Albumin Activates ERK Via EGF Receptor in Human Renal Epithelial Cells

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Emerging clinical and experimental evidence strongly implicates proteinuria in the progression of kidney disease. One pathway involves the activation of NfκB by albumin, and it has been demonstrated that the activation of NfκB induced by albumin is dependent on mitogen-activated protein kinase ERK1/ERK2. To study the effect of albumin on gene expression, primary human renal tubular cells were exposed in vitro to albumin (1%) for 6 h, and gene expression profiling was performed with the human oligonucleotide microarray, U133A Affymetrix Gene Chip. In all, 223 genes were differentially regulated by albumin, including marked upregulation of the EGF receptor (EGFR) and IL-8. Accordingly, the authors sought to delineate the signaling pathway linking albumin to the EGFR and activation of ERK1/ERK2. It was found that albumin led to a dose- and time-dependent activation of ERK1/ERK2. Treatment with albumin led to EGFR phosphorylation, but the activation of ERK1/ERK2 was prevented by pretreatment of the cells with AG-1478, the EGFR kinase inhibitor, at a dose that inhibited EGF-induced ERK1/ERK2 activation. Exogenously administered reactive oxygen species (ROS) were found to activate ERK1/ERK2 via the EGFR and src tyrosine kinase activity and pretreatment of cells with the antioxidant N-acetylcysteine (NAC) and the NADPH oxidase inhibitor DPI abrogated albumin-induced activation of ERK1/ERK2. The src tyrosine kinase inhibitor, PP2, also inhibited the albumin-induced activation of ERK1/ERK2. Finally, pretreatment with AG-1478, the MEK inhibitor UO126, and NAC prevented the albumin-induced increase in IL-8 expression. The authors conclude that the EGF receptor plays a central role in the signaling pathway that links albumin to the activation of ERK1/ERK2 and increased expression of IL-8. Gene profiling studies suggest that there may be a positive feedback loop through the EGFR that amplifies the response of the proximal tubule cell to albumin. Taken together, these results suggest that the EGFR may be an important treatment target for kidney disease associated with proteinuria.


In increasing numbers of patients present each year with end-stage renal failure. Many of the diseases causing this loss of kidney function are characterized by proteinuria. Although proteinuria was originally thought to be merely a marker of disease severity, emerging clinical and experimental evidence strongly implicate direct toxicity of proteinuria in the progression of kidney disease (1–4).

Animal models of proteinuria induced by renal injury as well as protein-overload models suggest that excessive protein re-absorption stimulates the recruitment of inflammatory leukocytes, resulting in interstitial injury and fibrosis (5–9). Experiments involving in vitro models have offered insight into the mechanism(s) by which proteinuria may cause direct tubular toxicity. One pathway implicated in the deleterious effects of proteinuria involves the activation of the transcription factor NfκB by albumin, and subsequent translation of NfκB-dependent pro-inflammatory genes such as monocyte chemoattractant protein-1 (MCP-1) and RANTES (regulated upon activation, normal T cell expressed and secreted) (10,11), contributing to the recruitment of inflammatory cells and subsequent release of potentially harmful inflammatory cytokines. The activation of this pathway has been confirmed in human studies of proteinuric kidney disease (12).

In human proximal tubular cells, it has been demonstrated that the activation of NfκB induced by albumin is dependent on the generation of reactive oxygen species (ROS) by NADPH oxidase (13,14). The production of ROS can be attenuated with inhibitors of protein kinase C, NADPH oxidase, and, to some extent, by inhibitors of the mitochondrial respiratory chain (14). Recently, an important link has been made between the induction of NfκB by albumin and the mitogen-activated protein kinase (MAPK) extracellular signal-regulated kinase, ERK1/ERK2 (15). Inhibition of ERK
allows the activation of NFκB in albumin-treated murine tubular cell culture (16). The involvement of the MAPK pathways has been previously demonstrated in several in vivo and in vitro models of renal injury (17). However, the mechanisms responsible for the activation of ERK by albumin have not been fully elucidated.

We first used a high-throughput screen of broad-based gene expression to determine which genes were activated by albumin in primary cultured human renal tubular cells. EGFR and IL-8 were two genes found to be markedly upregulated by albumin. Accordingly, we sought to link the EGFR signaling and IL-8 expression by testing the hypothesis that albumin-induced activation of ERK1/ERK2 was dependent on the EGFR, and that inhibition of the EGFR kinase would attenuate the increase in IL-8 expression.

