1) and regulated on activation, normal T cell expressed and secreted (RANTES).[28,29]

Numerous studies have shown that activation of the TGF-β signaling pathway contributes to glomerular sclerosis and tubulointerstitial fibrosis induced by various insults including hyperuricemia.[30–32] The functional actions of TGF-β in distinct injuries are thought to depend on its interaction with TGF-β receptors[33,34] and subsequent activation of Smad3. Activated Smad3, together with Smad4, is translocated to the nucleus, where it drives expression of TGF-β1–targeted genes. TGF-β1 can also initiate the profibrogenic processes independently of Smads *via* transactivation of EGF receptor (EGFR).[35,36] As a result, some downstream signaling pathways, including the extracellular signal–regulated kinases 1/2 (ERK1/2) pathway, phosphoinositide-3-kinase (PI3K)/Akt pathway, and the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway are activated, triggering gene transcription and biologic effects. In addition, other non-EGFR ligands, such as angiotensin II and endothelin 1, and some environmental stimuli, such as oxidative stress, can also induce EGFR transactivation.[37–40] Because all these stimuli can induce renal fibrogensis,[41–43] EGFR may act as a common mediator in transducing diverse signals that lead to renal fibrosis.

Although emerging evidence suggests that EGFR activation is critically involved in chronic renal injury and glomerular sclerosis,[44,45] whether EGFR mediates the development of HN remains unknown. In this study, we investigated the effect of EGFR inhibition with a highly selective inhibitor, gefitinib, on the development of HN and the mechanisms involved.

Results

Gefitinib Inhibits EGFR Activation in the Kidney of Hyperuricemic Rats

To investigate the role and mechanisms of EGFR in the development and progression of HN, we established a rat model of HN by oral administration of a mixture of adenine (0.1 g/kg) and potassium oxonate (1.5 g/kg) daily. As shown in Figure 1, rats with HN displayed an increase in renal EGFR phosphorylation. Administration of gefitinib, a compound that can specially inhibit EGFR activation,[46_48] significantly decreased the level of phosphorylated EGFR (p-EGFR) in the kidney. Densitometry analysis indicates a 94% reduction of p-EGFR in HN rats treated with gefitinib compared with those treated with vehicle (Figure 1, A and B). p-EGFR was only barely detectable in the kidneys of vehicle-treated rats (Figure 1A). Notably, total EGFR also increased in the kidney of hyperuricemic rats. Gefitinib treatment slightly reduced its expression but did not reach statistical significance (Figure 1, A and C). These data illustrate that hyperuricemia induces activation of EGFR.

Inhibition of EGFR Prevents Renal Dysfunction and Proteinuria and Alleviates Renal Histopathologic Changes in Hyperuricemic Rats

As an initial step toward understanding the role of EGFR in regulating HN, we first examined the onset and duration of HN in this model. After 0, 1, 2, 3, and 4 weeks of daily feeding of the mixture of adenine and potassium oxonate, we determined serum uric acid, creatinine, BUN, and urine microalbumin. As shown in Supplemental Figure 1, serum uric acid was significantly increased at day 7 and kept at the same level at day 14, and was further elevated at days 21 and 28. Serum creatinine and BUN were increased at day 14 and further increased at day 21; the elevated levels were sustained until at least 28 days. Urine microalbumin was increased at day 7 and remained constant at days 14, 21, and 28. Because increased urine microalbumin was detectable at day 7 after the animals were fed the mixture of adenine and potassium oxonate daily, this suggests that rats develop HN as early as 1 week in this model.

We next assessed the effect of EGFR inhibition on serum creatinine, BUN, and proteinuria, as well as on histopathologic changes in the kidneys of hyperuricemic rats. As shown in Figure 2, A–C, administration of gefitinib significantly reduced serum levels of creatinine and BUN and urine microalbumin levels in hyperuricemic rats. Periodic acid–Schiff staining showed that the kidneys of hyperuricemic rats developed severe glomerulosclerosis and tubulointerstitial damage with tubular atrophy, tubular dilatation, and interstitial fibrosis (Figure 2E). Gefitinib administration preserved kidney architecture and attenuated the

glomerular and tubulointerstitial damage. Seminal scoring analysis indicated that EGFR inhibition improved tubular injury by >50% ([Figure 2D](#)). No significant histopathologic changes were observed in the kidney of rats without feeding a mixture of adenine and potassium oxonate ([Figure 2E](#)). Taken together, inhibition of EGFR can improve renal function, reduce proteinuria, and alleviate glomerular and tubular damage in rats with HN.

Inhibition of EGFR Diminished Hyperuricemia-Induced Renal Tubular Injury

Lipocalin-2 (Lcn2) is a well known biomarker for AKI but is also essential for CKD progression in mice and humans.[49],[50] Thus, we also examined the effect of EGFR blockade on its expression in the kidney of hyperuricemic rats. Immunohistochemistry staining showed that Lcn2 expression was barely seen in the kidney of sham-operated kidney with or without administration of gefitinib, but it was clearly expressed in renal tubules of HN. Lcn2-positive staining dots were also observed in the lumen of some tubules of injured kidney, suggesting that they were debris from detached tubular cells. Treatment with gefitinib largely suppressed hyperuricemia-induced Lcn2 expression ([Figure 3A](#)). These results were also confirmed by immunoblot analysis ([Figure 3, B and C](#)). Thus, we suggest that EGFR activation contributes to renal tubular cell injury in HN.

Inhibition of EGFR Attenuates Progression of Hyperuricemia-Induced Renal Fibrosis

Renal tubulointerstitial fibrosis is the common final pathway of renal damage in CKD, regardless of its etiology.[51] To examine whether EGFR mediates hyperuricemia-induced renal fibrogenesis, extracellular matrix protein deposition and expression were examined in the kidneys of hyperuricemic rats. As shown in [Figure 4, A and B](#), kidneys of rats given adenine and potassium oxonate oral daily for 3 weeks displayed severe morphologic lesions characterized by tubular dilation with epithelial atrophy and interstitial expansion with collagen accumulation as evidenced by an increase in Masson trichrome–positive areas within the tubulointerstitium. In contrast, kidneys treated with gefitinib demonstrated a remarkable improvement of the morphologic lesions with less fibrosis in the interstitium ([Figure 4, A and B](#)). Because both collagen 1 and fibronectin are key components of the interstitial matrix, we further analyzed their expression by immunoblotting. As shown in [Figure 5, A–C](#), expression of both collagen 1 and fibronectin was upregulated in the kidney of hyperuricemic rats,

and gefitinib treatment reduced their expression. Hence, inhibition of EGFR may have therapeutic potential in treating fibrotic kidney diseases.

Inhibition of EGFR Blocks Renal Interstitial Fibroblast Activation in the Kidney of Hyperuricemic Rats

Activated interstitial fibroblasts (also called myofibroblasts) play a critical role in the initiation and progression of renal fibrosis,[51] which is characterized by expression of α-smooth muscle actin (α-SMA). To assess the effect of EGFR inhibition on myofibroblast activation *in vivo*, we investigated the effect of gefitinib on the expression of this protein in rats with HN. Immunoblot analysis of whole kidney lysates indicated increased expression of α-SMA in HN rats, and inactivation EGFR with gefitinib reduced α-SMA expression (Figure 6, A and B). Immunohistochemistry staining showed that α-SMA was primarily localized in tubular-interstitial area and that gefitinib treatment significantly decreased the number of α-SMA–positive cells (Figure 6C). Therefore, EGFR also mediates renal interstitial fibroblast activation in rats with HN.

EGFR Mediates Activation of the TGF-β/Smad3 Signaling Pathway in the Kidney of Hyperuricemic Rats

TGF-β signaling contributes to HN,[52] but whether EGFR mediates uric acid–induced activation of TGF-β signaling remains unclear. To address this issue, we first examined the effect of EGFR inhibition on the production of TGF-β in the kidney of HN rats by ELISA. Figure 7A showed that expression of TGF-β was increased in the kidney of hyperuricemic rats and suppressed with gefitinib treatment (Figure 7A). As Smad3 is the major downstream mediator of TGF-β signaling and regulates the transcription of TGF-β–targeted genes,[53] we also compared the level of phosphorylated Smad3 (p-Smad3) in the kidney of hyperuricemic rats treated or untreated with gefitinib. Clearly, in hyperuricemic rats, the level of renal p-Smad3 was elevated and gefitinib administration significantly reduced its expression. p-Smad3 was minimally expressed in the kidney of sham group both treated and untreated with gefitinib (Figure 7, B and C). The expression levels of total Smad3 were unchanged and were not affected by gefitinib in hyperuricemic rats. Taken together, these data suggest that EGFR activity may be critically involved in the activation of TGF-β signaling in hyperuricemia-associated kidney diseases.

Inhibition of EGFR Abrogates NF-κB Pathway Activation and Blocks Macrophage Infiltration in the Kidney of Hyperuricemic Rats

NF-κB is a pivotal transcription factor that regulates chemokine expression, and its activation is critically involved in the inflammatory responses.[54] Immunoblot analysis of whole kidney lysates showed that expression of phosphorylated NF-κB (p-NF-κB p65) was increased in the kidney of hyperuricemic rats (Figure 8A) and inhibition of EGFR significantly reduced its expression. p-NF-κB was not detectable in the kidney of sham groups both treated and untreated with gefitinib. The total NF-κB level was not changed in the kidney of each group of animals (Figure 8, A and B).

