Epiregulin promotes proliferation and migration of renal proximal tubular cells

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Zhuang S, Yan Y, Daubert RA, Schnellmann RG. Epiregulin promotes proliferation and migration of renal proximal tubular cells. Am J Physiol Renal Physiol 293: F219–F226, 2007. First published March 27, 2007; doi:10.1152/ajprenal.00082.2007.—Epiregulin is an epidermal growth factor (EGF) member that activates ErBb1 and ErbB4 homodimers and all possible heterodimeric ErbB complexes. Because its role in renal cell regeneration has not been investigated, we assessed the effect of exogenous epiregulin on regeneration of renal proximal tubular cells (RPTC) in primary culture. Epiregulin (10 ng/ml) was equivalent to EGF (10 ng/ml) in enhancing RPTC proliferation and migration. Epiregulin induced activation of the EGF receptor (EGFR), Akt, a downstream kinase of phosphoinositide 3-kinase 3-kinase (PI3K), and extracellular signaling-regulated kinase 1/2 (ERK1/2). Treatment with AG1478, a specific EGFR inhibitor, blocked phosphorylation of EGFR, Akt, ERK1/2, proliferation, and migration. Furthermore, inactivation of PI3K with LY-294002 blocked epiregulin-induced RPTC proliferation and, to a lesser extent, migration. However, blockade of ERK1/2 had no such effects. We suggest that epiregulin is a potent mitogen for renal epithelial cells and may contribute to renal regeneration through activation of EGFR and PI3/Akt pathways.

Acute renal failure (ARF) is the result of renal proximal tubular cell (RPTC) injury and death and can arise in a variety of clinical situations, especially after renal ischemia and drug or toxicant exposure. The return of kidney function after ARF depends on the ability of noninjured and sublethally injured RPTC to repair and regenerate (2). During the regenerative process, renal epithelial cells convert to the dedifferentiated phenotype and then migrate and proliferate to replace lost cells (2, 15). Finally, the regenerating cells redifferentiate and resume the structure and function of the intact epithelium (15). Although the mechanism by which renal epithelial cells regenerate after injury remains poorly understood, administration of various growth factors, including epidermal growth factor (EGF), has been reported to accelerate recovery in experimental models of ARF (3, 8, 12, 20). The EGF family consists of EGF, heparin-binding EGF-like growth factor (HB-EGF), transforming growth factor-α, epiregulin, amphiregulin, epigen, neuroregulin, and betacellulin (19). All of these ligands are synthesized as transmembrane precursors that are proteolytically cleaved to release biologically active, soluble growth factors (7, 19). These ligands bind and activate members of the ErbB receptor tyrosine kinase family, which includes the EGF receptor (EGFR), also known as ErbB1, along with ErbB2, ErbB3, and ErbB4 (19). Among these ErbB members, EGFR has been identified in the RPTC and mediates RPTC proliferation and recovery of renal function after ARF (28, 29).

EGFR is the primary receptor for all EGF-like ligands. However, epiregulin is unique in that it not only stimulates activation of EGFR but also ErbB4 and all possible heterodimeric ErbB complexes (11, 21). The broader receptor activity of epiregulin may result in a more potent mitogenic signal than EGF. For example, epiregulin was reported to be a more potent mitogen than EGF for rat primary hepatocytes, although its affinity for ErB was much lower than EGF (26). Furthermore, epiregulin has dual biological activities: it stimulates proliferation of fibroblasts, hepatocytes, smooth muscle cells, and keratinocytes, but it inhibits growth of several tumor-derived epithelial cell lines (22, 24, 25). Although the mechanism by which epiregulin exhibits different effects on normal and tumor cells is not clear, these unique functions may be beneficial in the development of epiregulin as an agent to promote renal regeneration.

There has been a concern that in vivo administration of exogenous growth factors might induce malignancy and promote growth of existing tumors in patients with tumors (14). Therefore, the efficacy of epiregulin in regulating renal regenerative responses (proliferation and migration) and the signaling mechanisms thereof needs to be examined.

