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Epidermal Growth Factor Receptor Inhibition Slows Progression of Diabetic Nephropathy in Association With a Decrease in Endoplasmic Reticulum Stress and an Increase in Autophagy

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Previous studies by us and others have reported renal epidermal growth factor receptors (EGFRs) are activated in models of diabetic nephropathy. In the present study, we examined the effect of treatment with erlotinib, an inhibitor of EGFR tyrosine kinase activity, on the progression of diabetic nephropathy in a type 1 diabetic mouse model. Inhibition of renal EGFR activation by erlotinib was confirmed by decreased phosphorylation of EGFR and extracellular signal-related kinase 1/2. Increased albumin/creatinine ratio in diabetic mice was markedly attenuated by erlotinib treatment. Erlotinib-treated animals had less histological glomerular injury as well as decreased renal expression of connective tissue growth factor and collagens I and IV. Autophagy plays an important role in the pathophysiology of diabetes mellitus, and impaired autophagy may lead to increased endoplasmic reticulum (ER) stress and subsequent tissue injury. In diabetic mice, erlotinib-treated mice had evidence of increased renal autophagy, as indicated by altered expression and activity of ATG12, beclin, p62, and LC3A II, hallmarks of autophagy, and had decreased ER stress, as indicated by decreased expression of C/EBP homologous protein, binding immunoglobulin protein, and protein kinase RNA-like ER kinase. The mammalian target of rapamycin (mTOR) pathway, a key factor in the development of diabetic

nephropathy and an inhibitor of autophagy, is inhibited by AMP-activated protein kinase (AMPK) activation. Erlotinib-treated mice had activated AMPK and inhibition of the mTOR pathway, as evidenced by decreased phosphorylation of raptor and mTOR and the downstream targets S6 kinase and eukaryotic initiation factor 4B. Erlotinib also led to AMPK-dependent phosphorylation of Ulk1, an initiator of mammalian autophagy. These studies demonstrate that inhibition of EGFR with erlotinib attenuates the development of diabetic nephropathy in type 1 diabetes, which is mediated at least in part by inhibition of mTOR and activation of AMPK, with increased autophagy and inhibition of ER stress.

In the industrialized world, diabetes mellitus represents the leading cause of end-stage renal disease (ESRD). Diabetic nephropathy is one of the major microvascular complications of diabetes and a major source of morbidity and mortality. The renal lesions are similar in type 1 and 2 diabetes (1). Both the incidence and prevalence of ESRD secondary to diabetes continue to rise. In the United States, >30% of patients receiving either dialytic therapy

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or renal transplantation have ESRD as a result of diabetic nephropathy, and >40% of the incident cases of ESRD are attributable to diabetes. Given the global epidemic of obesity in developed countries, an increasing incidence of diabetic nephropathy is being widely reported.

The underlying mechanisms predisposing to development and progression of diabetic nephropathy are an area of active investigation. Inadequate control of blood glucose and blood pressure undoubtedly contributes, and there is evidence for a genetic predisposition, although the modifier genes involved have yet to be conclusively identified. Studies in experimental animals have implicated a number of cytokines, hormones, and intracellular signaling pathways in either development or progression of diabetic nephropathy. Angiotensin II and transforming growth factor- β have been posited to play central roles in mediating the progressive glomerulopathy and tubulointerstitial fibrosis that characterize diabetic nephropathy. Blockade of angiotensin II production or signaling is the only specific intervention currently available for treatment of patients with diabetic nephropathy, and given that renin-angiotensin system inhibition can slow but usually not prevent progressive injury in diabetic nephropathy, it is imperative that additional, complementary therapeutic targets be identified. In previous studies, we reported that epidermal growth factor receptor (EGFR) phosphorylation increased in murine kidneys within 2 weeks of induction of diabetes by streptozotocin (STZ), which was inhibited by the EGFR tyrosine kinase inhibitor erlotinib. Erlotinib also inhibited renal extracellular signal-related kinase (ERK) activation and transforming growth factor- β expression and signaling in these animals (2). The current studies investigated whether prolonged EGFR signaling plays a role in mediating progressive glomerular and tubulointerstitial injury in diabetic nephropathy.

RESEARCH DESIGN AND METHODS

Cell Culture

Mesangial cells were isolated from wild-type mice crossed onto the immortal mouse as previously reported (3). The immortalized mesangial cells were propagated at 33°C in the presence of interferon- γ (100 IU/mL). The cells were cultured at 37°C without interferon- γ for 72 h before the experiments were performed to allow the conditionally immortalized mesangial cells to acquire a phenotype analogous to freshly isolated primary mesangial cells.

Animals

All protocols were approved by the Institutional Animal Care and Use Committee of Vanderbilt University. Wild-type and endothelial nitric oxide synthase (eNOS)^{-/-} mice on the C57BLKS/J (BKS) background were used. At 2 months of age, male mice received daily injections for 5 consecutive days of STZ (50 mg/kg i.p.) that was freshly prepared in 0.1 mol/L citrate buffer (pH 4.5). The onset of diabetes was evaluated by measuring fasting blood glucose. Mice were administered erlotinib (80 mg/kg) by daily gavage.

Measurements of Blood Glucose, Albuminuria, and Blood Pressure

Blood glucose was measured using a B-glucose analyzer (HemoCue, Lake Forest, CA) on blood samples after a 6-h fast initiated at 6:00 A.M. Blood was collected in conscious mice via the saphenous vein. Mice were trained three times in metabolic cages (Braintree Scientific, Braintree, MA) before 24-h urine collections. Briefly, a single mouse was put into a metabolic cage for 24 h and then returned to its original cage for 2 d before the next training period. The metabolic cages were moisturized to minimize the evaporation of urine sample when 24-h urines were collected. Urinary albumin and creatinine excretion was determined using Albuwell M kits (Exocell, Philadelphia, PA). Systolic blood pressure was measured in conscious, trained mice at room temperature using a tail-cuff monitor (BP-2000 Blood Pressure Analysis system; Visitech Systems).