Influx of inflammatory cells into the interstitium is a common pathologic feature of almost all kinds of CKD, including HN.[29] Infiltration of immune cells, in particular macrophages, is critically associated with the progression of uric acid nephropathy.[29] To elucidate the effect of EGFR inactivation on this process, we conducted immunohistochemistry staining using an antibody against CD68, a marker of active macrophages. As shown in Figure 8C, the number of CD68-postive macrophages in the injured kidney was remarkably increased in HN rats compared with sham-operated animals, and administration of gefitinib significantly reduced their infiltration (Figure 8, C and D).

Taken together, our data indicate that EGFR activity contributes to activation of the NF-κB signaling pathway and is required for macrophage infiltration into the kidney in hyperuricemia-associated kidney diseases.

EGFR Activation Is Essential for Release of Cytokines/Chemokines in the Kidney of Hyperuricemic Rats

Because release of proinflammatory cytokines/chemokines is essential for the development of hyperuricemia-associated renal injury,[22] we further assessed the role of EGFR in the expression of some proinflammatory cytokines/chemokines, including TNF-α IL-1β, MCP-1, and RANTES, in the kidney by ELISA. Our results showed that expression of all these cytokines was markedly upregulated in the kidney of hyperuricemic rats. Gefitinib treatment reduced renal expression of TNF-α more than 3-fold in HN rats compared with levels in HN animals that did not receive gefitinib (Figure 9A). Expression of IL-1β, MCP-1, and RANTES was also suppressed by >50% in the injured kidney subjected to gefitinib administration (Figure 9, B–D). Because RANTES is a major chemokine in attracting inflammatory cells, including monocytes/macrophages, to sites of inflammation,[55,56] we further evaluated its expression by immunohistochemistry. Supplemental Figure 2, A and B, illustrates that a low level of RANTES was observed in the tubules of sham-operated rats with or without administration with

gefitinib, but abundant expression of RANTES was observed in the kidney tubules of HN rats. Inhibition of EGFR markedly downregulated RANTES expression levels. Thus, these data indicate that EGFR activation is critically associated with production of multiple cytokines in hyperuricemia-associated renal injury.

Inhibition of EGFR Prevents a Rise of Serum Uric Acid and Reduces Serum XOD Activity in Hyperuricemic Rats

Because hyperuricemia is commonly associated with upregulation of serum XOD activity and increased XOD activity can activate production of urate, we examined the effect of EGFR inhibition on the production of uric acid and the activity of serum XOD in HN rats. After 3 weeks of daily feeding of the mixture of adenine and potassium oxonate, serum uric acid increased >3-fold over the value in the group not fed adenine and potassium oxonate (Figure 10A). Gefitinib treatment significantly reduced serum uric acid levels, which was consistent with its inhibitory effect on renal dysfunction (Figure 2, A and B). Figure 10B shows that activity of serum XOD nearly doubled in the kidney of hyperuricemic rats compared with that of animals without feeding of adenine and potassium oxonate. Administration of gefitinib was effective in reducing the activity of serum XOD in hyperuricemic rats. Thus, we suggest that EGFR may contribute to increased serum uric acid levels *via* regulation of metabolism of uric acid.

Inhibition of EGFR Preserves Expression of OAT1 and OAT3 in the Kidney of Hyperuricemic Rats

In addition to increased uric acid production, reduction of uric acid excretion is also associated with elevated serum uric acid levels.[2] Uric acid excretion requires specialized transporters that are located in renal proximal tubule cells, intestinal epithelial cells, and vascular smooth muscle cells. Because human urate transporters such as OAT1 and OAT3 are considered to play critical roles in uric acid homeostasis,[57] we examined the effect of EGFR inhibition on their expression in rats. As shown in Figure 11A, an abundance of OAT1 and OAT3 was expressed in the normal kidney and their levels were decreased in the kidney of hyperuricemic rats. Interestingly, treatment with gefitinib prevented OAT1 and OAT3 downregulation in the injured kidney (Figure 11, A–C). Consistent with these observations, we found that hyperuricemic rats had reduced levels of urinary uric acid relative to sham-operated animals, and gefitinib administration restored the injured kidney to excrete the urinary uric acid to the normal level (Figure 11D

). Collectively, these data suggest that EGFR activation may also lead to an increase in blood levels of uric acid via downregulation of OAT1 and OAT3.

EGFR Activity Is Required for Uric Acid–Induced Activation of Cultured Renal Interstitial Fibroblasts

Figure 6 indicates that gefitinib treatment inhibits expression of α-SMA in the kidney of hyperuricemic rats, suggesting that EGFR mediates uric acid-induced renal interstitial fibroblast activation. To verify the role of EGFR in the activation of renal interstitial fibroblasts, we examined the effect of EGFR inhibition on the activation of renal fibroblasts in response to uric acid in cultured renal interstitial fibroblasts (NRK-49F). Exposure of NRK-49F to uric acid at 200–800 μM resulted in an increase in the expression of α-SMA and collagen 1 as well as EGFR phosphorylation, with the maximum induction observed at 800 μM (Supplemental Figure 3). Treatment with gefitinib dose-dependently suppressed uric acid–induced expression of all these proteins. In conjunction with this observation, phosphorylation/activation of Smad3 was also increased in uric acid–treated NRK-49F, and presence of gefitinib inhibited Smad3 phosphorylation in a dose-dependent fashion (Figure 12, A–E). These data are consistent with our *in vivo* observations and provided further evidence for the role of EGFR in mediating activation of renal interstitial fibroblasts and TGF-β signaling.

Chronic progressive kidney diseases typically are characterized by active renal fibrosis and inflammation. ERK1/2 signaling pathways are critically involved in the development and progression of renal fibrogenesis and inflammation.[58] Thus, we also examined ERK1/2 activation in HN and cultured renal interstitial fibroblasts. As shown in Supplemental Figure 4, A–C, renal ERK1/2 phosphorylation was increased in HN rats and administration of gefitinib largely reduced their phosphorylation. In line with this observation, uric acid also induced ERK1/2 phosphorylation/activation in cultured renal interstitial fibroblasts (Supplemental Figure 4 D-E) and gefitinib treatment inhibited phosphorylation of these kinases in a dose-dependent manner. To further examine whether ERK1/2 mediate activation of renal interstitial fibroblasts, we treated NRK-49F with a highly selective ERK1/2 inhibitor, U0126, at the concentrations of 5–20 μM. Our results showed that U0126 was effective in inhibiting uric acid–induced expression of α-SMA and collagen 1 in a dose-dependent manner (Supplemental Figure 5, A and B). Thus, we suggest that EGFR inhibition may alleviate HN progression *via* a mechanism involved in abrogating the ERK1/2 signaling pathway.

Discussion

Although accumulating evidence indicates that hyperuricemia is an independent risk factor for CKD and contributes to kidney fibrosis,[1] the underlying mechanisms are largely unknown. In this study, we examined the role of EGFR in chronic kidney injury in a rat model of HN induced by oral administration of a mixture of adenine and potassium oxonate. Our results demonstrated that administration of a special EGFR inhibitor, gefitinib, improved renal function and attenuated glomerular sclerosis and renal interstitial fibrosis in hyperuricemic rats. Inactivation of EGFR also inhibited uric acid–induced activation of TGF-β signaling, expression of multiple proinflammatory cytokines/chemokines, and upregulation of XOD activity. Furthermore, EGFR inhibition preserved expression of OAT1 and OAT3, two critical membrane transporters that promote uric acid secretion from blood to renal tubular lumen in the injured kidney. To our knowledge, this study is the first to demonstrate that EGFR is critically involved in the pathogenesis of HN.

The mechanism by which uric acid induces EGFR activation remains unclear. To date, no report shows that uric acid can directly induce EGFR activation. However, unlike many other growth factor receptors, EGFR can be activated by stimuli that do not directly interact with the EGFR ecto domain. For example, G protein–coupled receptor ligands (*i.e.,* angiotensin II), cytokines (*i.e.,* IL-1), and oxidants (*i.e.,* H_2O_2) can induce activation of EGFR through a mechanism known as transactivation.[44,45,47] EGFR transactivation is involved in metalloprotease-mediated shedding of EGF-like ligands from cellular membranes, releasing their soluble form and subsequently binding to the EGFR.[59] In this context, uric acid has been reported to stimulate renin-angiotensin system expression in adipocytes and induce oxidative stress.[10] Thus, uric acid may indirectly trigger EGFR activation *via* activation of the renin-angiotensin system and/or production of reactive oxygen species. Another possibility is that uric acid can directly stimulate production of EGFR ligands. In this study, we have shown that hyperuricemia resulted in increased production of TGF-β. Other studies have also shown that uric acid induces production of several cytokines, such as TNF-α and IL-1α. Because EGFR can be activated by multiple ligands, such as TGF-α, heparin-binding EGF-like growth factor, amphiregulin, and epiregulin, and some of these factors are expressed in renal tubular cells (*i.e.,* heparin-binding EGF and TGF-α),[47] further investigation is required to examine whether uric acid can stimulate production of EGFR ligands in kidney cells and, if so, which ligand(s) plays a primary role in this process.