Materials and Methods

Cell Culture

Primary human renal epithelial cells were purchased from Clonetics (Cambrex, Walkersville, MD). The cells were cultured according to manufacturer’s instructions using Renal Epithelial Basal Medium with recommended supplements included in the REGM Singlequots Bulletkit (Cambrex). Passage 5 to 10 cells were used for all studies.

RNA Extraction and Microarray Analyses

Cells were grown to 80% confluence in 75 cm² flasks. On day 1, medium was changed in all flasks, 24 h before performing the experiment. The medium recommended by the cell supplier (REBM; Cambrex) was used and mixed with the recommended supplements. On the second day, medium was changed in all flasks in half; cells were exposed to medium alone in the other half; and cells were exposed to medium containing 1% bovine serum albumin. After 6 h of exposure, cells were washed with PBS and medium, and trypsinized. RNA was then extracted using the Qiagen RNeasy kit (Valencia, CA). The RNA obtained from cells grown in two flasks was used for each experimental sample, and each experiment was performed in quadruplicate (total 16 flasks for eight microarrays—four arrays from control cell RNA and four arrays from albumin-treated cell RNA). The RNA for each microarray was therefore pooled from cells obtained from two flasks. RNA quality was verified using the Agilent bioanalyzer (Agilent Technologies, Palo Alto, CA).

Synthesis of cDNA and array hybridization, washing, and scanning were performed by the Affymetrix Gene Chip core facility at The Centre for Applied Genomics at Toronto’s Hospital for Sick Children (Ontario, Canada) according to Affymetrix-recommended protocols (Santa Clara, CA). The human genome U133A Affymetrix Gene Chip was used. Hybridization and imaging were performed using an Affymetrix Fluidics station.

The microarray data were examined and visualized using Affymetrix Microarray Suite 5.0 software (18) in Bioconductor (19). The calculation of expression values from probe intensities and normalization of arrays was performed using the RMA method (20,21). Differential expression of genes across the two treatments was assessed using the significance analysis of microarrays (SAM) technique in Bioconductor (22). A set of 223 genes was selected to which SAM assigned an estimated false discovery rate of 0.000.

Cell Treatment for Inhibitor Studies

Cells were transferred to six-well plates and grown for 48 h to 72 h to 80% confluence. Before experiments, cells were incubated in basal medium in the absence of supplements overnight. On the day of experiment, cells were pretreated by incubation for 1 h at 37°C with one of the following inhibitors: β-cyclodextrin (5 mM), filipin III (2 μg/ml), or calphostin C (0.5 μM). Incubation with AG-1478 (0.2 μM) and PD158780 (Calbiochem Catalogue #513035) was performed for 20 min, N-acetylcysteine (NAC) was applied for 1 h (20 mM), diphenylene iodonium (DPI) was applied at the indicated times and concentrations, and PP2 and PP3 (Calbiochem) treatment was performed for 30 min at the doses indicated in figures. Pre-incubation with the MEK inhibitor, UO126, was performed for 2 h. Cells were then treated with albumin at the specified concentrations for the indicated time points at 37°C. BSA for the signaling experiments was purchased from BioShop. BSA (Catalogue #A3059), HSA (Catalogue #A3782), transferring (Catalogue #T18158), IgG (catalogue #I5406), EGF, filipin III, DPI, and NAC were purchased from Sigma (St. Louis, MO), and AG-1478, calphostin C, β-cyclodextrin, and PP2 were purchased from Calbiochem (San Diego, CA). Phospho-EGF receptor (Tyr 1068 and Tyr 845) antibody was purchased from Cell Signaling Technology (Beverley, MA).