Signaling by all EGF-like ligands is mediated by rapid tyrosine phosphorylation of their respective receptors. The phosphorylated tyrosine residues become binding sites for a group of cytoplasmic signaling proteins including phosphatidylinositol 3-kinase (PI3K) and the guanosine triphosphatase (GTPase)-activating protein Ras. Ras is the upstream activator of the extracellular signal-regulated kinase (ERK) pathway and initiates ERK activation through sequential activation of Raf and MEK. Activation of the PI3K/Akt and ERK pathways is required for migration and/or proliferation in a variety of cell types (4, 6, 9, 10, 23).

Our recent studies showed that RPTC display a remarkable capacity for EGFR-dependent autocrine proliferation and migration after different forms of injury (29). The aims of this study were to assess the potency and efficacy of exogenous epiregulin in regulating RPTC proliferation and migration and to deduce the signaling pathways for these actions.

Materials and Methods

Chemicals and reagents. Human-recombinant epiregulin was obtained from R&D Systems (Minneapolis, MN). LY-294002 and U-0126 were purchased from Cell Signaling Technology (Beverly, Massachusetts). AG1478, U-0126, and the MEK inhibitor were purchased from Calbiochem (La Jolla, CA). The MEK inhibitor was dissolved in DMSO, and the other inhibitors were dissolved in DMSO or ethanol as indicated in the figure legend. U-0126 was used at 10 μM, AG1478 was used at 10 μM, and LY-294002 was used at 10 μM.

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MA), AG-1478 and PPI were obtained from Biomol (Plymouth Meeting, PA). All other chemicals were purchased from Sigma (St. Louis, MO). In all experiments using pharmacological inhibitors, control cells were treated with an equivalent amount of vehicle. Antibodies to phospho-EGFR, phospho-Akt, Akt, and phospho-ERK1/2 were obtained from Cell Signaling Technology. Antibodies to ERK1/2 and EGFR were purchased from BD Laboratories (San Diego, CA) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively.

Isolation and culture of renal proximal tubules. Female New Zealand White rabbits were purchased from Myrtle’s Rabbitry (Thompson Station, TN). RPTC were isolated using the iron oxide perfusion method and grown in six-well tissue culture dishes under improved conditions as previously described (16). The culture medium was a 1:1 mixture of DMEM/Ham’s F-12 (without glucose, phenol red, or sodium pyruvate) supplemented with 15 mM HEPES buffer, 2.5 mM L-glutamine, 1 mM pyridoxine HCl, 15 mM sodium bicarbonate, and 6 mM lactate. Hydrocortisone (50 nM), selenium (5 ng/ml), human transferrin (5 μg/ml), bovine insulin (10 nM), and L-ascorbic acid-2-phosphate (50 μM) were added daily to fresh culture medium.

Boyden chamber migration assay. The Boyden chamber migration assay is based on a chamber with two medium-filled compartments separated (29). The number of cells in S-phase of the cell cycle was determined using flow cytometry after staining with propidium iodide as previously described (28). The percent of cells in S-phase was evaluated by 10.2 ± 0.32(246) on July 6, 2017. http://ajprenal.physiology.org/ Downloaded from

RESULTS

Epiregulin promotes RPTC proliferation following plating and mechanical injury. It was reported that epiregulin has a greater effect than EGF in stimulating cell proliferation and migration of other epithelial cells such as hepatocytes and keratinocytes (22, 26). We compared the ability of epiregulin to EGF in stimulating RPTC proliferation and migration. RPTC were cultured in defined media until 40–50% confluent and then incubated with 0–20 ng/ml epiregulin or EGF for 24 h. The percent of RPTC in the S-phase of the cell cycle was used as a marker of proliferation. Epiregulin and EGF increased RPTC proliferation in a concentration-dependent manner (Fig. 1A). At a concentration of 1 ng/ml or lower, neither epiregulin nor EGF increased the number of RPTC in the S-phase. Treatment with 10 ng/ml epiregulin or EGF resulted in ~34 and 32% of RPTC in the S-phase, respectively. Higher concentrations of epiregulin or EGF (20 ng/ml) did not further increase RPTC proliferation.

We then examined the effect of epiregulin and EGF at their optimum concentration (10 ng/ml) on RPTC proliferation after mechanical injury. Mechanical injury was initiated and the remaining RPTC were allowed to proliferate in the absence or presence of epiregulin or EGF. Approximately 9% of RPTC were in the S-phase 24 h subsequent to mechanical injury and treatment with epiregulin led to 17% RPTC in the S-phase, and this was equivalent to the degree of proliferation induced by EGF (15%; Fig. 1B). These data suggest that epiregulin and EGF have equivalent effects on RPTC proliferation after plating and mechanical injury.