Numerous studies have demonstrated that TGF-β1 is critically involved in chronic renal damage.[30_32] Studies by our group and others also demonstrated that activation of EGFR signaling is a critical step for production of TGF-β1 in murine models of renal fibrosis induced by unilateral ureteral obstruction (UUO) injury or chronic angiotensin II infusion[30] and activation of TGF-β signaling. In line with those observations, we here demonstrated that EGFR inhibition blocked increased production of TGF-β1 in the kidney of hyperuricemic rats. In addition, EGFR mediates uric acid–induced activation of Smad3, a key molecule in TGF-β signaling in the injured kidney and *in vitro* cultured renal interstitial fibroblasts. These data suggest that a cross-talk between EGFR and TGF-β signaling exists in the kidney of HN. Because Chen *et al*.[45] reported that EGFR mediated sustained TGF-β expression through activation of ERK1/2, we also examined whether uric acid induced ERK1/2 phosphorylation and whether ERK 1/2 mediates renal interstitial fibroblast activation. Our results demonstrated that hyperuremia induced renal ERK1/2 phosphorylation *in vivo* and uric acid also induced phosphorylation of these signaling molecules in cultured renal interstitial fibroblasts. Further, inhibition of EGFR suppressed ERK1/2 phosphorylation *in vitro* and *in vivo*, and blocking ERK pathways also inhibits uric acid–induced activation of cultured renal interstitial fibroblasts. Therefore, TGF-β–mediated tissue fibrosis may rely on an unremitting feed-forward mechanism of EGFR/ERK1/2 activation. Additional studies are needed to further establish the role of ERK1/2 in linking EGFR to activation of TGF-β signaling in HN.

It has been reported that the hyperuricemia-induced inflammatory response mediates kidney injury.[1] Our previous studies in a UUO model demonstrated that EGFR activation contributes to a proinflammatory response and infiltration of inflammatory cells into the interstitium.[46] In the current study, inhibition of EGFR with gefitinib also reduced macrophage infiltration and expression of multiple proinflammatory cytokines/chemokines, including TNF-α, IL-6, MCP-1, and RANTES, in the kidney of hyperuricemic rats. Moreover, EGFR inactivation resulted in a decrease in renal phosphorylation of NF-κB induced by hyperuricemia. These data suggest that inhibition of the inflammatory response may serve as another mechanism by which EGFR inhibition attenuates the pathogenesis and renal fibrosis in this model. On the other hand, uric acid inhibits renal proximal tubule cell proliferation *via* activation of NF-κB,[60] suggesting that inactivation of NF-κB pathways by EGFR inhibition may be beneficial to renal tubular cell regeneration. Currently, the mechanism by which EGFR is coupled to the activation of NF-κB signaling and initiation of proinflammatory responses in HN remains unclear. In a subcutaneous air pouch gout model, Toll-like receptors

(TLR2, TLR4) and myeloid differentiation factor 88–deficient bone marrow–derived macrophages were insufficient in sensing crystals and crystal-induced generation of proinflammatory cytokines.[60] Thus, TLR-dependent signaling may mediate EGFR-elicited inflammatory responses in the kidney of HN. This hypothesis needs to be tested in future studies.

Serum uric acid levels are largely determined by uric acid production, excretion, and reabsorption.[1,2] Our data indicate that administration of gefitinib partially reduces the level of serum uric acid and XOD activity, suggesting that EGFR in part mediates HN through regulation of uric acid production. XOD is an enzyme that catalyzes the oxidation of purine substrates, xanthine and hypoxanthine, producing both uric acid and reactive oxygen species. Thus, besides uric acid, reactive oxygen species may also induce chronic kidney damage through transactivation of EGFR and activation of multiple signaling pathways, such as NF-κB and STAT3.[61] In this regard, we have recently shown that STAT3 is a critical profibrotic mediator in renal interstitial fibrosis after UUO injury.[62]

Uric acid excretion requires special transporters expressed in renal tubule cells. The recently identified human urate transporters, such as OAT1 and OAT3, play a critical role in this process.[18,19] They are localized on the basolateral membrane of epithelial cells, with the ability to transport uric acid from the renal interstitium into tubular epithelial cells, which then secrete it into the renal tubular lumen. Therefore, increased OAT1 and OAT3 expression would promote uric acid secretion. Previous studies have shown that expression of OAT1 was reduced in the kidney of CKD induced by 5/6 nephrectomy.[63] The current study demonstrated that expression of OAT1 and OAT3 was reduced in the kidney of HN. Interestingly, gefitinib inhibition of EGFR protected kidneys from downregulation of these two transporters in hyperuricemic rats, implying that EGFR inhibition may also reduce serum uric acid levels through preservation of OAT1 and OAT3 in the injured kidney. In addition to OAT1 and OAT3, several other excretion transporters (*i.e.,* MRP4, ABCG2, and GLUT9) are also expressed in renal tubules; further investigation is necessary into whether they are also subject to regulation by EGFR and contribute to hyperuricemia in HN.

Uric acid metabolism varies from species to species and is different in humans and rats. The physiologic level of uric acid in rats is lower because of the existence of uricase, which can convert uric acid into allantoin.[64] However, because this metabolic pathway is not present in humans as a result of a lack of uricase, uric acid is mostly excreted directly in urine and is easily accumulated in the human kidney.[65–67] The higher basal level of uric acid in humans may trigger

an adaptive mechanism that protects the kidney against injury when uric acid level is slightly increased. However, excessive production/accumulation of uric acid would still cause HN, which is characterized by both tubulointerstitial fibrosis and glomerular injury. Currently, the mechanism of uric acid–evoked glomerular injury remains unclear, but renal vascular damage and persistent glomerular hypertension may contribute to this process.[68],[69]

Hyperuricemia is a common finding in CKD because of decreased uric acid clearance. Although the role of uric acid in the causation or progression of CKD has been debated, increasing evidence supports uric acid as a cause or exacerbating factor for kidney fibrosis and progressive CKD. In rats with hyperuricemic nephropathy, allopurinol significantly ameliorated uric acid–induced renal fibrosis and renal function impairment.[25] Moreover, a recent systemic review and meta-analysis indicated that three of eight randomized controlled trials with 476 participants showed a benefit from allopurinol treatment in abrogating increases of serum creatinine.[70] This suggests that uric acid–lowering therapy may help prevent or attenuate CKD progression. Because EGFR inhibition not only reduces uric acid levels but also blocks several profibrotic processes, we anticipate that application of an EGFR inhibitor or both an EGFR inhibitor and uric acid–lowering drug such as allopurinol would have a better therapeutic effect in the prevention or treatment of CKD than administration of allopurinol alone. Future clinical trials will address this issue.

In summary, we have demonstrated that inhibition of EGFR attenuated development of hyperuricemia-induced nephropathy in a rat model. This effect was associated with blockade of TGF-β signaling, suppression of inflammation, and reduction of uric acid levels through preservation of uric acid transporter expression and inhibition of XOD activity. Therefore, EGFR inhibition may hold a therapeutic potential for treatment of uric acid nephropathy.

Concise Methods

Chemicals and Antibodies

Antibodies to p-Smad3, Smad3, p-ERK1/2, ERK1/2, p-EGFR, p-NF-κB (p65), and NF-κB (p65) were purchased from Cell Signaling Technology (Danvers, MA). Antibodies to OAT1, OAT3, fibronectin, collagen 1(A2), glyceraldehyde 3-phosphate dehydrogenase, EGFR, and CD68 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Primers were synthesized from Invitrogen (Carlsbad, CA). Gefitinib was purchased from LC Laboratories (Woburn, MA).

Serum XOD kit was from Jiancheng Technology (Nanjing, China). TNF-α, IL-1β, MCP-1, RANTES, and TGF-β1 ELISA kits were from R&D systems (Minneapolis, MN). Vectastain ABC kit was from Vector Laboratories (Burlingame, CA). Antibodies to α-SMA and all other chemicals were from Sigma-Aldrich (St. Louis, MO).

Cell Culture and Treatments

NRK-49F cells were cultured in DMEM (Sigma-Aldrich) containing 5% FBS, 0.5% penicillin, and streptomycin in an atmosphere of 5% CO_2 and 95% air at 37°C. To determine the role of EGFR and ERK1/2 in uric acid–induced renal fibroblast activation, NRK-49F cells were starved with 0.5% FBS for 24 hours and then exposed to various concentrations of uric acid (0–800 μM) for 36 hours in the absence or presence of gefitinib or U0126. Then, cells were harvested for immunoblot analysis.

HN Model and Gefitinib Administration

Male Sprague–Dawley rats (6–8 weeks old) weighing 200–220 g were purchased from Shanghai Super–B&K Laboratory Animal Corp. Ltd. Animals were housed in stainless steel cages in a ventilated animal room at the Experimental Animal Center of Tongji University. Room temperature was maintained at 20±2°C, relative humidity at 60%±10%, and a 12-hour light/dark cycle. Distilled water and sterilized food for rats were available *ad libitum*. The rats were acclimated to this environment for 7 days before experiments. Twenty-four male rats were randomly assigned to four groups of six rats: sham group, sham treated with gefitinib (80 mg/kg) group, HN group, and HN treated with gefitinib (80 mg/kg) group. The HN rat model was established by oral administration of a mixture of adenine (0.1 g/kg) and potassium oxonate (1.5 g/kg) daily consistently for 3 weeks. To assess the efficacy of gefitinib in HN rats, gefitinib at 80 mg/kg in 50 μl of DMSO was given daily by peritoneal injection. Animals treated with DMSO alone were used as controls. After 3 weeks, the animals were euthanized and the kidneys were collected for protein analysis and histologic examination. Twenty-four–hour urine samples were collected in metabolic cages at day 0 and weekly for determination of urinary levels of protein. Blood was also taken once a week for the measurement of serum uric acid, BUN, creatinine, and other biochemistry indices.

To observe the onset and duration of HN in this model, we conducted further time-dependent experiments. At 0, 7, 14, 21, and 28 days after daily feeding of the mixture of adenine and potassium oxonate, blood was taken once a week for the

measurement of serum uric acid, BUN, creatinine, and other biochemistry indices. Urine was collected and urine microalbumin was measured as mentioned above. All the animal experiments were performed according to the policies of the Institutional Animal Care and Use Committee at Tongji University.