Western Blot Analyses

After treatment with albumin, cells were washed twice with ice-cold PBS and transferred to microcentrifuge tubes for centrifugation. Cells were lysed in a commercial buffer (Cell Signaling, Beverly, MA) containing 20 mmol Tris-HCl (pH 7.5), 150 nM NaCl, 1% Triton X-100, 1 mmol Na3EDTA, 1 mmol EGTA, 1% Triton, 2.5 mmol sodium pyrophosphate, 1 mmol β-glycerophosphate, 1 mmol Na3VO4, 1 μg/ml leupeptin, and 1 mmol phenylmethyl sulfonyl fluoride. After 20 min of incubation in the lysis buffer, a 10-min centrifugation at 14,000g at 4°C was performed. The supernatant was collected, and a 6x SDS sample loading buffer containing 7 ml 4x Tris Cl/SDS (0.28M) pH 6.8, 3 ml glycerol, 1 g SDS, 1.2 mg bromophenol blue, and 0.93 g DTT (0.5M), or 1:20 B-mercaptoethanol was added to each sample and boiled for 5 min. Equal amounts of cell lysate (10 μg) were separated by a 10% SDS-PAGE gel and then transferred onto Immobilon polyvinylidene fluoride membrane (Millipore, Bedford, MA). After blocking with 5% milk, primary antibodies were applied (phospho-ERK 1:1000, total-ERK 1:1000, purchased from Cell Signaling, Beverly MA) and allowed to incubate overnight at 4°C. Signals were detected by use of an ECL Western blotting kit (Amersham Biosciences, Piscataway, NJ).

Fluorescence Imaging

Cells were transferred to six-well plates containing an autoclaved glass cover slip and then grown to 80% confluence. Cells were serum-starved overnight before treatment. After treatment, cells were washed three times with ice-cold PBS. Cells were then fixed with 4% paraformaldehyde for 30 min at room temperature. Cells were quenched for 10 min with 100 mmol glycine in PBS. Cells were then washed again three times with PBS, permeabilized with 0.1% Triton X-100 over 30 min, and then washed again. Blocking with 5% goat serum was performed at room temperature over 1 h, and cells were then incubated overnight at 4°C with the primary antibody (1:50, 1:100) in 1% goat serum and 0.01% Triton-X in PBS. After incubation, cells were washed five times with PBS, and then the secondary Alexa-488 goat anti-rabbit antibody (1:50) (Molecular Probes Eugene, OR) was applied for 1 h. After washing the cells with PBS and then distilled water, the cover slip was placed directly on a glass slide using DAKD fluorescence mounting medium. Cells were then imaged with a Zeiss confocal laser-scanning microscope (LSM 510, Dusseldorf, Germany).

MAPK Activity Assay

The assay was performed according to manufacturer’s recommendations (Cell Signaling, Beverly, MA). A monoclonal phospho-antibody to...
p44/42 (Thr202 and Tyr204) was used to immunoprecipitate active ERK1/ERK2 from cell lysate. The precipitate was then incubated with an Elk-1 fusion protein in the presence of ATP and kinase buffer, allowing active ERK1/ERK2 to phosphorylate Elk-1. Phosphorylation of Elk-1 at Ser383 was then measured by Western blotting using an antibody against phospho-Elk-1.

After treatment with the appropriate inhibitors and albumin, cells were washed twice with ice-cold PBS and centrifuged at 14,000 × g for 30 s. The supernatant was discarded, and cells were resuspended in commercial cell lysis buffer (Cell Signaling, Beverly, MA) and then incubated on ice for 20 min. The tubes were then centrifuged at 14,000 × g for 10 min at 4°C, and the supernatant was transferred to a fresh MC tube. Equal amounts of protein were then used, and immunoprecipitation of active MAPK was performed using immobilized phospho-ERK1/ERK2 monoclonal antibody overnight. The samples were then centrifuged at 3000 × g for 30 s and washed twice with 1x kinase buffer (included in kit) and kept on ice. The pellet was resuspended in 50 μl 1x kinase buffer supplemented with 200 μmol ATP and 4 μg Elk-1 fusion protein, and then incubated for 30 min at 30°C. The reaction was terminated with 10 μl 6x SDS sample buffer and boiled for 5 min. Samples were then vortexed and centrifuged again in a microcentrifuge for 2 min. Finally, the samples were loaded on 10% SDS gels and incubated overnight with a primary antibody against phospho-Erk-1 (1:1000). Signals were then detected using the ECL blotting kit as described previously.

Semiquantitative Reverse-Transcription PCR
RNA was extracted from cells using the Qiagen RNeasy kit (Valencia, CA); reverse-transcription was then performed using the Qiagen Omniscript RT kit, both according to product specifications. IL-8 and β-actin reverse-transcription PCR primers were purchased from R&D Systems (Minneapolis, MN), and cycle parameters were programmed according to manufacturer's specifications using the GeneAmp PCR System 4500 (Perkin Elmer, Wellesley, MA). IL-8 and β-actin were detected on a 1.5% agarose gel.