Antibodies

The primary antibodies that were used for immunohistochemistry and immunoblotting included goat anti-human connective tissue growth factor (CTGF), goat anti-p-EGFR (1173), and rabbit anti-nitrotyrosine (marker of oxidative stress) from Santa Cruz Biotechnology; rabbit anti-murine collagen type I and type IV from Rockland Immunochemicals; rat anti-mouse F4/80 (marker of macrophages) from AbD Serotec; and rabbit anti-phosphorylated (p)-EGFR (Tyr¹⁰⁶⁸), p-EGFR (Tyr⁸⁴⁵), p-AMP-activated protein kinase α (AMPK α ; Thr¹⁷²), p-AMPK β 1 (Ser¹⁰⁸), p-ERK, p-Ulk1 (Ser³¹⁷), p-Ulk1 (Ser⁷⁵⁷), p-p70 S6 kinase (S6K; Thr³⁸⁹), p-mammalian target of rapamycin (mTOR; Ser²⁴⁴⁸), p-raptor (Ser⁷⁹²), p-eukaryotic initiation factor 4B (eIF-4B; Ser⁴²²), LC3A, LC3B, ATG12, beclin, protein kinase RNA-like endoplasmic reticulum kinase (PERK), binding immunoglobulin protein (BIP)/78-kDa glucose-regulated protein, p62, and mouse-anti C/EBP homologous protein (CHOP) from Cell Signaling Technology.

Immunohistochemistry

Animals were anesthetized with Nembutal (pentobarbital; 70 mg/kg i.p.) (Abbott Laboratories, North Chicago, IL), given heparin (1,000 units/kg i.p.) to minimize coagulation, and perfused with 3.7% formaldehyde, 10 mmol/L sodium m-periodate, 40 mmol/L phosphate buffer, and 1% acetic acid through the aortic trunk cannulated by means of the left ventricle (4). The fixed kidney was dehydrated through a graded series of ethanols, embedded in paraffin, sectioned (4 μ m), and mounted on glass slides. Immunohistochemical staining was carried out as in previous reports (5).

Immunoblotting

Kidney samples were homogenized with buffer containing 10 mmol/L Tris-HCl (pH 7.4), 50 mmol/L NaCl, 2 mmol/L EGTA, 2 mmol/L EDTA, 0.5% Nonidet P-40, 0.1% SDS, 100 μ mol/L Na₃VO₄, 100 mmol/L NaF, 0.5% sodium deoxycholate, 10 mmol/L sodium pyrophosphate, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μ g/ml aprotinin, and 10 μ g/ml leupeptin. The homogenate was

centrifuged at $15,000 \times g$ for 20 min at 4°C . An aliquot of the supernatant was taken for protein measurement with BCA protein assay kit (Thermo Scientific, Rockford, IL). The supernatant was then mixed with $2\times$ SDS sample buffer and heated to 95°C for 8 min, and the proteins were separated on different SDS gels under reducing conditions according to the size of interest of the probed protein and transferred to Immobilon-P Transfer Membranes (Millipore, Bedford, MA). The blots were blocked for 3 h with 50 mmol/L Tris-Cl (pH 7.4), 100 mmol/L NaCl, 0.5% Tween-20, containing 5% nonfat dry milk or 5% BSA (for phosphoproteins), followed by incubation for 16 h with primary antibody. The primary antibody was detected using corresponding horseradish peroxidase-conjugated secondary antibody and exposed on film using enhanced chemiluminescence (Amersham).

Histological Analysis

Periodic acid-Schiff-stained slides were evaluated for glomerular injury without knowledge of the identity of the various groups. A semiquantitative index was used to evaluate the degree of glomerular sclerosis. Each glomerulus on a single section was graded from 0–4, where 0 represents no lesion, and 1, 2, 3, and 4 represent sclerosis, involving

≤ 25 , 25–50, 50–75, or $>75\%$ of the glomerular tuft area, respectively (6).

Quantitative Analysis

Immunoblotting was quantitated with an IS-1000 digital imaging system (Alpha Innotech, San Leandro, CA). The immunoreactive band density of the protein of interest from vehicle-treated kidney was designated as 1 and that from erlotinib-treated kidney was expressed as fold of control. On the basis of the distinctive density and color of immunoreactivity of proteins of interest in video images, the number, size, and position of stained cells were quantified by using the BIOQUANT True Colors Windows system (R & M Biometrics, Nashville, TN) equipped with digital stage encoders that allow high-magnification images to be mapped to global coordinates throughout the whole section. Collagen I and IV levels were expressed as ratio of immunoreactive area versus glomerulus area, CTGF and nitrotyrosine levels were expressed as immunoreactive area versus kidney cortex area, and macrophage infiltration was expressed as cells per high-magnification field ($\times 160$). Sections from three regions of each kidney were analyzed, and the average was used as data from one animal sample (4).