Assessment of Serum Uric Acid, Renal Function, and Other Biochemistry Indices

Urinary microalbumin, urinary uric acid excretion, serum uric acid, creatinine, and BUN were determined by automatic biochemistry assay (P800; Modular). Briefly, collected blood was centrifuged at 2500 rpm/min for 5 minutes and 200 μl of serum was put in an automatic biochemistry analyzer (P800) for analysis.

Assessment of Serum Activity of XOD

Serum activity of XOD was examined according to the protocol provided by the manufacture (20100818, Jiancheng, Nanjing, China).

Immunoblot Analysis

Immunoblot analysis of tissue samples were performed as described previously.[46] The densitometry analysis of immunoblot results was conducted using ImageJ software (National Institutes of Health, Bethesda, MD).

Immunohistochemical Staining

Formalin-fixed kidneys were embedded in paraffin and prepared in 3-μm-thick sections. Immunohistochemical staining was conducted on the basis of the procedure described in our previous studies.[46] For evaluation of renal fibrosis, Masson trichrome staining was performed according to the protocol provided by the manufacture (Sigma-Aldrich). The collagen tissue area (blue color) was quantitatively measured using Image Pro-Plus software (Media-Cybernetics, Silver Spring, MD) by drawing a line around the perimeter of positive staining area, and the average ratio to each microscopic field (×400) was calculated and graphed. For general histology, sections were stained with periodic acid–Schiff. To assess the extent of tubular injury, morphologic damage (epithelial necrosis, luminal necrotic debris, and tubular dilation) in three to four sections per kidney and 10–12 fields per section were quantified using the following scale: none=0; <10=1; 11%–25%=2; 26%–75%=3; and >75%=4. Severity of inflammation was graded by counting the absolute number of CD68-positive cells in each field and

reported as the mean of 20 random high-power (×400) fields each rat in six rats per group.

ELISA Analysis

To examine renal expression of cytokines, such as MCP-1, RANTES, TNF-α, IL-1β, and TGF-β1, rat kidneys were homogenized in the extraction buffer containing 20 mM Tris–HCl, pH 7.5, 2 M NaCl, 0.1% Tween 80, 1 mM ethylene diamine tetraacetate, and 1 mM phenylmethylsulfonyl fluoride. The supernatant was recovered after centrifugation at 19,000 g for 20 minutes at 4°C. Renal tissue multiple cytokine level was determined using the commercial Quantikine ELISA kit in accordance with the protocol specified by the manufacturer (ELISA kit, R&D Systems, Minneapolis, MN). Total protein levels were determined using a bicinchoninic acid protein assay kit. The concentration of cytokines in kidneys was expressed as picograms per milligram of total proteins.

Statistical Analyses

All the experiments were performed at least three times. Data depicted in graphs represent the mean±SEM for each group. Intergroup comparisons were made using one-way ANOVA. Multiple means were compared using the Tukey test. The differences between two groups were determined by t test. Statistical significant difference between mean values was marked in each graph. $P<0.05$ is considered to represent a statistically significant difference.

Disclosures

None.

Supplementary Material

Supplemental Data:

Acknowledgments

Footnotes

Published online ahead of print. Publication date available at www.jasn.org.

This article contains supplemental material online at
http://jasn.asnjournals.org/lookup/suppl/doi:10.1681/ASN.2014080793/-/DCSupplemental.

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Figures and Tables

Figure 1.
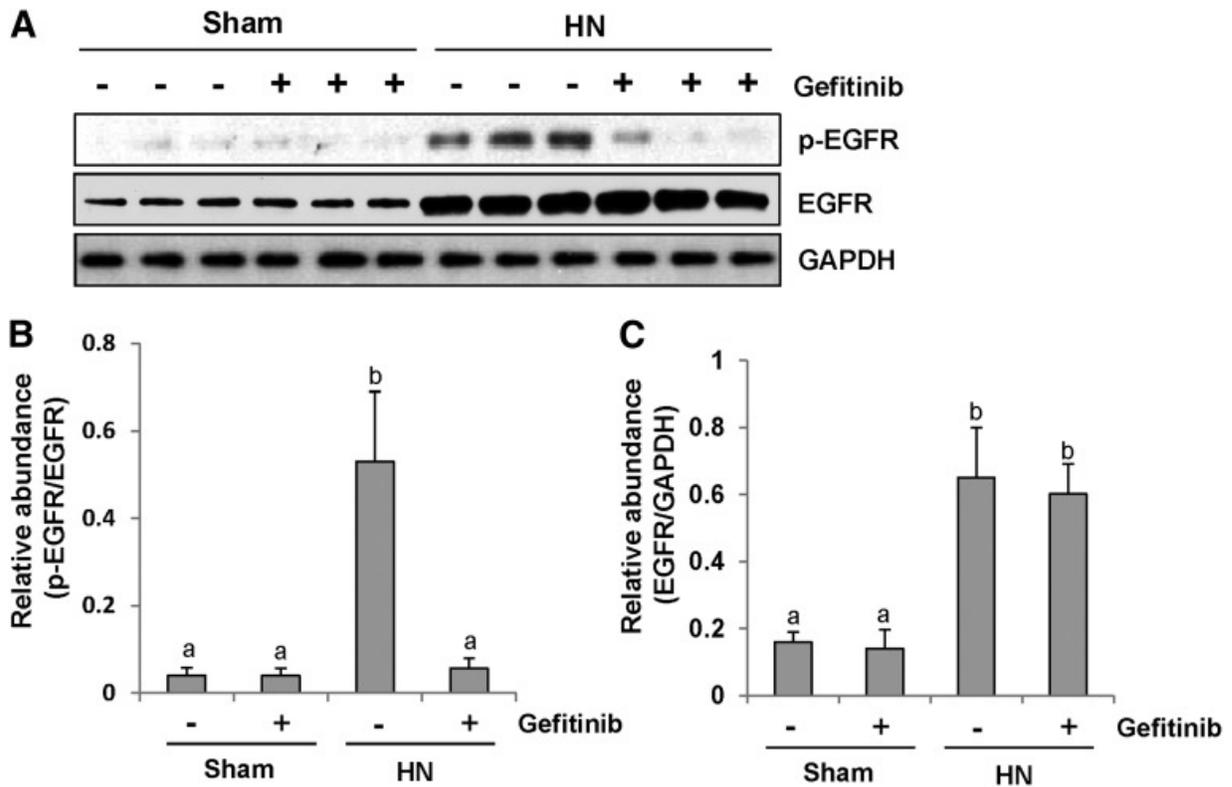
Gefitinib inhibits EGFR activation in the kidney of HN rats. (A) Rat model of HN was established by oral administration of a mixture of adenine and potassium oxonate daily. In some rats, gefitinib were simultaneously administrated intraperitoneally. After 3 weeks, the kidneys were taken for immunoblot analysis of p-EGFR, EGFR, or glyceraldehyde 3-phosphate dehydrogenase (GAPDH). (B) Expression levels of p-EGFR and EGFR were calculated by densitometry, and the ratio between p-EGFR and total EGFR was determined. (C) Total EGFR levels were normalized with GAPDH. Data are represented as the mean±SEM (n=6). Means with different superscript letters are significantly different from one another ($P<0.05$).

Figure 2.

PAS Staining
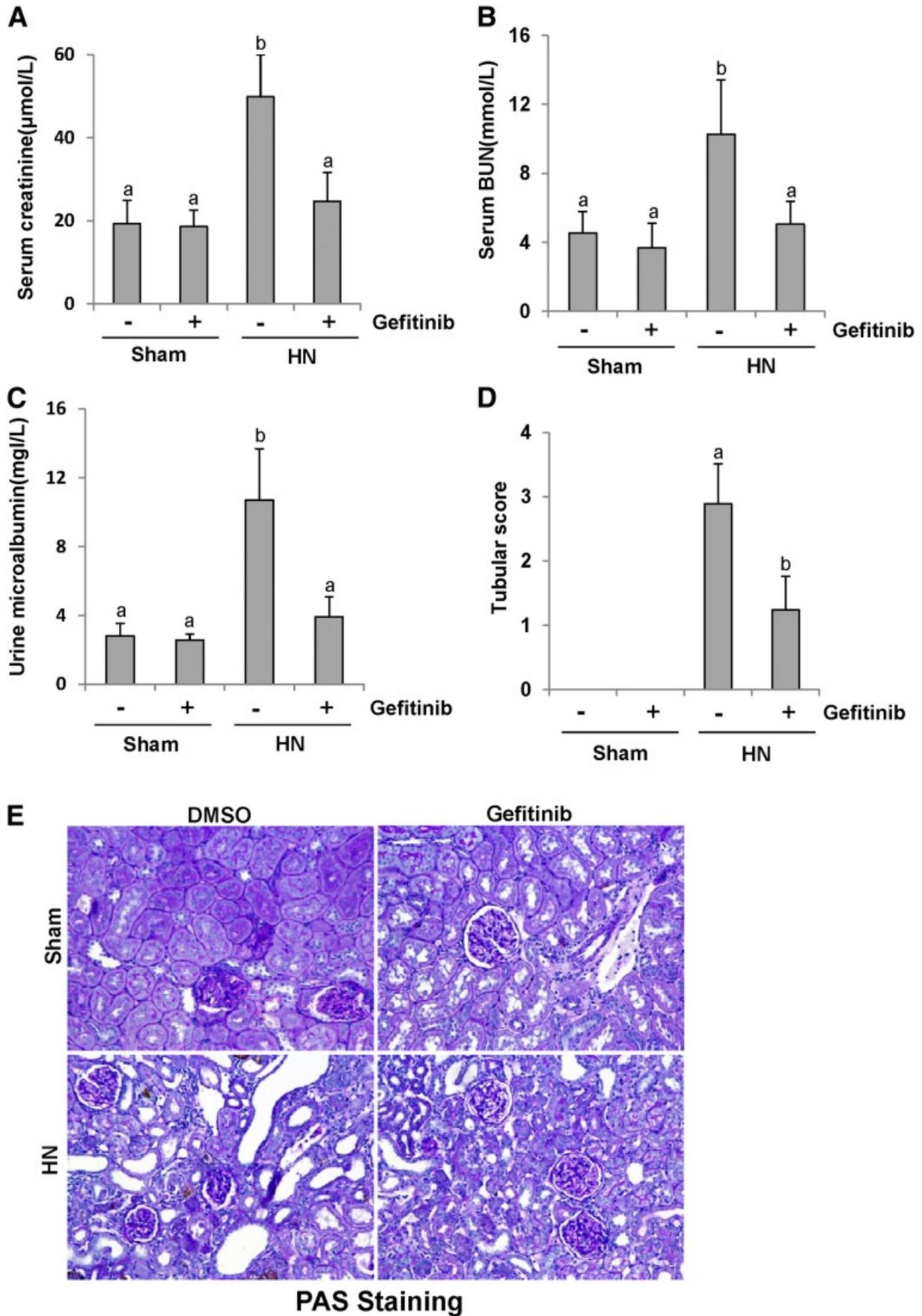
Gefitinib halts progression of proteinuria and improves renal function and kidney pathology in hyperuricemic rats. (A) Expression level of serum creatinine was examined using automatic biochemistry assay. (B) Serum BUN. (C) Urine microalbumin. (D) Photomicrographs (original magnification, ×200) illustrate periodic acid–Schiff staining of the kidney tissues in control or HN rats with or without gefitinib. (E) Morphologic changes were scored on the basis of the scale described in the Concise Methods section. Data are represented as the mean±SEM (n=6). Means with different superscript letters are significantly different from one another ($P<0.05$).