Real-Time PCR
RNA was extracted from cells as described. First-strand cDNA synthesis was performed using the Invitrogen Superscript First Strand Synthesis System, according to manufacturers' instructions, using random hexamers. Real-time PCR was performed using the Applied Biosystems Prism 7900 HT Sequence Detection System (Foster City, CA) in a 384-well plate. All reactions were performed in triplicate. The PCR reaction was performed using the Taqman Universal Mastermix (Applied Biosystems). Primers and probes were designed using referenced sequences available through PubMed Nucleotide (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi). Primer Express software (Applied Biosystems) was then used to align and select sequences. After a BLAST search, it was verified that there was no overlap for selected sequences with other known genes. Finally, selected forward and reverse primer sequences as well as probes were purchased from Integrated DNA Technologies (Coralville, IA). The sequences are selected and provided in Table 1. The quantitation of PCR product was performed by constructing a standardized curve using dilutions of a known concentration of cDNA obtained from RNA extracted from untreated cells.

Statistical Analyses
Statistical tests for densitometry results were performed using Microsoft Excel and displayed graphically using both Excel and Graphpad Prism.

Table 1. Primer sequences used for real-time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>F-Primer</th>
<th>R-Primer</th>
<th>Probe</th>
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<tbody>
<tr>
<td>EGF-R</td>
<td>5'-CGCAAGTGTAAGAAGTGC-3'</td>
<td>5'-CTGTACATTATGGAAGTTG-3'</td>
<td>5'-FAM-CCCGGAAAGTGTGAACG-3'</td>
</tr>
<tr>
<td>IL-8</td>
<td>5'-GCCTTCGATTTCGAGCT-3'</td>
<td>5'-TGCCTACGCACTTTAAG-3'</td>
<td>5'-FAM-TGTTGGAAGTGTCAGTAC-3'</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5'-GAAGATGGTGAAGTG-3'</td>
<td>5'-GAAGATGGTGAAGTG-3'</td>
<td>5'-56-FAM-CCAGCTTCCGTTCTACCC-3BH1-3'</td>
</tr>
</tbody>
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Figure 1. Real-time PCR confirming microarray results. Cells were exposed to 1% albumin for 6 h or control conditions. Albumin exposure results in increases of EGFR mRNA levels (A) and IL-8 mRNA levels (B). Values are expressed as relative units as they have been corrected for GAPDH concentration obtained from a standard curve constructed from untreated cells. The data are representative of three separate experiments. Values are mean ± SEM, *P<0.05.
Results

Gene Expression Profiling

RNA was extracted from human renal epithelial cells exposed to either medium alone or medium containing 1% albumin for 6 h. The RNA was then hybridized to an U133A Affymetrix Human Microarray Chip, and gene expression was analyzed as described. In total, 223 genes were determined to be differentially expressed when cells are exposed to albumin. Two of the genes that were found to be markedly upregulated were the EGF receptor (EGFR) and the pro-inflammatory cytokine, IL-8. The microarray analyses for EGFR and IL-8 mRNA levels were confirmed with real-time PCR. As shown in Figure 1A and 1B, there was a three-fold increase in EGFR mRNA levels and a 20-fold increase in IL-8 mRNA levels 6 hours after exposure to 1% albumin, respectively. Accordingly, subsequent studies were designed to link EGFR signaling to the effect of albumin on IL-8 expression.

Activation of ERK and Role of EGFR

Figure 2A demonstrates the activation (phosphorylation) of ERK1/ERK2 induced by 1% albumin in a time-dependent fashion. The maximal activation of ERK1/ERK2 occurred 5 min after exposure to albumin. Figure 2B shows that the activation of ERK1/ERK2 by albumin was also dose-dependent, and subsequent studies were performed with 1% albumin, similar to previously published dose-response experiments that examined the induction of NFkB by albumin (11). Figure 3 shows that the phosphorylation ERK1/ERK2 after treatment with HSA, BSA from Sigma (BSA1), and BSA2 from Bioshop was similar. In contrast, IgG and transferrin did induce the phosphorylation of ERK1/ERK2 in the cultured human kidney epithelial cells.
We then studied the effect of the EGFR kinase inhibitor, AG-1478, on the albumin-induced phosphorylation of ERK1/ERK2. Pretreatment with AG-1478, a selective inhibitor of the EGFR, markedly attenuated the phosphorylation of ERK1/ERK2, indicating a central role for the EGFR in the activation of ERK by albumin (Figure 4). Lipid rafts may be important in signal transduction events dependent on the EGFR, so we also studied the effect of the cell membrane cholesterol-depleting drugs, β-cyclodextrin and filipin III, on the albumin-induced activation of ERK1/ERK2. Both of these reagents attenuated the phosphorylation of ERK1/ERK2 by albumin (Figure 4). In addition, multiple PKC isoforms have been localized to caveolae, and we found that the PKC inhibitor, calphostin C, also attenuated albumin-induced ERK1/ERK2 phosphorylation.