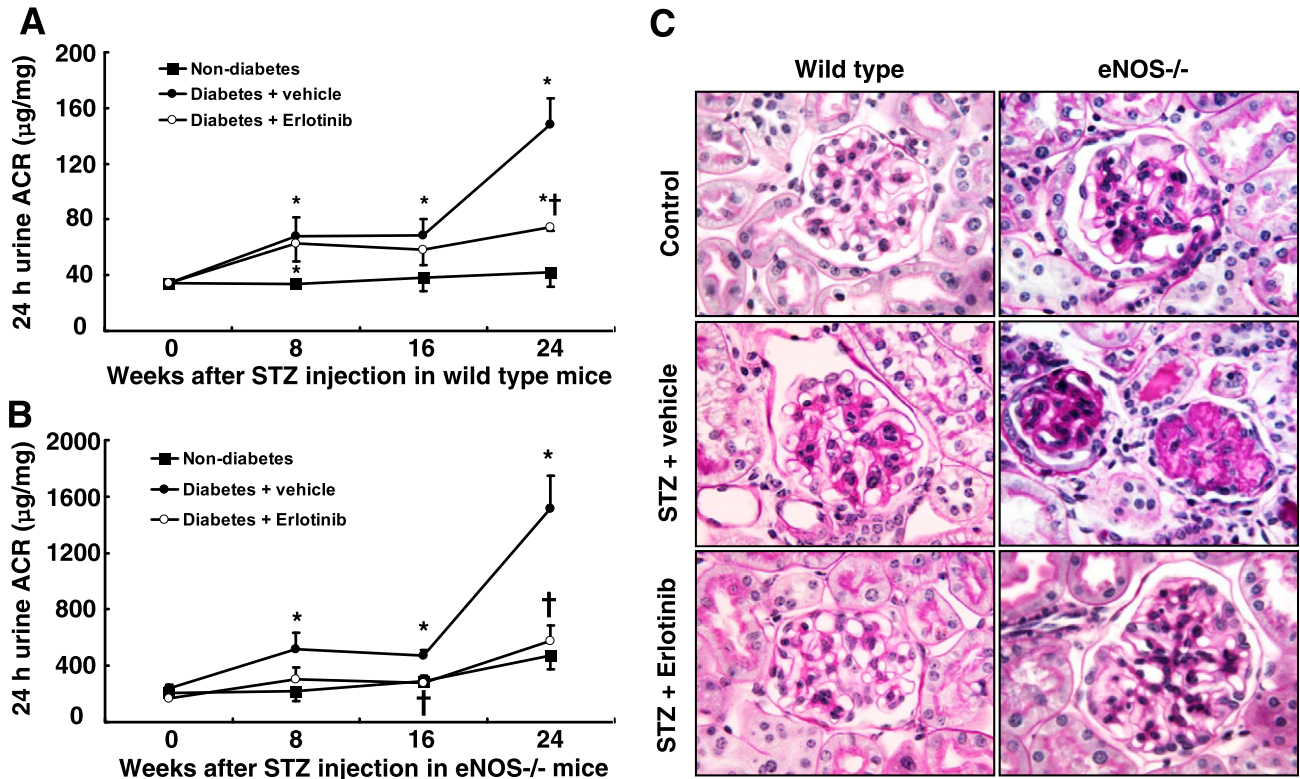


Figure 1—EGFR inhibition with erlotinib attenuated progression of diabetic nephropathy. Albuminuria, measured by 24-h urinary albumin/creatinine ratio (ACR), was markedly attenuated by erlotinib treatment in both STZ-wild-type (A) and STZ-eNOS^{-/-} mice (B). C: Periodic acid-Schiff staining indicated that mesangial expansion in STZ-wild-type mice and mesangial expansion, mesangiolytic, and glomerulosclerosis in STZ-eNOS^{-/-} mice were markedly attenuated with erlotinib treatment (original magnification $\times 400$). * $P < 0.05$ vs. corresponding nondiabetic mice; † $P < 0.05$ vs. corresponding STZ + vehicle group; $n = 4-6$.

Micrography

Bright-field images from the Leitz Orthoplan microscope with Optronics DEI-750 three-chip red-green-blue color video camera were digitized by the BIOQUANT TCW system (Bioquant Image Analysis Corporation, Nashville, TN) and saved as computer files. Contrast and color level adjustments (Adobe Photoshop; Adobe Systems) were performed for the entire image (i.e., no region- or object-specific editing or enhancements were performed).

Statistics

All data are presented as mean \pm SE. ANOVA and *t* tests were used for data analysis. A *P* value <0.05 was considered significant.

RESULTS

We used an STZ model of type 1 diabetes in mice. Wild-type diabetic mice on the BKS background (STZ-wild-type) developed mesangial expansion and moderate albuminuria after 24 weeks of diabetes (Fig. 1A and C). As we have previously reported (7), deletion of STZ-eNOS^{-/-} markedly exacerbated development of diabetic nephropathy (Fig. 1B and C). Compared with STZ-wild-type,

STZ-eNOS^{-/-} mice, killed 24 weeks after induction of diabetes, demonstrated a >10 -fold increase in albuminuria (albumin/creatinine ratio: $1,516 \pm 233$ vs. 148 ± 19 μ g/mg of creatinine; *n* = 4 in each group), marked mesangial expansion, mesangiolytic, and glomerulosclerosis (Fig. 1C).

The EGFR axis is activated in early diabetes (2), and inhibition of EGFR phosphorylation has been reported to attenuate diabetes-associated early kidney hypertrophy and glomerular enlargement (8). However, the effect of long-term EGFR inhibition on the development of diabetic nephropathy is unclear. We treated STZ-wild-type and STZ-eNOS^{-/-} mice with erlotinib, an EGFR tyrosine kinase inhibitor, from 2–24 weeks after initiation of diabetes. At the time of sacrifice, erlotinib treatment dramatically decreased EGFR phosphorylation in STZ-eNOS^{-/-} mice as indicated by immunoblotting and immunostaining (Fig. 2A and B). The activation of p44/p42 ERKs, a downstream signaling pathway activated by EGFR phosphorylation (9), was also markedly inhibited in erlotinib-treated STZ-eNOS^{-/-} kidney (Fig. 2C). Similar inhibition of EGFR-ERK signaling was