Figure 3.

A

Lcn2 Immunohistochemistry

B

C

Inhibition of EGFR decreases expression of Lcn2 in the kidney of hyperuricemic rats. (A) Photomicrographs illustrating Lcn2 immunochemistry staining of kidney tissue collected at day 21 after feeding of the mixture of adenine and potassium oxonate with or without gefitinib. (B) The kidney tissue lysates were subjected to immunoblot analysis with specific antibodies against Lcn2 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). (C) Expression level of Lcn2 was quantified by densitometry and normalized with GAPDH. Data are represented as the mean±SEM (n=6). Means with different superscript letters are significantly different from one another ($P<0.05$).

Figure 4.

Trichrome Masson Staining

Gefitinib treatment attenuates the accumulation of collagen fibrils in renal interstitium of HN rats. (A) Photomicrographs illustrating Masson trichrome staining of kidney tissue collected at day 21 after feeding of the mixture of adenine and potassium oxonate with or without

gefitinib. (B) The graph shows the percentage of Masson-positive tubulointerstitial area (blue) relative to the whole area from 10 random cortical fields (original magnification, ×400). Data are represented as the mean±SEM (n=6). Means with different superscript letters are significantly different from one another (P<0.05).

Figure 5.
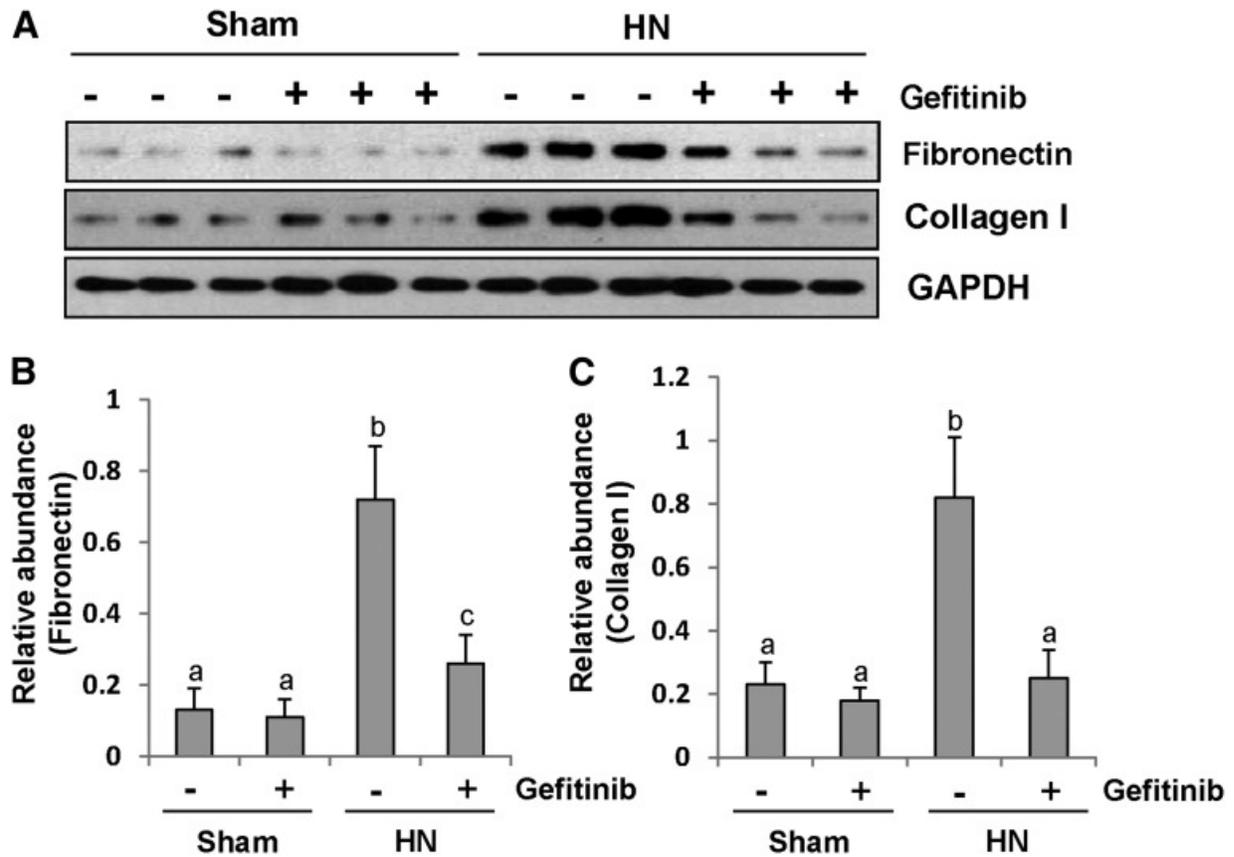
Gefitinib inhibits renal expression level of fibronectin and collagen 1 in hyperuricemic rats. (A) The kidney tissue lysates were subjected to immunoblot analysis with specific antibodies against fibronectin, collagen 1, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). (B) Expression level of fibronectin was quantified by densitometry and normalized with GAPDH. (C) Expression level of collagen 1 was quantified by densitometry and normalized with GAPDH. Data are represented as the mean±SEM. Means with different superscript letters are significantly different from one another ($P<0.05$).