ERK1/ERK2 activity was also measured by immunoprecipitating phospho-ERK, and determining the ability of the immunoprecipitate to phosphorylate the transcription factor, Elk-1. Elk-1 is a transcription factor that, when activated, binds the serum response element mediating gene transcription in response to a variety of stimuli (23–25). The phosphorylation of Elk-1 was detected by Western blot with an antibody specifically targeted against the phospho-Elk-1. Figure 5 shows that the phosphorylation of ERK1/ERK2 induced by albumin was associated with an increase in ERK1/ERK2 activity. ERK1/ERK2 activity is increased three-fold when cells are exposed to albumin, and this response was abrogated when the cells are pretreated with inhibitor of the EGFR AG-1478. In parallel with the Western blot analyses of phospho-ERK1/ERK2, β-cyclodextrin, filipin III, and calphostin C all decreased albumin-induced ERK1/ERK2 activation.

Fluorescence microscopy showed that the increased phosphorylation and activation of ERK1/ERK2 was associated with translocation of phospho-ERK1/ERK2 to the cell nucleus. Cells were probed with antibodies specific for phospho-ERK1/ERK2, as described. As illustrated in Figure 6, there was little nuclear staining for phospho-ERK1/ERK2 under control conditions (Figure 6A), whereas cells exposed to albumin demonstrated increased nuclear fluorescence compared with controls, indicating activation and translocation of phospho-ERK1/ERK2 (Figure 6B). In Figure 6C, cells were pretreated with AG-1478, which prevented the activation and translocation of ERK1/ERK2 induced by albumin.

Because AG-1478 attenuated albumin-induced phosphorylation, activation, and translocation of ERK1/ERK2, we also sought to show that the dose of AG-1478 used would prevent
EGF-dependent activation of ERK1/ERK2 in our cultured primary kidney cells. As shown in Figure 7, EGF led to rapid phosphorylation of ERK1/ERK2 that was completely abrogated by AG-1478. Albumin treatment (1%) also led to the phosphorylation of the EGFR on tyrosine 1068 and tyrosine 845, and this response was attenuated by the EGFR tyrphostins, AG1478 and PD158780 (Figure 8). In addition, pretreatment with AG 1478, PD 157780, and U0126 prevented the albumin-induced phosphorylation of ERK1/ERK2 (Figure 9). Taken together these results show that EGFR is expressed in our cultured primary kidney cells, and that albumin-induced activation of ERK1/ERK2 is dependent on MEK and ligand-independent transactivation of the EGF receptor.

Role of ROS

It has been reported that albumin treatment increases the generation of ROS in kidney tubule cells, and ROS can signal through the EGF receptor in some cell types (13,14,26). We therefore sought to determine if the activation of ERK1/ERK2 by albumin was dependent on ROS and whether ROS-induced activation of ERK1/ERK2 was dependent on the EGFR in our cultured kidney tubule cells. Pretreatment with the antioxidant NAC (Figure 10A) or pretreatment with the NADPH oxidase inhibitor DPI (Figure 10B) both attenuated ERK1/ERK2 activation. Because ERK1/ERK2 activation by ROS has been shown to proceed through the EGFR (27,28), we then studied the effect of AG-1478 on ROS-induced activation of ERK1/ERK2.

We first confirmed that exogenous treatment with hydrogen peroxide, as well as pretreatment with AG-1478, prevented the albumin-induced phosphorylation of ERK1/ERK2. As shown in Figure 7, EGF treatment induced phosphorylation of ERK1/ERK2, which was abrogated by pretreatment with AG-1478. *P<0.05 compared with control cells. The data are representative of three separate experiments.

Figure 6. Albumin induced nuclear localization of phospho-ERK in human renal tubule cells. Primary antibody is directed against phospho-ERK1/ERK2. (A) Untreated cells. (B) Cells exposed to 1% albumin for 5 min. (C) Cells were pretreated for 20 min with 0.2 µM AG-1478 before exposure to 1% albumin for 5 min, and are shown at higher magnification, highlighting absence of the nuclear localization of phospho-ERK1/ERK2. As a negative control, albumin-treated cells were imaged in the absence of anti-phospho–ERK1/ERK2 antibody, and no immunofluorescence was observed (data not shown).