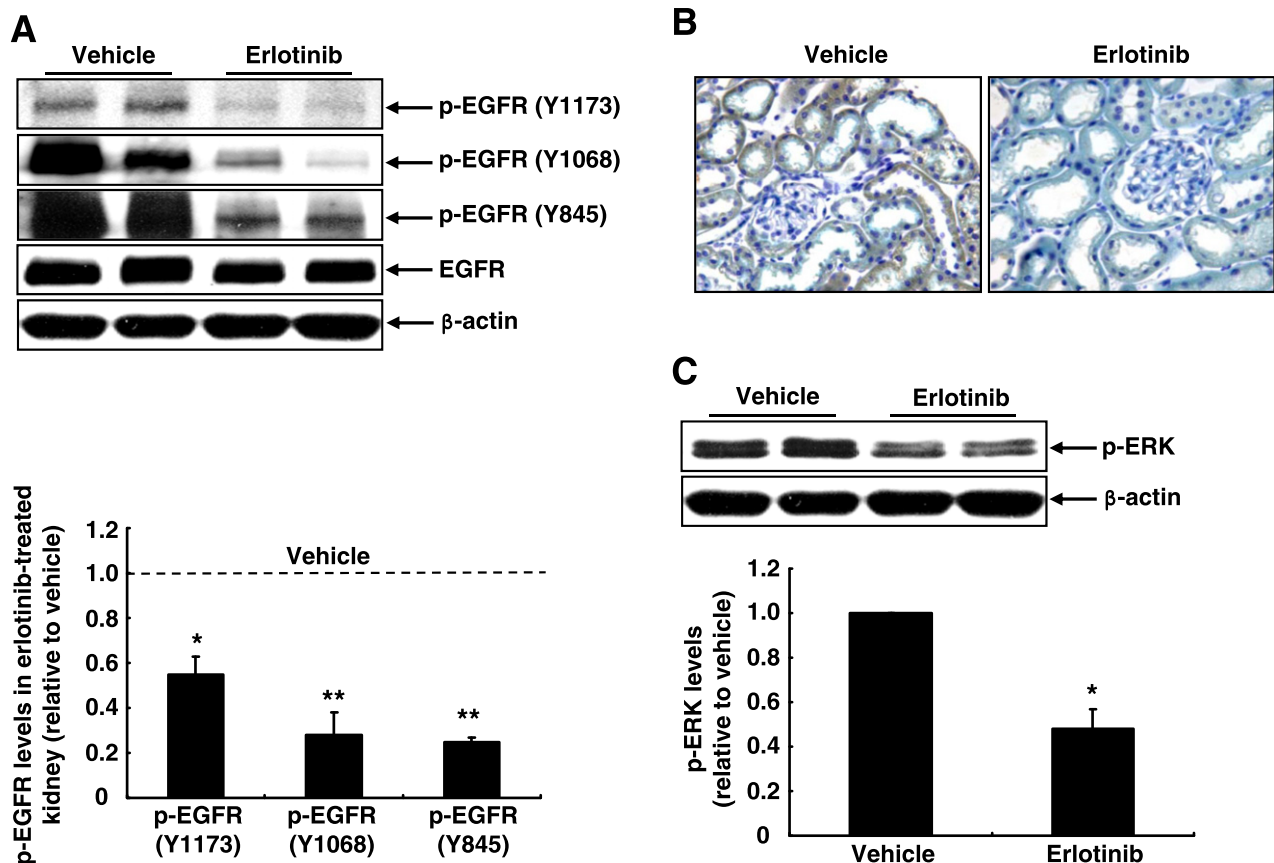


Figure 2—A: Erlotinib treatment markedly inhibited kidney EGFR phosphorylation at the indicated tyrosine residues in STZ-eNOS^{-/-} mice. B: Immunostaining of p-EGFR (Y1068) was primarily restricted to tubular epithelial cells in STZ-eNOS^{-/-} mice and reduced by erlotinib treatment (original magnification $\times 250$). C: Erlotinib also markedly inhibited kidney ERK1/2 phosphorylation in STZ-eNOS^{-/-} mice. **P* < 0.05 ; ***P* < 0.01 vs. vehicle group; *n* = 3 in vehicle group and *n* = 4 in erlotinib group.

found in erlotinib-treated STZ-wild-type kidney (data not shown).

In both STZ-wild-type and STZ eNOS^{-/-} mice, erlotinib inhibited diabetes-induced increases in albuminuria (Fig. 1A and B). Erlotinib attenuated mesangial expansion in STZ-wild-type mice (Fig. 1C) and markedly decreased the extent of glomerular pathology in STZ eNOS^{-/-} mice (glomerulosclerosis index: 0.50 ± 0.29 vs. 1.75 ± 0.25 in vehicle; *P* < 0.05; *n* = 4) (Fig. 1C). In STZ-eNOS^{-/-} mice, erlotinib treatment also led to significantly decreased

expression of markers of renal injury, including CTGF, collagen I, and collagen IV (Fig. 3A). Furthermore, erlotinib treatment markedly reduced renal oxidative stress and inhibited renal macrophage infiltration in STZ-eNOS^{-/-} kidney (Fig. 3B). However, erlotinib treatment did not affect hyperglycemia or blood pressure in either STZ-wild-type or STZ-eNOS^{-/-} mice (Table 1).

Recent studies have indicated a role for the unfolded protein response/ER stress in progression of diabetic nephropathy. We found that administration of erlotinib