Figure 6.
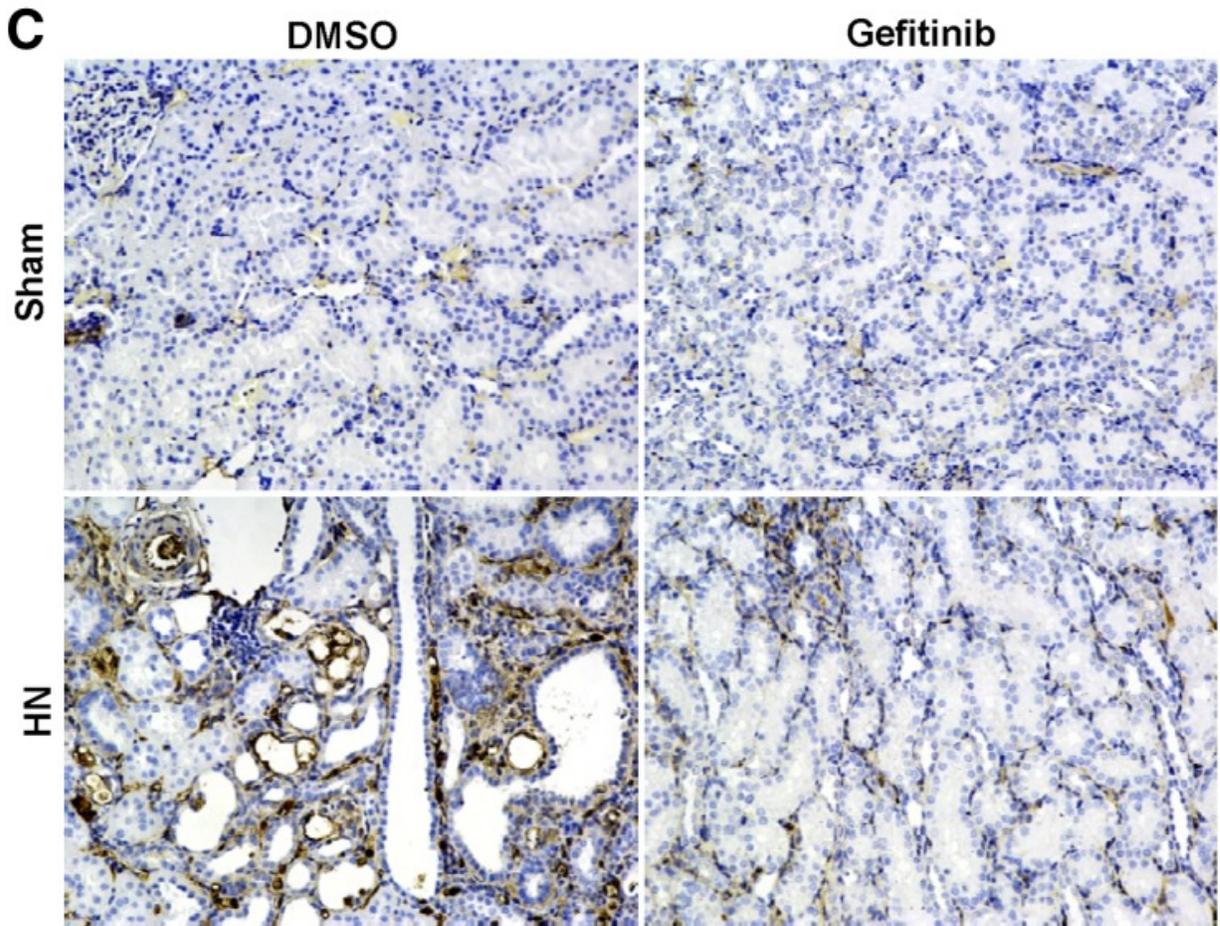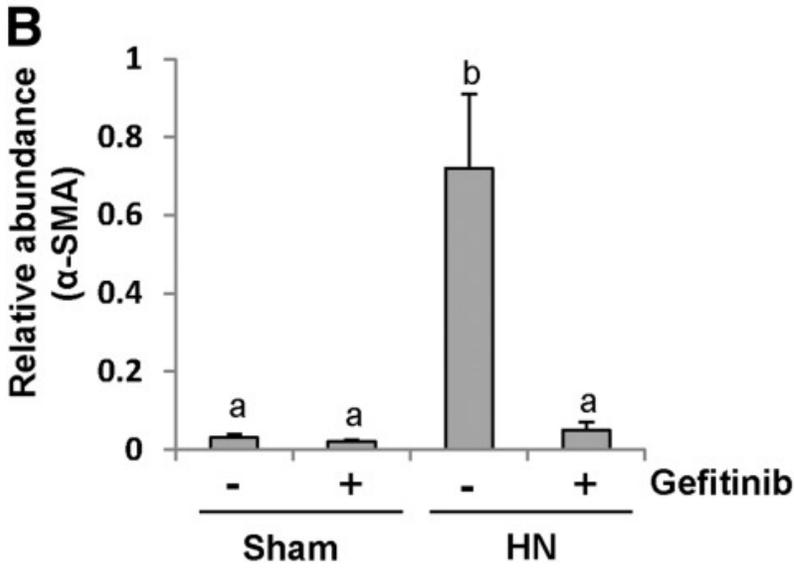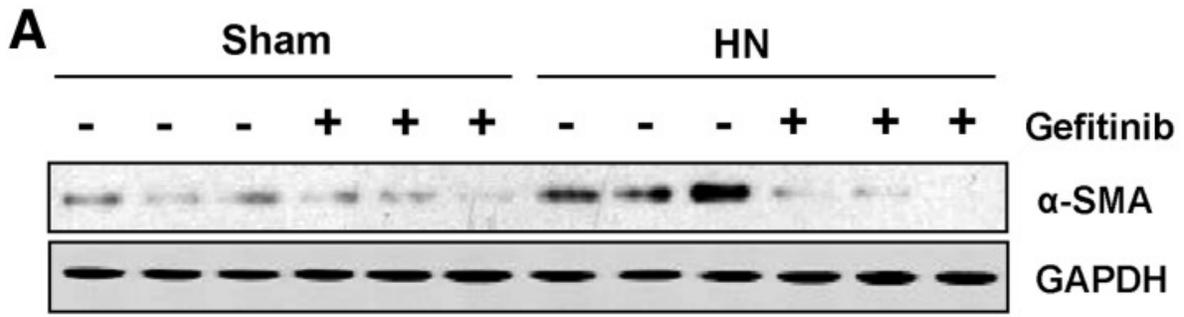
α-SMA Immunohistochemistry

Inhibition of EGFR blocks expression of α-SMA in the kidney of hyperuricemic rats. (A) The kidney tissue lysates were subjected to immunoblot analysis with specific antibodies against α-SMA and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). (B) Expression level of α-SMA was quantified by densitometry and normalized with GAPDH. (C) Photomicrographs (original magnification, ×200) illustrate immunochemistry α-SMA staining of the kidney tissues. Data are represented as the mean±SEM. Means with different superscript letters are significantly different from one another ($P<0.05$).

Figure 7.
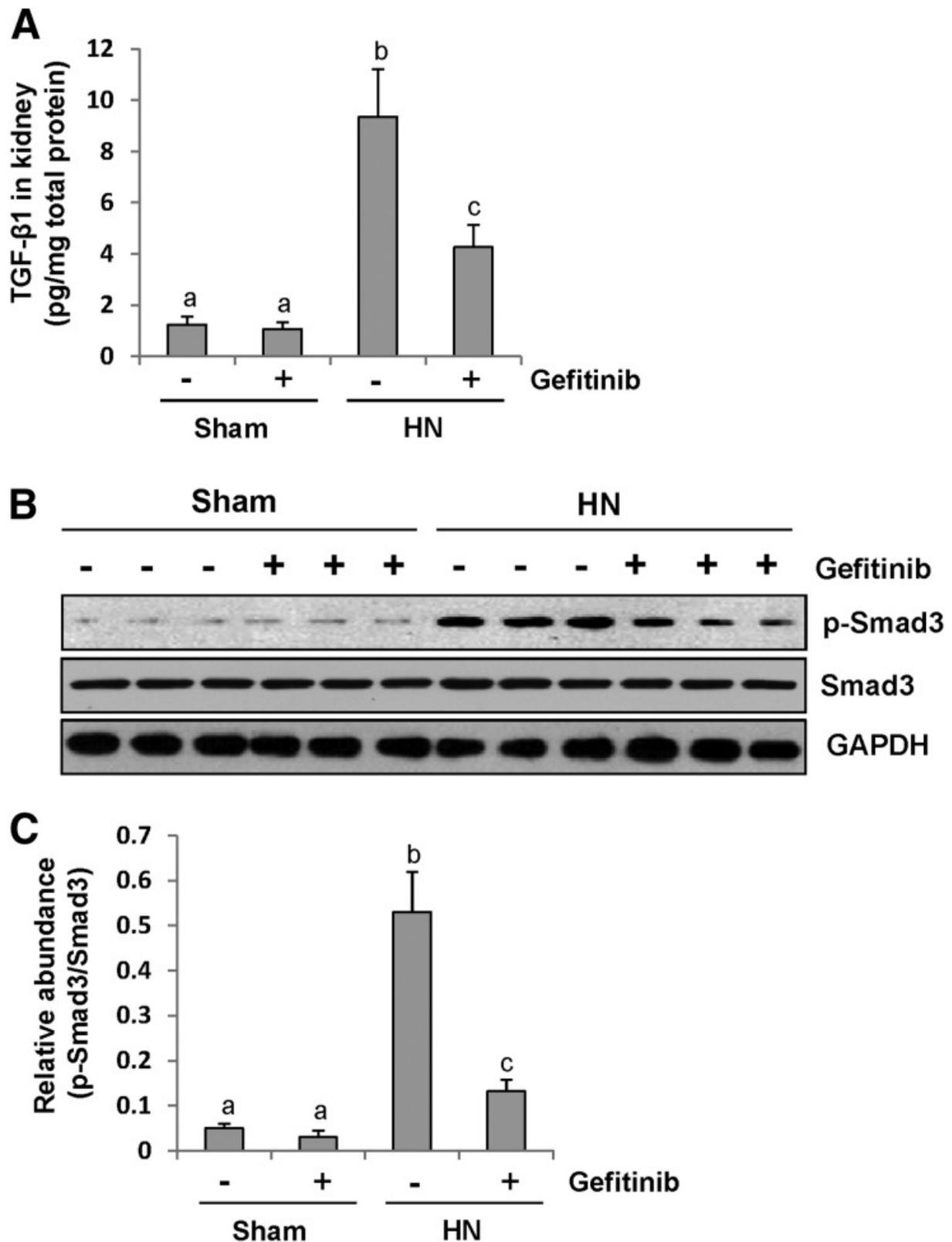
Pharmacologic blockade of EGFR activity suppresses TGF-β signaling in the kidney of hyperuricemic rats. (A) Protein was extracted from the kidneys of rats after feeding of the mixture of adenine and potassium oxonate with or without gefitinib treatment and subjected

to ELISA as described in the Concise Methods section. Protein expression level of TGF-β1 was indicated. (B) The kidneys were taken for immunoblot analysis of p-Smad3, Smad3, or glyceraldehyde 3-phosphate dehydrogenase. (C) Expression levels of p-Smad3 and Smad3 were calculated by densitometry and the ratio between p-Smad3 and Smad3 was determined. Data are represented as the mean±SEM. Means with different superscript letters are significantly different from one another ($P<0.05$).