Figure 7. The effect of AG-1478 on EGF-induced phosphorylation of ERK1/ERK2. Cells were pre-incubated with the indicated concentrations of AG-1478 for 20 min and then treated with 100 ng/ml EGF. Cell lysates were immunoblotted with antibodies directed against total and phosphorylated ERK1/ERK2. Densitometry was performed and results are expressed as fold increase compared with cells treated with serum-free medium alone (control). EGF treatment induced phosphorylation of ERK1/ERK2, which was abrogated by pretreatment with AG-1478. *P<0.05 compared with control cells. The data are representative of three separate experiments.
peroxide led to the phosphorylation of ERK1/ERK2 in a time-
and dose-dependent manner (Figure 11A and 11B, repectively). As shown in Figure 12, pretreatment of cells with AG-
1478 attenuated the phosphorylation of ERK1/ERK2 by exog-
enous hydrogen peroxide. To further define the signaling
mechanism linking ROS and ERK1/ERK2 in our cultured pri-
mary cells, we studied the effect of the src tyrosine kinase
inhibitor, PP2, on H2O2-induced activation of ERK1/ERK2 by
exogenous H2O2. There was a dose-dependent effect of PP2 on
H2O2-induced phosphorylation of ERK1/ERK2, as illustrated
in Figure 13. To implicate src tyrosine kinase activity in the
cellular response to albumin, we then studied the effect of PP2
on albumin-induced phosphorylation of ERK1/ERK2. As
shown in Figure 14, the src tyrosine kinase inhibitor, PP2, also
decreased the phosphorylation of ERK1/ERK2 by albumin.
PP3, the inactive analogue of the src kinase inhibitor, PP2, did
not attenuate the albumin-induced phosphorylation of ERK1/
ERK2 (Figure 15).

The gene expression profiling studies revealed that the EGFR
was a target gene for albumin, and the signaling studies con-
firmed a role for the EGFR in the cellular response to albumin
and ROS. Recent studies have shown that IL-8 expression is
increased in kidney tubule cells treated with albumin (13), and
our gene expression profiling study confirmed that IL-8 is a
target gene for albumin. We therefore sought to link EGFR
signaling and IL-8 expression, and we studied the effect of
AG-1478 on the albumin-induced increase in IL-8 mRNA levels.
As shown in Figure 16A, AG-1478 attenuated the increase in
albumin-induced IL-8 mRNA levels as assessed by semiquan-
titative reverse-transcription PCR and confirmed quantitatively
by real-time reverse-transcription PCR (data not shown). Fi-
nally, to relate the increase in IL-8 mRNA levels to the activa-
tion of ERK1/ERK2 and the generation of ROS, we studied the
effect of the MEK inhibitor, U0126, and antioxidant, NAC, on
albumin-induced IL-8 mRNA levels by semiquantitative re-
verse-transcription PCR. As shown in Figure 16B, both of these
inhibitors attenuated the albumin-induced increase in IL-8
mRNA levels.

Discussion
Proteinuria is emerging as a major mediator of renal injury
and is an important determinant of the progression of kidney
disease. From a clinical perspective, increasing degree of pro-
teinuria has long been known to be a negative prognostic
indicator in patients with a variety of renal diseases (4,29,30). It
has been hypothesized that proteinuria occurs as a result of
changes in the permeability of the glomerular filtration barrier caused by either direct injury or exposure to chronically elevated intraglomerular pressure that occurs because of hyperfiltration that develops as a compensatory mechanism for nephron loss (31,32). In addition, there is accumulating evidence that proteinuria, particularly albumin, is capable of exerting direct pro-inflammatory and pro-fibrotic effects in tubular cells (4,33,34). These effects likely directly contribute to chronic tubulointerstitial injury, one of the best prognostic indicators of renal outcome (33).

The activation of NFkB and dependent genes in tubular cells is one of the major potentially injurious pathways that has been linked to albumin (10–12) in renal tubular cells. It has been demonstrated that the activation of this pathway is dependent on the generation of ROS (13,14). Albumin has been demonstrated to activate various MAPKs including p38 and ERK, and the induction of NFkB by albumin has also been linked to activation of the MAPK, ERK1/ERK2 (16).