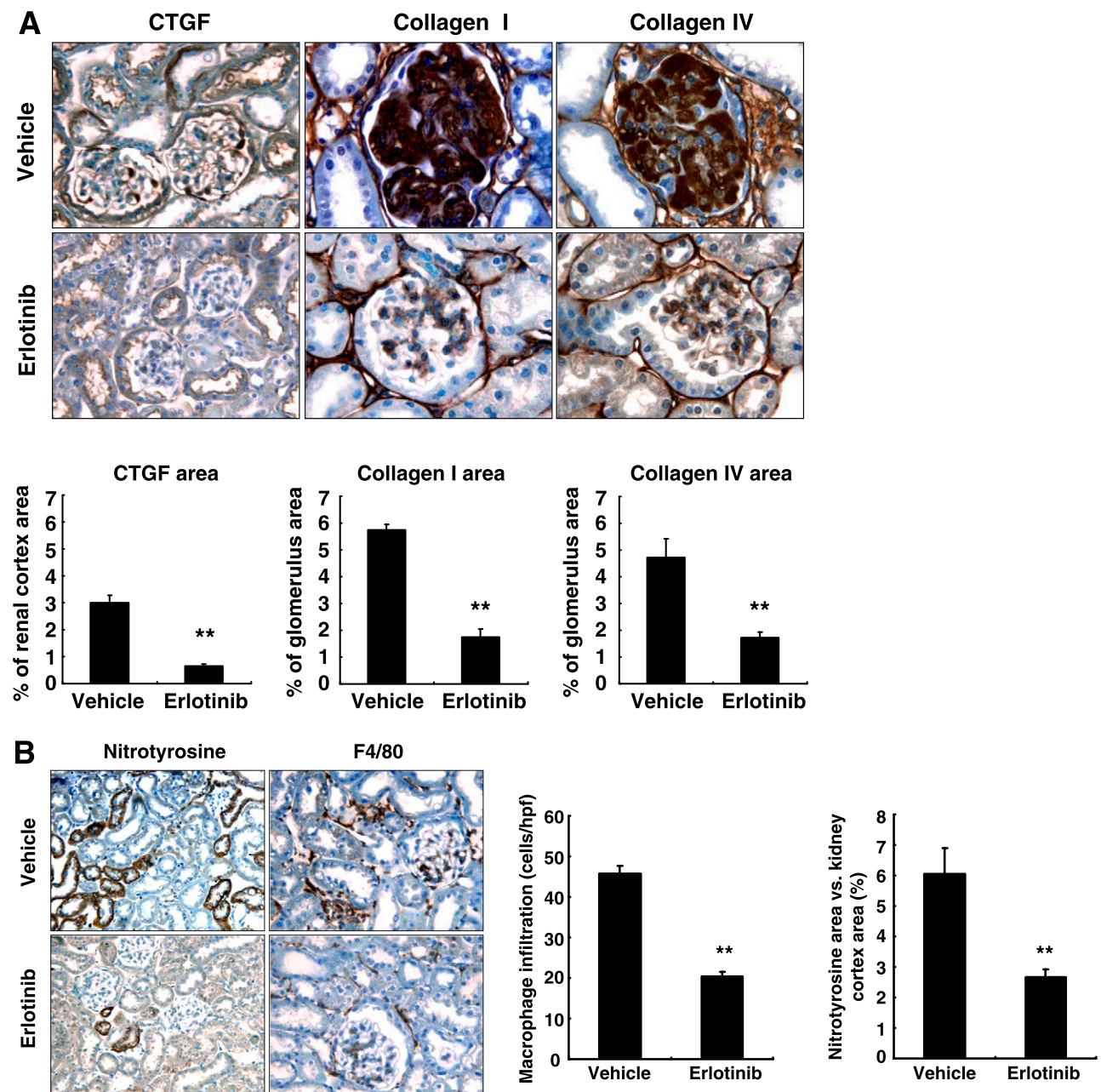


Figure 3—A: Erlotinib treatment markedly reduced renal expression of CTGF, collagen I, and collagen IV in STZ-eNOS^{-/-} mice. Original magnification: CTGF, ×250; collagen I and collagen IV, ×400. B: Erlotinib treatment also reduced kidney macrophage infiltration (indicated by F4/80 immunoreactivity) and oxidative stress (indicated by nitrotyrosine immunostaining) in STZ-eNOS^{-/-} mice. Original magnification: nitrotyrosine, ×160; F4/80, ×250. ***P* < 0.01 vs. vehicle group; *n* = 4. hpf, high-power field.

Table 1—Blood glucose and blood pressure in experimental mice

	Blood glucose (mg/dL)	SBP (mmHg)
Wild-type mice		
Nondiabetes	124 ± 11	111 ± 2
Diabetes + vehicle	386 ± 66**	96 ± 5*
Diabetes + EGFR I	363 ± 36**	95 ± 1*
eNOS ^{-/-} mice		
Nondiabetes	129 ± 7	151 ± 2
Diabetes + vehicle	383 ± 43**	125 ± 6*
Diabetes + EGFR I	439 ± 24**	130 ± 6*

n = 4 in each group. SBP, systolic blood pressure. **P* < 0.05 vs. nondiabetic group; ***P* < 0.01 vs. nondiabetic group.

to STZ-eNOS^{-/-} mice led to marked decreases in renal expression of CHOP, which has been associated with a predisposition for cell death (10) (Fig. 4A). Immunolocalization indicated that CHOP was primarily localized to tubule

epithelial cells and glomeruli in kidneys from STZ-eNOS^{-/-} mice (Fig. 5A). In addition, two other markers of ER stress, BIP and PERK, were also primarily localized to glomeruli, and their expression was markedly decreased with erlotinib treatment (Fig. 5A).

Stimulation of autophagy in the pancreatic islets of diabetic *Akita* mice has been reported to reduce ER stress (11). Therefore, we investigated whether erlotinib treatment might stimulate renal autophagy in STZ-eNOS^{-/-} mice. As indicated in Fig. 4B, erlotinib treatment significantly increased expression of components of the autophagy pathway, including ATG12 and beclin and decreased expression of p62. The stimulation of autophagy by erlotinib treatment was further confirmed by increased LC3A II levels. Immunolocalization indicated that the increased expression of LC3A was most intense in proximal tubules but was also detected in the glomeruli (Fig. 5B).

In yeast, the ATG1 kinase, which forms a complex with ATG13 and ATG17, regulates initiation of autophagy. In