Epiregulin promotes RPTC migration. We also compared the effect of epiregulin and EGF on RPTC migration using a Boyden chamber assay. As shown in Fig. 2A, treatment with epiregulin increased the numbers of RPTC that migrated from the upper surface to the underside of the transwell membrane. This migratory effect occurred in a concentration-dependent manner, and 10 ng/ml epiregulin and EGF equivalently induced a 300% increase in RPTC migration compared with controls (Fig. 2B). A greater concentration (20 ng/ml) of epiregulin or EGF did not further increase RPTC migration. These data reveal that epiregulin is equivalent to EGF in stimulating RPTC migration.

Inhibition of EGFR blocks epiregulin-induced proliferation and migration. To determine the importance of EGFR signaling in RPTC proliferation and migration after plating and mechanical injury, the EGFR-specific inhibitor AG1478 was used. Treatment of cells with AG1478 decreased RPTC proliferation in the absence and presence of epiregulin after plating and mechanical injury (Fig. 3).

To determine the effect of EGFR inhibition on RPTC migration induced by epiregulin, both wound healing and Boyden

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chamber assays were used. After wounding, RPTC adjacent to the wound flattened, elongated, and migrated to partially cover the wound area at 24 h subsequent to mechanical injury (Fig. 4, A and B). Exogenous epiregulin enhanced RPTC migration and wound closure, and this was blocked by AG1478.

In the Boyden chamber assay, epiregulin exposure increased the number of RPTC that migrated from the upper surface to the underside of the transwell membrane by 3.2-fold and this increase in migration was completely blocked by AG1478 (Fig. 4, C and D). These data reveal that EGFR is required for epiregulin-stimulated RPTC proliferation and migration.

Epiregulin induces EGFR phosphorylation. To determine the effect of epiregulin on EGFR phosphorylation, we performed immunoblot analysis using an antibody against the phosphorylated Tyr1068 of EGFR. Total EGF receptor content was measured using immunoblot analysis coupled with an antibody that recognizes EGFR independent of its phosphorylation state. Treatment of RPTC with epiregulin increased EGFR phosphorylation within 5 min and was persistent at least

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Fig. 1. Epiregulin (EPI) promotes renal proximal tubular cell (RPTC) proliferation after plating and mechanical injury. A: primary RPTC were cultured to 40–50% confluence and then incubated with different concentrations of EPI or epidermal growth factor (EGF) for 24 h. B: confluent monolayers of RPTC were scraped to create mechanical injury and then incubated for 24 h in the absence or presence of 10 ng/ml EPI or 10 ng/ml EGF. The number of RPTC in the S-phase of the cell cycle was determined. Data are expressed as means ± SE, n = 4. Bars with different letters are significantly different from each other (P < 0.05).

Fig. 2. EPI induces RPTC migration. A: RPTC were plated in the top chamber and grown to 40–50% confluence. EPI or EGF was then added to the bottom chamber at 10 ng/ml (A) or the indicated concentrations (B) and incubated for an additional 24 h. Cells that migrated to the underside of the filters were counted from the images of 9 randomly selected fields. Data are means ± SE of 3 independent experiments. Bars with different letters are significantly different from each other (P < 0.05).
Data are expressed as means ± SE, n = 5. Bars with different letters are significantly different from each other (P < 0.05).

60 min (Fig. 5A). Epiregulin-induced EGFR phosphorylation was completely blocked by AG1478 (Fig. 5B). Total EGFR levels did not change under these experimental conditions. These data reveal that epiregulin activates the EGFR in RPTC.

In additional experiments, immunoblot analysis was conducted to detect ErbB4 expression using an anti-ErbB4 antibody. However, we did not detect ErbB4 expression in RPTC after plating or mechanical injury (data not shown).