Our goal was to investigate mechanism(s) by which albumin contributes to the pathogenesis of renal tubular cell injury by examining broad-based gene expression with microarrays. We extracted RNA from primary human renal tubular cells ex-
posed to albumin or control conditions. The expression of the cytokine IL-8 was increased in primary cultured human tubular epithelial cells, as described previously; however, in addition, we also found that the EGFR mRNA levels were markedly increased. The increased expression of IL-8 and EGFR was confirmed by real-time PCR. The EGFR has been shown to function as an important component of the signaling pathways linking G-protein–coupled receptor activation and a variety of physical stimuli to the activation of MAPK, including ERK1/ERK2. Furthermore, disruption of EGFR signaling has been targeted as a potential therapeutic maneuver in animal models of polycystic kidney disease (35,36). We therefore sought to delineate how the EGFR may be involved in albumin-induced signaling in cultured human tubule cells, and whether the EGFR could be linked to albumin-induced cytokine expression.

Our first major observation was that the phosphorylation and activation of ERK1/ERK2 by albumin in primary human renal tubular cells was inhibited by the highly selective and potent EGFR kinase inhibitor, AG-1478 (37). In accord with these observations, AG-1478 inhibited the nuclear translocation of ERK1/ERK2. The activation of MAPKs including ERK1/ERK2 and p38 has been previously described (16); however, this is the first indication that the EGFR plays an important role in this signaling pathway and, taken together with the gene expression profiling data, suggest that there may be an important positive feedback loop linking albumin exposure and EGFR gene expression.

In vivo, tubular epithelial cells are polarized, and the surface expression of proteins is tightly regulated (38–40). Most EGFR is expressed on the basolateral surfaces of tubular cells, where it would not be expected to interact directly with receptor-mediated albumin uptake. However, approximately 10% of EGFR is expressed on the luminal membrane, where it may function as a scaffold to facilitate transmembrane signaling in response to albumin (38). Interestingly, the EGFR may have a greater role in signaling from the luminal membrane in at least two settings. First, overexpression of EGFR in polarized kidney epithelial cells leads to an increase in EGFR on the apical cell surface, where it can contribute to the activation of ERK1/ERK2 (41). The finding of increased EGFR expression in our gene expression profiling suggests that this mechanism may be of
importance in proteinuric kidney diseases. An albumin-induced increase in EGFR expression therefore might be associated with increased apical EGFR. The second instance in which EGFR may have an important role in signaling from the luminal membrane is in situations of tubular epithelial cell injury. This may be associated with loss of cell-to-cell contact, and albumin–EGFR interactions may be facilitated by the resultant disruption of polarity (42).

Recent advances have extended our understanding of the role of the EGFR in the cellular response to a variety of stimuli. Physical stimuli such as ultraviolet light are known to be capable of activating the EGFR in the absence of ligand (43,44). Receptor tyrosine kinase transactivation via G-protein–coupled receptors has also emerged as an important mechanism by which stimuli may induce the activation of the EGFR and, subsequently, p44/42 ERK (45). Transactivation of the EGFR has been demonstrated in vascular smooth muscle-like cells and endothelial cells for a variety of G-protein receptor stimuli, including known effectors of renal injury, like endothelin-1 and angiotensin II (46–49). In addition, the cellular response to ROS has been associated with EGFR transactivation and MAPK signaling events, including the activation of ERK1/ERK2. In fact, G-protein–coupled receptor signaling and ROS-mediated signaling may overlap because G-protein receptor stimulation often leads to production of H_2O_2 via NADPH oxidase (50). For example, ROS generation mediates EGFR transactivation by angiotensin II in vascular smooth muscle cells (26), although the necessity of ROS generation for the subsequent activation of ERK1/ERK2 through the EGFR may be cell-specific (51). ROS have also been shown to activate ERK1/ERK2 and induce downstream products c-fos and c-jun (27). Furthermore, it has been demonstrated that ROS play a critical role in angiotensin II-induced ERK1/ERK2 and c-src activation in vascular smooth muscle cells (28). More recently, this pathway linking ROS to EGFR transactivation and ERK1/ERK2 activation has been defined in studies of cultured rabbit renal proximal tubule cells (56).