Figure 8.
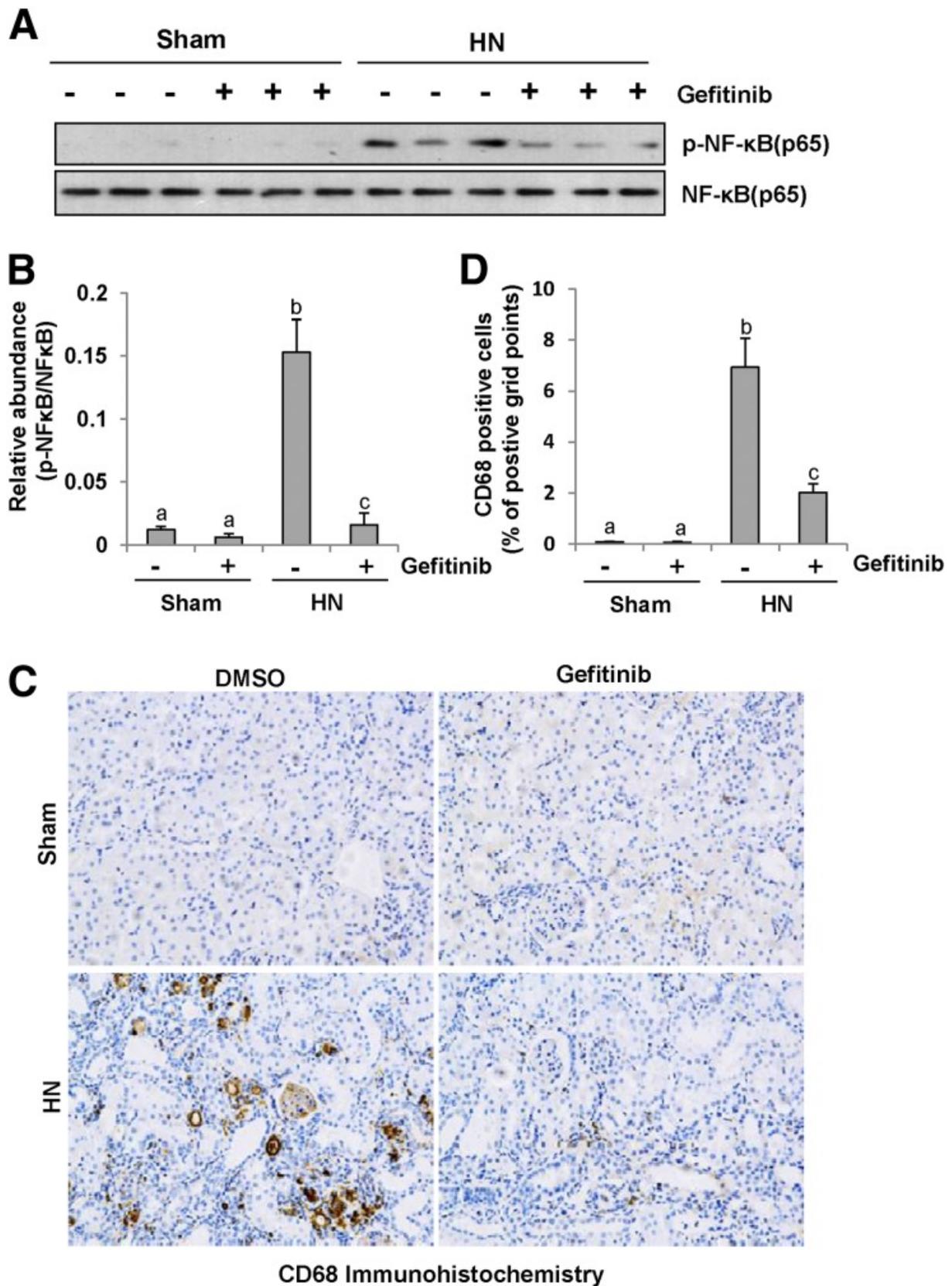
Gefitinib inhibits NF-κB pathway activation and macrophage infiltration in the kidney of hyperuricemic rats. (A) The kidney tissue lysates were subjected to immunoblot analysis with

specific antibodies against p-NF-κB (p65 and NF-κB (p65). (B) Expression level of p-NF-κB (p65) was quantified by densitometry and normalized with NF-κB (p65). (C) Photomicrographs (original magnification, ×200) illustrate CD68 staining of the kidney tissues. (D) CD68 staining graphic presentation of quantitative data. Data are represented as the mean±SEM. Means with different superscript letters are significantly different from one another ($P<0.05$).

Figure 9.
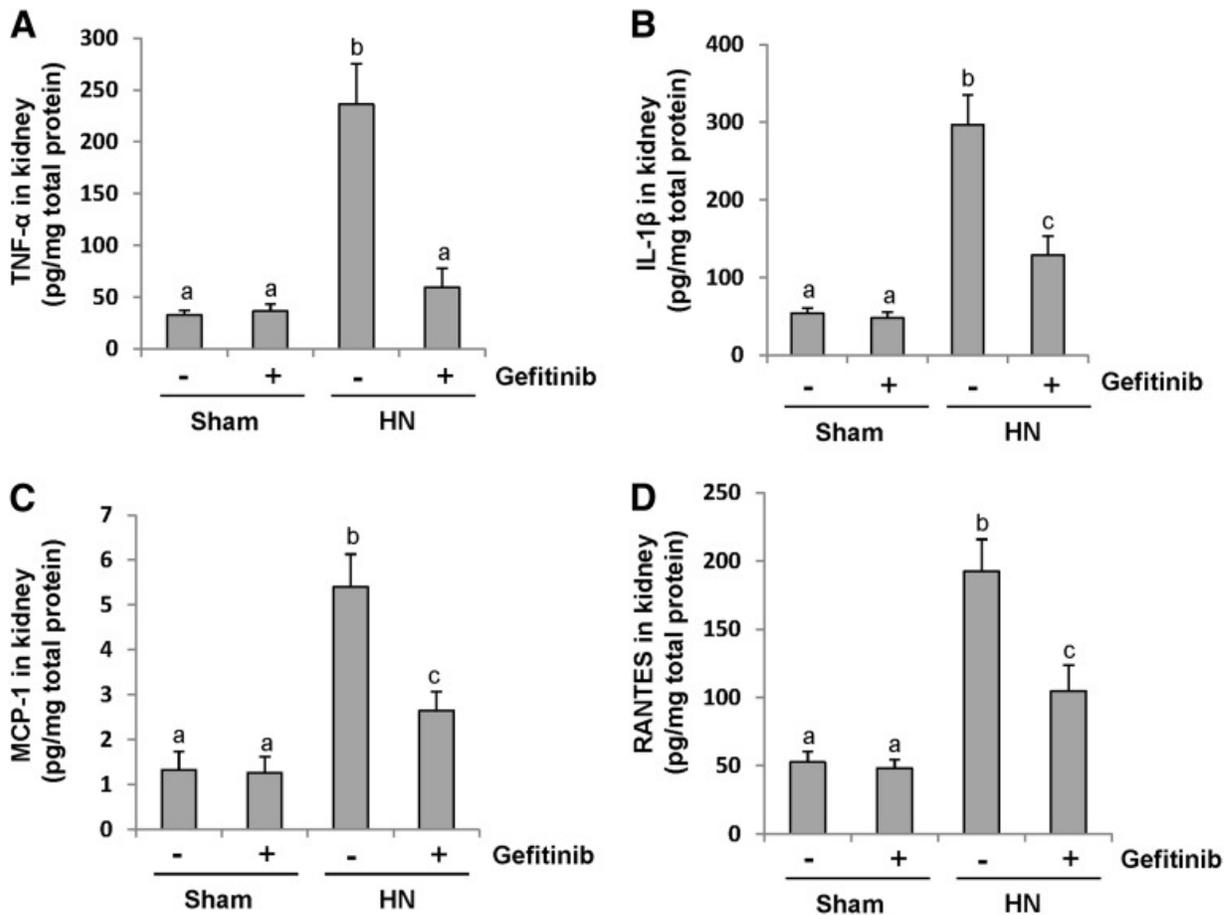
Gefitinib reduces the expression of TNF-α, IL-1β, MCP-1, and RANTES in the kidney of hyperuricemic rats. (A) Protein was extracted from the kidneys of rats after feeding of the mixture of adenine and potassium oxonate with or without gefitinib treatment and subjected to ELISA as described in the Concise Methods section. Protein expression level of TNF-α was indicated. (B) IL-1β. (C) MCP-1. (D) RANTES. Data are represented as the mean±SEM. Means with different superscript letters are significantly different from one another (P<0.05).

Figure 10.
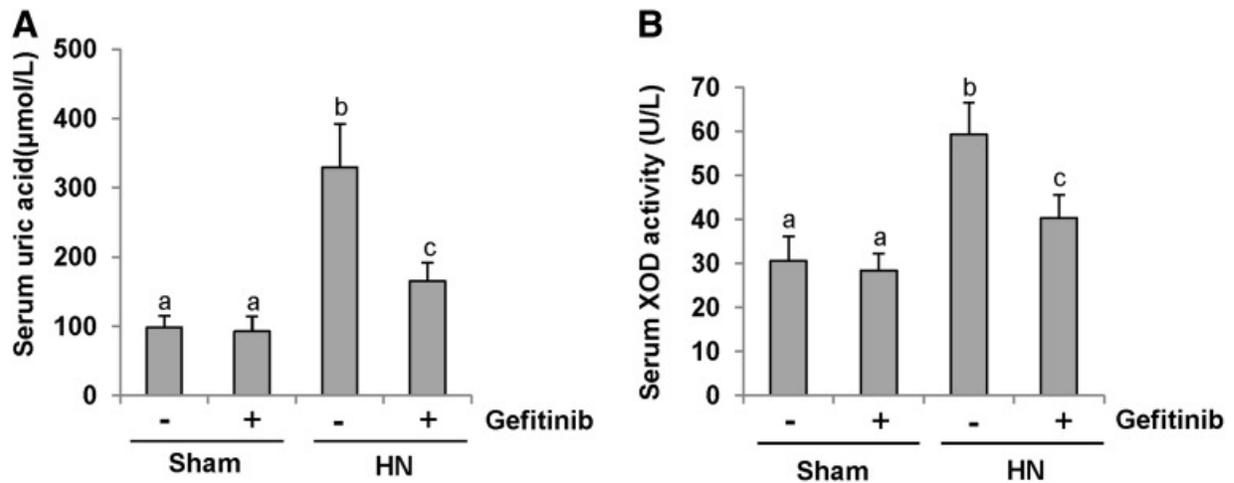
EGFR inhibition prevents a rise of serum uric acid and downregulates the activity of serum XOD in hyperuricemic rats. (A) Expression level of serum uric acid was examined using automatic biochemistry assay (P800; Modular). (B) Serum XOD activity was examined by XOD kit. Data are represented as the mean±SEM. Means with different superscript letters are significantly different from one another ($P<0.05$).