Epiregulin induces Akt and ERK phosphorylation. EGFR activation initiates multiple intracellular signaling pathways, and among them, the PI3/Akt and ERK pathways have been reported to be involved in proliferation and migration in RPTC and other cell types (17, 29). To determine whether epiregulin induced proliferation and migration through activation of these two pathways, we first examined the effect of epiregulin on Akt and ERK phosphorylation using immunoblot analysis and antibodies that recognize phosphorylated Akt at Ser473 (a target of PI3K) and ERK1/2. Akt and ERK1/2 phosphorylation was observed in RPTC within 5–10 min of epiregulin exposure and remained elevated through 120 min (Fig. 6A). A concentration-response study showed that epiregulin-induced Akt and ERK phosphorylation was barely detectable at 1 ng/ml, and maximal induction was observed at 10 ng/ml (Fig. 6B). In the presence of AG1478, epiregulin-induced phosphorylation of Akt and ERK was completely blocked (Fig. 6C). These data reveal that epiregulin induces PI3/Akt and ERK activation through EGFR activation.

PI3K mediates epiregulin-induced RPTC proliferation and migration. To determine the role of the PI3K and ERK pathways in mediating epiregulin-induced RPTC proliferation and migration, migration in RPTC and other cell types (17, 29). To determine whether epiregulin induced proliferation and migration through activation of these two pathways, we first examined the effect of epiregulin on Akt and ERK phosphorylation using immunoblot analysis and antibodies that recognize phosphorylated Akt at Ser473 (a target of PI3K) and ERK1/2. Akt and ERK1/2 phosphorylation was observed in RPTC within 5–10 min of epiregulin exposure and remained elevated through 120 min (Fig. 6A). A concentration-response study showed that epiregulin-induced Akt and ERK phosphorylation was barely detectable at 1 ng/ml, and maximal induction was observed at 10 ng/ml (Fig. 6B). In the presence of AG1478, epiregulin-induced phosphorylation of Akt and ERK was completely blocked (Fig. 6C). These data reveal that epiregulin induces PI3/Akt and ERK activation through EGFR activation.

DISCUSSION

Our recent studies show that autocrine signaling through EGFR is central to both the migration and proliferation that contribute to RPTC monolayer formation (29). In this manuscript, we provide data to indicate that epireglin, a new EGFR ligand, further stimulates activation of EGFR and subsequent Akt and ERK and that inactivation of EGFR blocked both epiregulin-induced proliferation and migration. Blockade of the PI3K/Akt pathway inhibited RPTC proliferation and, to a lesser extent, migration, whereas inhibition of ERK did not significantly affect these responses. We suggest that exogenous epiregulin promotes RPTC regeneration through activation of the EGFR-PI3K pathway.

Epiregulin is a pan-ErbB ligand and was reported to be more effective than EGF or TGF-α in promoting proliferation of other epithelial cells such as epidermal keratinocytes and hepatocytes (22, 24–26). Initially, we postulated that epiregulin may be a more potent and/or efficacious EGFR agonist than EGF in stimulating RPTC proliferation and migration. Interestingly, we observed that epiregulin and EGF were comparable in stimulating these biological responses (Figs. 1 and 2). The discrepancy between RPTC and other cell types with respect to the potency and efficacy of epiregulin remains unclear. It is possible that RPTC only express an EGF receptor that is not detected by our immunoblot analysis.
Fig. 4. Effect of EPI and AG1478 on RPTC migration after mechanical injury. A: confluent monolayers of RPTC were scraped to create mechanical injury and incubated with 10 ng/ml EPI for 24 h in the presence and absence of 10 μM AG1478. B: RPTC migration into the wounded area was quantified by measuring the migration of cells from the wound edge. Each bar represents means ± SE, n > 8. C: RPTC were plated in the top chamber and grown to 40–50% confluence. AG1478 was added to the culture and incubated for 1 h. EPI was added to the bottom chamber and incubated for an additional 24 h. D: cells that had migrated to the underside of the filters were counted from the images of 9 randomly selected fields. Data are means ± SE of 3 independent experiments. Bars with different superscripts are significantly different from one another (P < 0.05).