It has been suggested in other cell systems that the activation of NFκB and NFκB-dependent genes such as IL-8 and MCP-1 by albumin is a function of the generation of ROS, and that the activation of NFκB might be ERK1/ERK2-dependent (13,14). The activation of ERK1/ERK2 by albumin was inhibited in our cells by pretreatment with the antioxidant, NAC, and the inhibitor of cellular NADPH oxidase, DPI. We therefore sought to determine whether ROS activated ERK1/ERK2 via the EGFR and src kinase in our human kidney cells. Our second major observation was that exogenously administered H_2O_2 activates ERK1/ERK2 in a similar manner to albumin, PDF and that the hydrogen peroxide-induced activation of was dependent on src tyrosine kinase activity. Taken together, these observations support the hypothesis that the generation of ROS after albumin exposure is an important proximate event in the albumin-induced cell signaling.

Although the EGFR was the focus of the current study, a number of other signaling molecules may play a role in the activation of ERK1/ERK2. For example, lipid rafts may be important in the signal transduction events dependent on the
EGFR, and the EGFR, as well as other components of MAPK pathways, have been localized to caveolae (49,52–54). In this regard, it is tempting to speculate that caveolae may serve to facilitate the propagation of albumin-induced cell signaling because treatment with membrane cholesterol-depleting drugs, β-cyclodextrin and filipin III, attenuated albumin-induced activation of ERK1/ERK2 in our cells. In addition, multiple PKC isoforms have also been localized to caveolae (55), and we found that the PKC inhibitor, calphostin C, attenuated ERK1/ERK2 phosphorylation and activation. However, further studies will be necessary to better define the role of caveolae and specific protein kinase C isoforms in the renal tubular response to albumin.

Finally, to confirm the central role of the EGFR in the signaling induced by albumin and to link the EGFR to cytokine expression and inflammation, we studied the effect of AG-1478 on albumin-induced expression of IL-8. We observed that AG-1478 attenuated the expression of the cytokine IL-8. It has recently been demonstrated that albumin is a stimulus for IL-8 expression in proximal tubular cells (13), and the expression of IL-8 in this study was found to be dependent on ROS production. Our findings suggest that the EGFR may be another important component of the signaling cascade linking albumin and the expression of potentially injurious cytokines. The linkage between the EGFR and IL-8 in both microarray studies and signaling studies suggests that gene expression profiling may be one way to identify cellular interactions and/or networks that play an important role in pathogenesis of kidney disease. However, it is important to note that the early signaling re-

Figure 15. Effect of PP3, the inactive analogue of the src tyrosine kinase inhibitor PP2, on the activation of ERK1/ERK2 by albumin. Cells were pre-incubated with 10 μM PP3 for 30 min, and then exposed to 1% albumin. *P<0.05 compared with control-treated cells. The data are representative of three separate experiments.

Figure 16. (A) Effect of AG-1478 on albumin-induced IL-8 expression. IL-8 mRNA levels were measured with semiquantitative reverse-transcription PCR. Cells were treated pretreated with 0.2 μM AG-1478 for 30 min when indicated, and then treated with 1% albumin for 6 h. *P<0.05 compared with control (untreated) cells. †P<0.05 compared with cells treated with BSA alone. (B) Effect of U0126 and NAC on albumin-induced IL-8 expression. IL-8 mRNA levels were measured with semiquantitative reverse-transcription PCR. Cells were treated pretreated with 10 μM U0126 for 2 h, or 10 mM NAC for 60 min, and then treated with 1% albumin for 6 h. *P<0.05 compared with control (untreated) cells. The data are representative of three separate experiments.
response linking albumin to increased IL-8 mRNA levels was not dependent on the effect of albumin on EGFR mRNA levels. Rather, the observations of the microarray experiment suggest that a later effect of albumin on EGF mRNA levels may serve to increase the response of the tubular cells to albumin and thereby function, at least in part, as a positive feedback loop.

In summary, we have demonstrated that albumin induces the phosphorylation, activation, and translocation of ERK1/ERK2, and that these events are dependent on signaling through the EGFR in primary human renal tubular epithelial cells. ROS are capable of activating ERK1/ERK2 through a similar EGFR-dependent mechanism. The activation of ERK1/ERK2 by albumin is also dependent on src tyrosine kinase activity and the production of ROS. Finally, these signaling events are upstream of IL-8 expression. EGFR expression is also increased by exposure to albumin, suggesting a possible positive feedback loop, which may propagate cell injury. Taken together, these studies suggest that the EGFR may be an important treatment target in kidney disease associated with proteinuria.

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