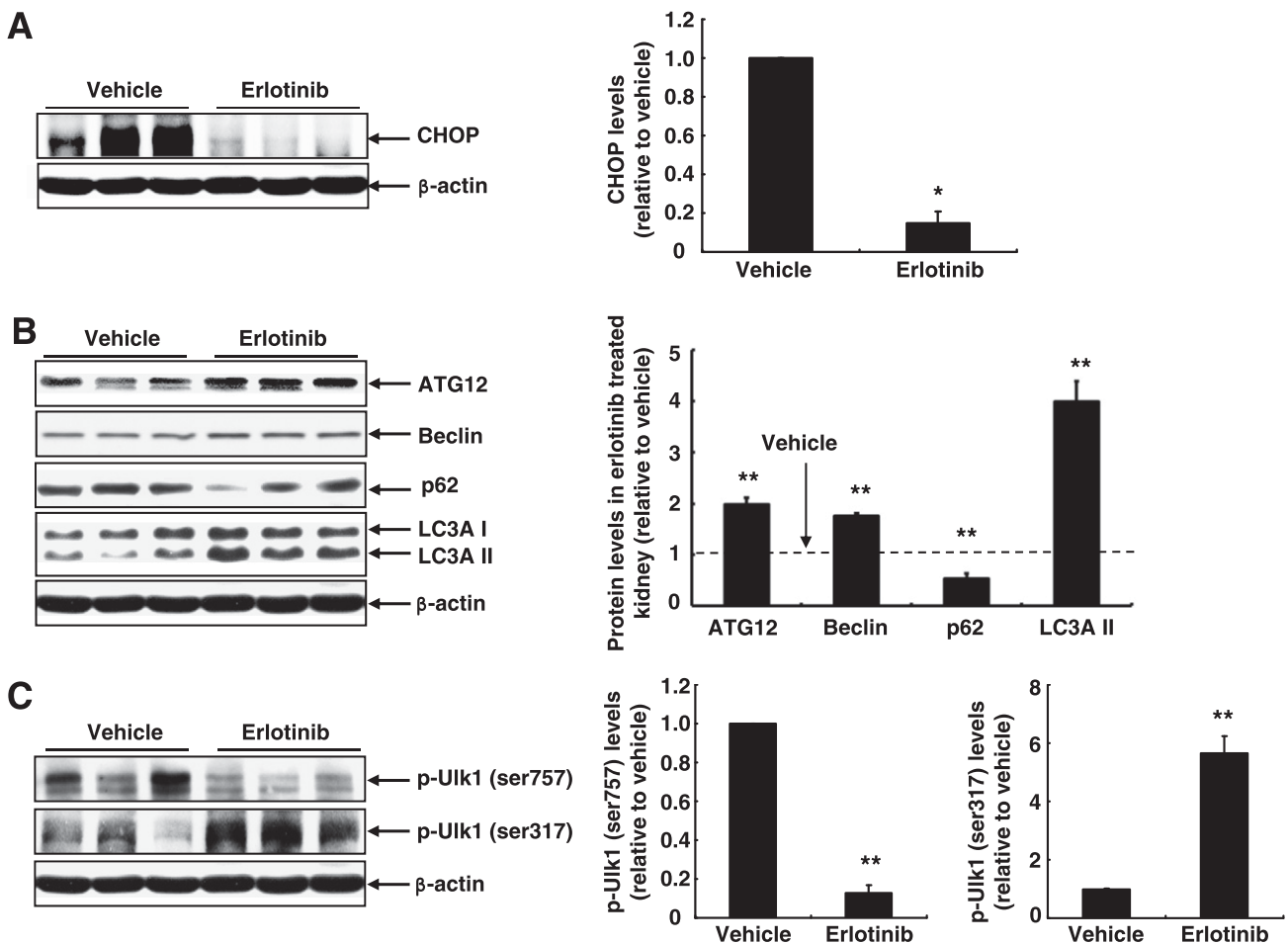


Figure 4—Erlotinib reduced kidney ER stress but stimulated the autophagic pathway in STZ-eNOS^{-/-} mice. A: Erlotinib inhibited kidney CHOP expression in STZ-eNOS^{-/-} mice. **P* < 0.05 vs. vehicle group; *n* = 3 in vehicle group and *n* = 4 in erlotinib group. B: Erlotinib increased expression of ATG12 and beclin and decreased expression of p62. Erlotinib-induced activation of autophagic pathway was indicated by increased expression levels of LC3A II, a membrane-bound form of LC3A produced during formation of autophagosomes. ***P* < 0.01 vs. vehicle group; *n* = 3–5. C: Erlotinib treatment increased Ulk1 phosphorylation on the AMPK phosphorylation site Ser³¹⁷, but decreased Ulk1 phosphorylation on the mTOR-dependent phosphorylation site Ser⁷⁵⁷. ***P* < 0.01 vs. vehicle group; *n* = 3 in vehicle group and *n* = 4 in erlotinib group.

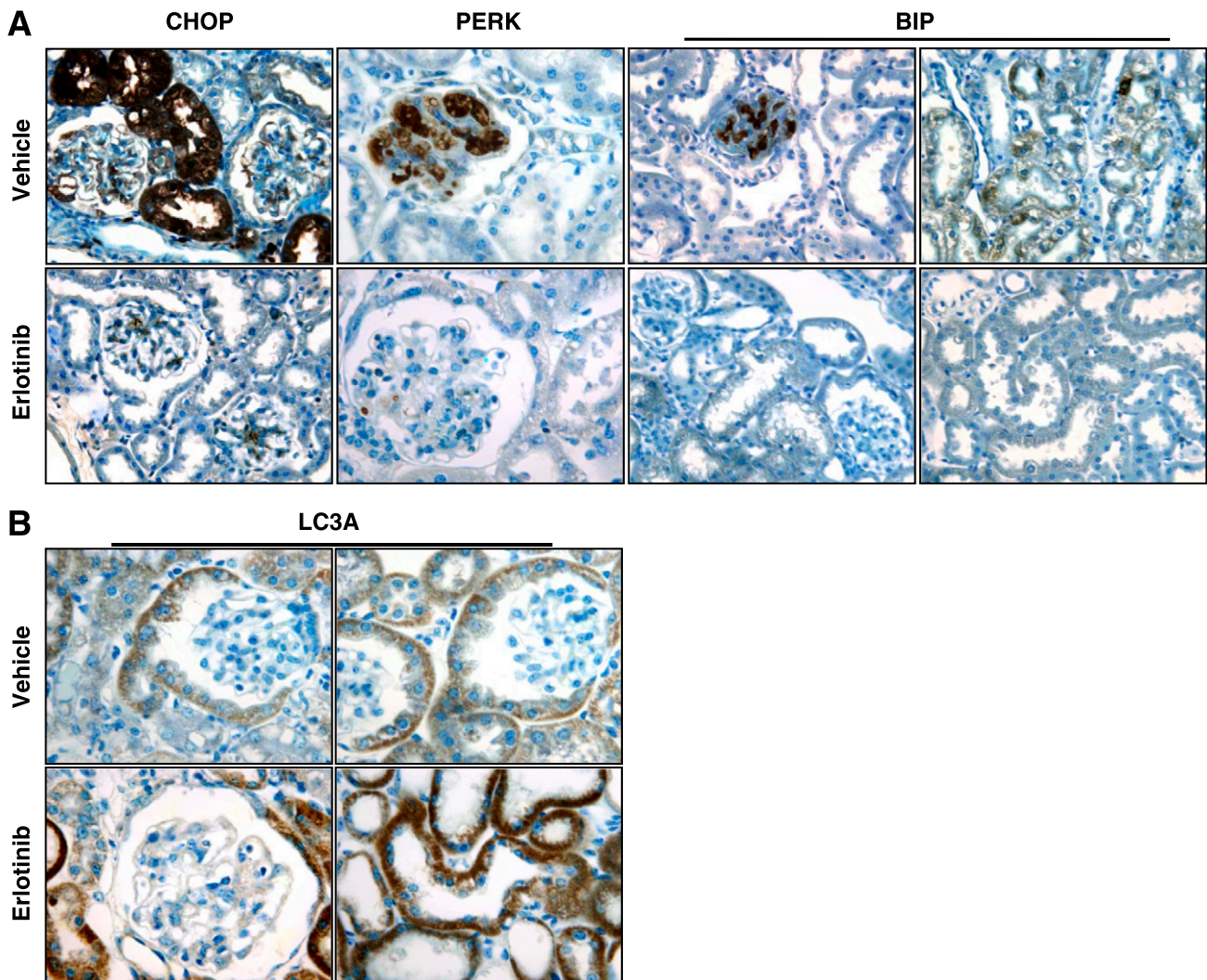


Figure 5—*A*: Erlotinib treatment decreased kidney ER stress, as indicated by decreased glomerular and tubule epithelial expression of CHOP, PERK, and BIP in STZ-eNOS^{-/-} mice. *B*: LC3A immunostaining was detected in tubular epithelial cells, but not in glomerulus, in vehicle-treated STZ-eNOS^{-/-} mouse kidneys. With erlotinib treatment, LC3A expression was detectable in glomerulus and was markedly increased in tubular epithelial cells. Original magnification: CHOP and BIP, $\times 250$; PERK and LC3A, $\times 400$.

mammals, the ATG1 homolog Ulk1 plays a similarly important role in autophagy initiation (12). Ulk1 has been reported to be activated by AMPK phosphorylation of Ser³¹⁷ and to be inhibited by mTOR phosphorylation of Ser⁷⁵⁷ (13). Kidney p-AMPK α levels were markedly decreased in STZ-eNOS^{-/-} mice compared with nondiabetic BKS mice, while p-mTOR and p-Ulk (Ser⁷⁵⁷) levels were markedly increased (fold of BKS control: p-AMPK α : 0.38 ± 0.04 , $P < 0.01$; p-mTOR: 2.20 ± 0.11 , $P < 0.01$; p-Ulk1 [Ser⁷⁵⁷]: 2.26 ± 0.025 , $P < 0.01$; $n = 3$ in each group). As indicated in Fig. 4C, erlotinib treatment in STZ-eNOS^{-/-} mice led to marked decreases in Ulk1 phosphorylation on Ser⁷⁵⁷ and marked increases in Ulk1 phosphorylation on Ser³¹⁷, suggesting that both mTOR and AMPK pathways might be involved in regulation of renal Ulk1 activity in erlotinib treated STZ-eNOS^{-/-} mice.