Fig. 5. EPI stimulates phosphorylation of EGFR and the effect of AG1478. RPTCs were cultured for 3 days and then treated with 10 ng/ml epiregulin for 0–60 min (A) or pretreated with 10 μM AG-1478 for 1 h and then exposed to epiregulin for 10 min (B). Cell lysates were subjected to immunoblot analysis using anti-phospho-EGFR antibody (Tyr 1068) or anti-EGFR antibody. Protein loading was monitored using total EGFR levels.
family member that is shared by EGF and epiregulin. In this regard, our previous studies showed that EGFR, a primary receptor for both EGF and epiregulin, is expressed in RPTC (29). Our current studies showed that inhibition of EGFR by AG1478 blocked epiregulin-induced proliferation and migration (Figs. 3 and 4). Although epiregulin also binds and activates ERbB4, we did not detect expression of ErbB4 in RPTC using immunoblot analysis (data not shown). Furthermore, the combination of epiregulin and EGF was not additive in RPTC proliferation (data not shown). These results suggest

Fig. 6. EPI induces activation of Akt and ERK1/2 and the effect of AG1478. RPTCs were cultured for 3 days and then treated with 10 ng/ml EPI for 0–120 min (A), treated with 0–20 ng/ml for 10 min (B), or pretreated with 10 μM AG-1478 for 1 h and then exposed to 10 ng/ml EPI for 10 min (C). Cell lysates were subjected to immunoblot analysis using antibodies to phospho-Akt, phospho-ERK1/2, total Akt, or total ERK1/2. Protein loading was monitored using total Akt and ERK1/2 levels.

Fig. 7. Effects of ERK1/2 and PI3K inhibition on EPI-stimulated RPTC proliferation after plating and mechanical injury. RPTCs were cultured for 3 days (A) or cultured until confluent and then scraped to produce mechanical injury (B) and then incubated with 10 ng/ml EPI for 24 h in the presence and absence of 20 μM LY-294002 and 20 μM U-0126. The number of RPTC in the S-phase of the cell cycle was determined. Data are expressed as means ± SE, n = 3. Bars with different superscripts are significantly different from one another (P < 0.05).
that EGFR may be the principal receptor for epiregulin in regulating RPTC proliferation and migration.

Our results revealed that epiregulin also promotes RPTC proliferation and migration after mechanical injury (Figs. 1B and 4). Enhancing these renal regenerative responses by epiregulin after injury suggests that epiregulin has the potential to accelerate renal regeneration after ARF. Although the growth promoting role of other EGFR ligands such as EGF and HB-EGF has been documented in renal epithelial cells (18), epiregulin displays dual biological functions in vitro: it stimulated proliferation of some epithelial cells such as hepatocytes, but inhibited growth of several tumor-derived epithelial cell lines (22, 24, 25). This unique role of epiregulin may render it more suitable for treatment of ARF, for which there has been concern that clinically used growth factors may promote tumorgenesis (14). In addition, epiregulin can induce mRNA synthesis for multiple EGF family growth factors including Hb-EGF and TGF-α in keratinocytes (22). Thus epiregulin may not only induce regenerative response via directly binding its receptors but may also effectively amplify its mitogenic signals through the production of other growth factors.

Epiregulin was originally isolated from the medium of transformed fibroblasts (15, 25). Its expression is relatively restricted: except for macrophages and placenta, other human tissues contain very low or no epiregulin transcripts. In the kidney, epiregulin was detected in mesangial cells using high-density oligonucleotide microarray analysis (13), but there are no reports about its expression in other kidney tissues. In our initial studies, we examined epiregulin protein expression in primary cultures of RPTC using immunoblot analysis and failed to detect epiregulin in the cell lysate. Furthermore, neutralization of epiregulin using an epiregulin neutralizing antibody did not inhibit RPTC proliferation (data not shown). These data support the hypothesis that epiregulin may contribute to renal regeneration through a paracrine or endocrine, but not autocrine, mechanism.

We examined the signaling pathways downstream from EGFR that are responsible for RPTC proliferation and migration induced by epiregulin. Our data revealed that the PI3K, but not the ERK pathway, mediates RPTC proliferation (Fig. 7). In addition, the PI3K pathway partially mediate RPTC migration (Fig. 8). Thus additional pathways contribute to migration. In this respect, it has been reported that the PI3K-Akt
pathway contributes partially to cell migration and that the main contribution is through EGFR-mediated recruitment of JAK1/2 to phosphorylate STAT1 and STAT3 in primary esophageal keratinocytes (1). Whether these two signaling molecules also mediate RPTC migration requires further investigation.

In conclusion, our findings suggest that exogenous epiregulin stimulates RPTC proliferation and migration by activating EGFR and that EGFR signaling to the PI3K-Akt pathway is required for RPTC proliferation and is partially necessary for migration.

REFERENCES