Figure 11.
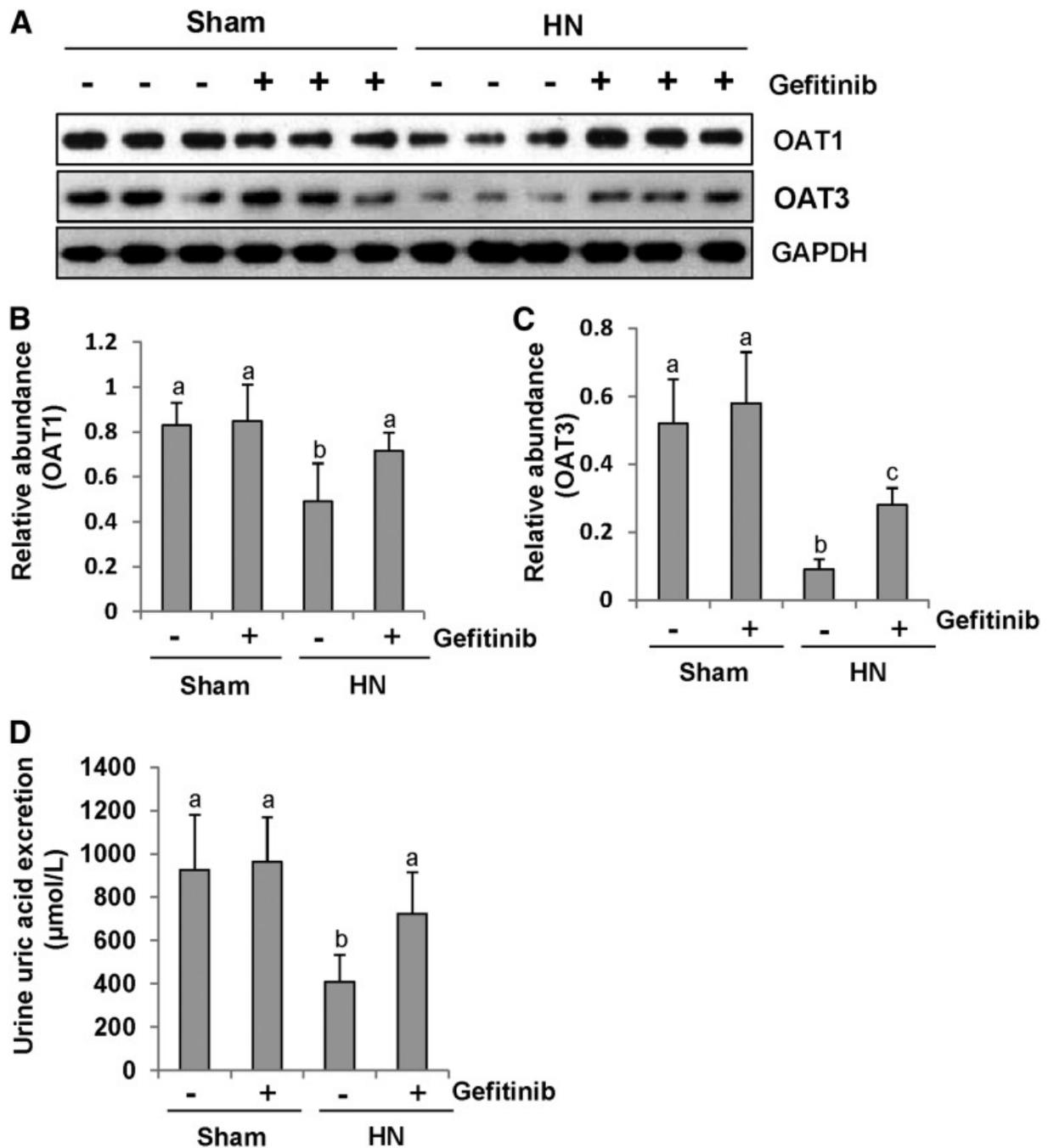
Gefitinib administration preserves the expression of two key urate transporters. (A) The kidney tissue lysates were subjected to immunoblot analysis with specific antibodies against OAT1, OAT3, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). (B) Expression level of OAT1 was quantified by densitometry and normalized with GAPDH. (C) Expression level of OAT3 was quantified by densitometry and normalized with GAPDH. (D) Excretion level of urine uric acid was examined by using automatic biochemistry assay. Data are represented as the mean±SEM. Means with different superscript letters are significantly different from one another ($P<0.05$).

Figure 12.
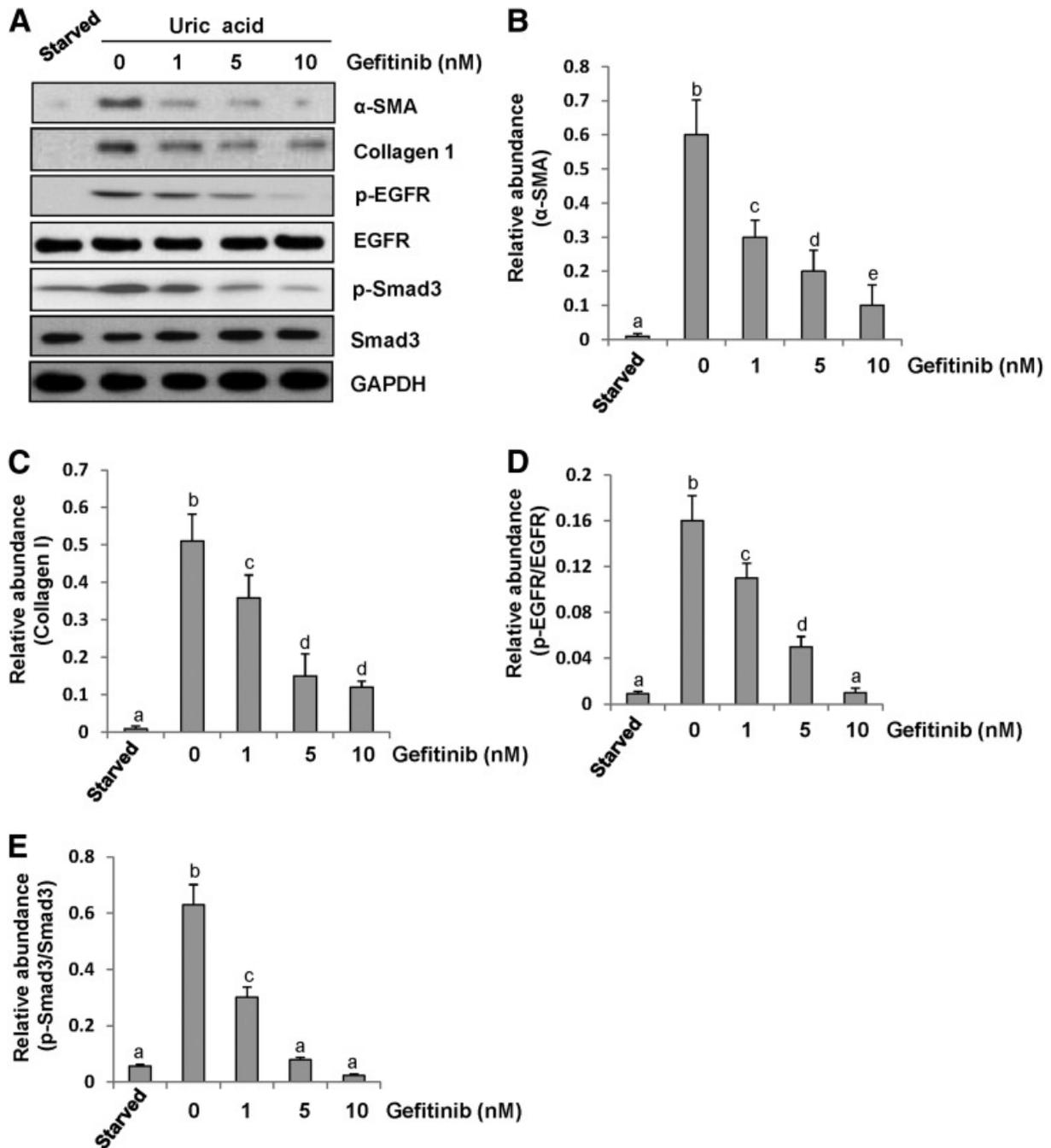
Gefitinib treatment inhibits uric acid–induced activation of cultured renal interstitial fibroblasts. Cultured NRK-49F cells were starved for 24 hours and then exposed to 800 μM uric acid for 36 hours in the absence or presence of gefitinib (0–10 nM). Cell lysates were subjected to immunoblot analysis using antibodies to α-SMA, collagen 1, p-EGFR, EGFR, p-Smad3, Smad3, or glyceraldehyde 3-phosphate dehydrogenase (GAPDH). (A) Representative immunoblots from three experiments. Expression levels of all these proteins were quantified by densitometry and expressed as means±SEM. α-SMA (B) and collagen 1 (C) were normalized with GADPH. The ratios of p-EGFR/EGFR (D) and p-Smad3/Smad3 (E) are

shown. Data are represented as the mean±SEM. Means with different superscript letters are significantly different from one another ($P<0.05$).

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