Consistent with the studies of Ulk1, phosphorylation of mTOR and its partner raptor were markedly lower in erlotinib-treated than vehicle-treated STZ-eNOS^{-/-} kidney (Fig. 6A). In addition, erlotinib treatment led to decreases in p-p70 S6K and p-eIF-4B, downstream targets of mTOR signaling (Fig. 6A). In contrast, erlotinib treatment led to increased AMPK kinase activity, as indicated by increased levels of p-AMPK α and p-AMPK β (Fig. 6B). Immunolocalization indicated that p-AMPK α , due to erlotinib treatment, was increased in both renal epithelial cells and glomeruli (Fig. 6C).

To investigate whether inhibition of EGFR activity affected the AMPK pathway and mTOR pathway in vitro, mesangial cells cultured in high-glucose medium (25 mmol/L) were treated with the EGFR inhibitor AG1478 (300 nmol/L). As indicated in Fig. 7A, AG1478 effectively inhibited EGFR phosphorylation. Inhibition of EGFR activity

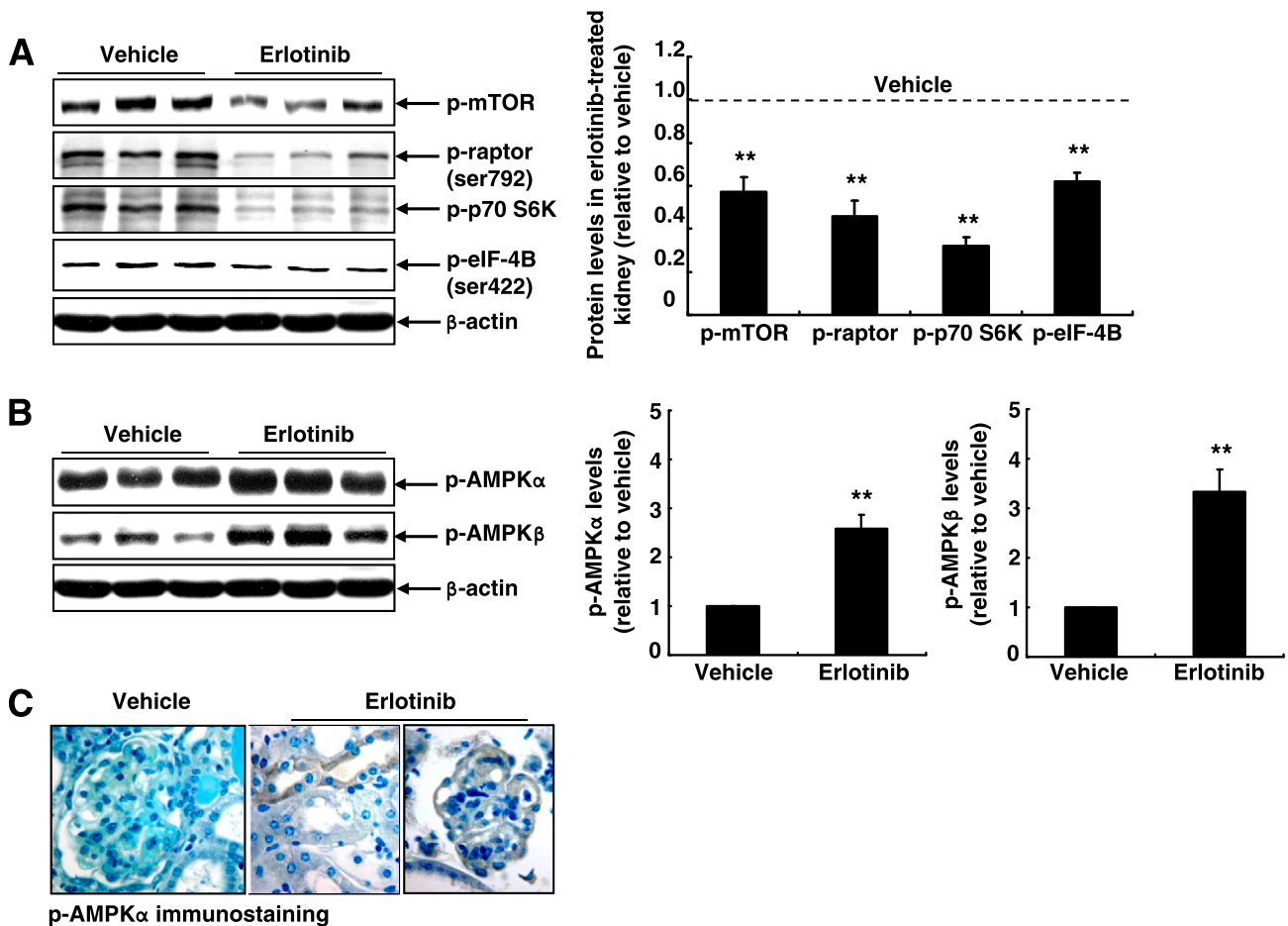


Figure 6—EGFR inhibition with erlotinib inhibited the kidney mTOR pathway but stimulated AMPK activation in STZ-eNOS^{-/-} mice. **A**: Erlotinib inhibited phosphorylation of mTOR, raptor, p70 S6K, and eIF-4B. **B**: Erlotinib stimulated phosphorylation of AMPK α and AMPK β . **C**: Erlotinib treatment increased kidney AMPK α activity in both epithelia and glomerulus (original magnification $\times 400$). $**P < 0.01$ vs. vehicle group; $n = 3$ –5.

with AG1478 markedly inhibited S6K activity and stimulated AMPK activity (Fig. 7B).

DISCUSSION

The present studies demonstrated that increased renal EGFR phosphorylation persisted for at least 24 weeks of STZ-induced diabetes. A pathologic role for this persistent EGFR activation was indicated by the effect of chronic treatment with the specific EGFR receptor tyrosine kinase inhibitor, erlotinib, which markedly decreased structural and functional evidence of progressive diabetic nephropathy. Furthermore, erlotinib treatment decreased mTOR activation and ER stress and increased both AMPK activity and expression of markers of autophagy.

The EGFR is a member of the family of ErbB receptors (ErbBs), which consists of four transmembrane receptors belonging to the receptor tyrosine kinase superfamily and includes EGFR (ErbB1/HER1), ErbB2/Neu/HER2, ErbB3/HER3, and ErbB4/HER4 (14). Among the four ErbBs, EGFR is the prototypical receptor, and receptor activation leads to phosphorylation on specific tyrosine residues within the

cytoplasmic tail. These phosphorylated residues serve as docking sites for a variety of signaling molecules, for which recruitment leads to the activation of intracellular pathways, including mitogen-activated protein kinase, Janus kinase/signal transducer and activator of transcription, src kinase, and phosphoinositide 3-kinase (PI3K) pathways, controlling cell proliferation, differentiation, and apoptosis (14–16). EGFR is widely expressed in mammalian kidney, including glomeruli, proximal tubules, and cortical and medullary collecting ducts (17–19), and expression increases in both glomeruli and tubules in response to diabetes. Given recent studies indicating tubule–glomerular interactions underlying diabetic nephropathy (20), it is likely that EGFR may be playing a pathogenic role in multiple cell types of the nephron.

Studies by our laboratory and others support a role for EGFR activation as an important mediator of renal repair following acute injury (9), but results by us and others have also ascribed a detrimental role to persistent EGFR activation in progressive renal fibrosis induced by subtotal nephrectomy (21), unilateral ureteral obstruction (22),

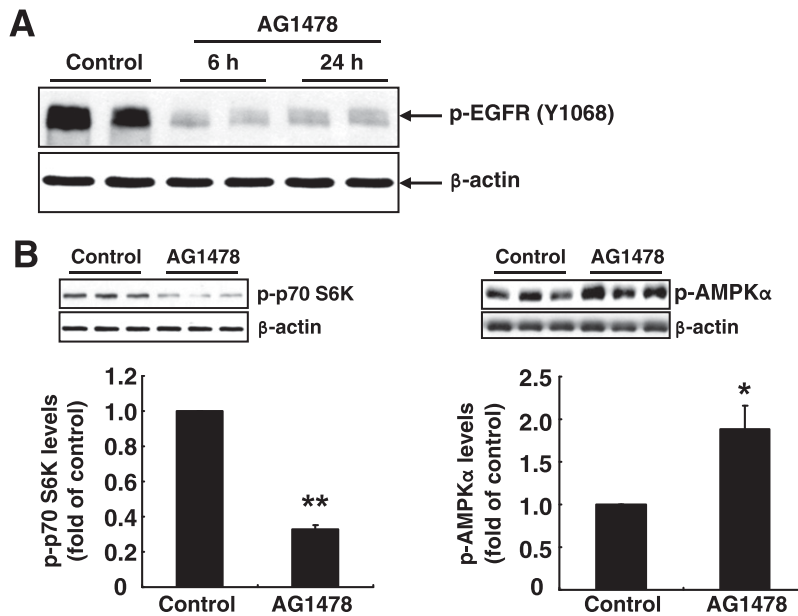


Figure 7—EGFR inhibition stimulated AMPK activity but inhibited S6K activity in mesangial cells. **A**: AG1478 (300 nmol/L) effectively inhibited EGFR phosphorylation in mesangial cells cultured in high-glucose medium (25 mmol/L). **B**: AG1478 treatment for 6 h led to inhibition of S6K activity and stimulation of AMPK activity. * $P < 0.05$; ** $P < 0.01$ vs. control group; $n = 3$.

renovascular hypertension (23), or renal injury induced by angiotensin II (2) or endothelin (24). The current studies indicate an important role for EGFR activation in mediating diabetic nephropathy as well. Our finding of a protective role for erlotinib concurs with a previous study in renin-transgenic rats, in which PKI 166, a structurally different EGFR inhibitor, was also found to inhibit diabetic nephropathy (25). In preliminary studies, we also found similar protection against progression of diabetic nephropathy with a third EGFR inhibitor, gefitinib.

Increased ER stress has been linked to the development of diabetic nephropathy, and chemical chaperones, which reduce misfolded proteins and thereby mitigate ER stress, have been shown to ameliorate STZ-induced diabetic nephropathy (26). The role of autophagy in diabetic nephropathy is still incompletely understood. Although some investigators have suggested that autophagy might play a pathogenic role (27), others have suggested that autophagy is protective (28). Podocytes have high basal levels of autophagy (29), and in this regard, we and others have recently reported that inhibition of podocyte autophagy by targeting autophagy-specific class III PI3K leads to progressive glomerulosclerosis (30).

mTOR activity increases in podocytes in diabetic mice and correlates with increased ER stress and progressive glomerulosclerosis (31). In addition to glomeruli, persistent mTOR activation has also been associated with apoptosis of renal tubule cells in diabetes (32). Renal mTOR activation in poorly controlled diabetes may result from a combination of AKT inhibition of tuberous sclerosis complex 2, hyperglycemia-induced AMPK inhibition, and

increased glucose uptake through glucose transporter 1, in which the resulting increased glycolysis and activation of GAPDH can lead directly to Rheb activation of mTOR by reducing Rheb binding to GAPDH (33,34). EGFR activation is a well-described mediator of mTOR activity through activation of the PI3K/AKT pathway (35,36). In addition, EGFR activation inhibits renal gluconeogenesis and stimulates glycolysis in proximal tubule (37,38) and has been reported to increase glucose transporter 1 expression in mesangial cells (39). A recent study has shown that erlotinib can activate AMPK and inhibit mTOR in small cell lung cancer cells with activating EGFR mutations (40), although the mechanism by which EGFR inhibits AMPK has yet to be determined.

Therefore, these studies provide strong evidence for an important pathological role of persistent EGFR receptor activation in the development and progression of diabetic nephropathy. They further indicate that the detrimental effects of EGFR activation result from increased ER stress and decreased autophagy secondary to persistent activation of the mTOR signaling pathway and inhibition of AMPK activity. That inhibition of EGFR activity by the EGFR kinase inhibitor erlotinib led to such marked amelioration of the observed nephropathic changes indicates that the direct inhibition of EGFR activity and/or inhibition of signaling pathways activated by the receptor may be viable targets for prevention of progressive kidney injury resulting from diabetes.

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Author Contributions. M.-Z.Z. and R.C.H. researched data and wrote the manuscript. Y.W. and P.P. researched the data. R.C.H. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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