Bradykinin Decreases Podocyte Permeability through ADAM17-Dependent Epidermal Growth Factor Receptor Activation and ZO-1 Rearrangement

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ABSTRACT

Recent data show that increases in bradykinin (BK) concentration contribute to the beneficial effects of angiotensin converting enzyme inhibitor (ACEI) treatment in chronic kidney disease (CKD). However, the possible role of BK in attenuated proteinuria, often seen in ACEI-treated patients, is not well studied. Here, we report that BK decreases mouse podocyte permeability through rearrangement of the tight junction protein zonula occludens-1 (ZO-1) and identify some of the major signaling events leading to permeability change. We show that BK2 receptor (BK2R) stimulation transactivates the epidermal growth factor receptor (EGFR). EGFR transactivation is mediated by a disintegrin and metalloenzyme (ADAM) family members which are required for both extracellular signal regulated kinase (ERK) and EGFR activation by BK. Using a gene silencing approach we observed that both BK-induced ERK activation and BK-induced permeability decrease in podocytes is attenuated by ADAM17 downregulation, and identified epiregulin (ER) as the EGFR ligand participating in ADAM-dependent BK2R-EGFR crosstalk. EGFR inhibition attenuated both ZO-1 rearrangement and BK-induced permeability decreases in podocyte. We propose that ZO-1 redistribution is an important element of BK-induced permeability change and that the signaling events involved in ZO-1 rearrangement include transactivation of the EGFR via ADAM17 activation and ER shedding. Our data indicate that ADAM17 and the EGFR may be potential novel therapeutic targets in diabetic nephropathy and other chronic kidney diseases.
INTRODUCTION

National Kidney Foundation’s data show that 1 of every 9 Americans currently has chronic kidney disease (CKD) (www.nkf.org), and end-stage renal disease (ESRD) is increasing worldwide. The leading causes of CKD are diabetes (diabetic nephropathy) and hypertension. An important predictor of glomerular function and disease progression is proteinuria. In the kidney, glomerular podocytes, endothelial cells, and the glomerular basement membrane provide the main filtration barrier for macro-molecules (Pavenstadt, 2000). Recently, podocytes have gained substantial attention, for their potential roles in glomerular function. Their distinctive “multi-legged” structure enables them to encircle the vascular wall, and by regulating the slit diaphragm, the intercellular junction between their foot processes, they are capable of regulating molecular flow from the blood into the urinary space (Pavenstadt, 2000). Renal injury is characterized by foot process effacement, with concomitant changes in cellular and slit architecture that correlate with the development of proteinuria.

The mediators and the exact mechanisms underlying the progression of kidney disease are not fully understood; however, the role of angiotensin II in disease progression is well documented. Angiotensin II (ANGII) affects both inflammatory and fibrotic processes in the kidney (Ruiz-Ortega et al., 2006) and contributes to the development of proteinuria (Langham et al., 2004). Extensive clinical data support the renoprotective effects of angiotensin converting enzyme inhibitors (ACEIs): they reduce proteinuria, delay renal fibrosis and preserve kidney function (Macconi et al., 2000). ACEIs inhibit not only the formation of active ANGII, but also kinin degradation, thereby increasing the level of the potent vasodilator, bradykinin (BK). Despite some conflicting data (Nabokov et al., 1998), evidence is increasing that the renoprotective effects of ACEIs are partly mediated by BK B2 receptor (BK2R) activation (Yokota et al., 2003). BK2R activation reduced fibrosis in animal models of tubule-interstitial fibrosis (Seccia et al., 2006) and investigators observed increased albuminuria and glomerular sclerosis in a BK2R-
deficient Akita mouse model of diabetes (Kakoki et al., 2004). Moreover, retrospective studies have shown that polymorphisms in the BK2R predispose individuals to increased urinary albumin excretion (Maltais et al., 2002) and that certain kinin polymorphisms are more frequent among patients with end stage renal disease (Zychma et al., 1999). Similarly, recent data suggest that BK exerts an important renoprotective effect through BK2R activation and could be an important therapeutic target in diabetic nephropathy [for review see (Doggrell, 2006)].

Inter-receptor crosstalk between angiotensin 1 receptors (ATR1) and the epidermal growth factor receptor (EGFR) has been implicated in ANGII-induced progression of chronic kidney disease and ADAM17, a member of the "adisintegrin and metalloenzyme" family of matrix metalloproteases that process EGFR ligands to their mature form was identified as a potential therapeutic target (Lautrette et al., 2005). Several in vivo studies showed that ACEIs preserve expression of glomerular nephrin (Benigni et al., 2001), which is one of the main components of the slit diaphragm. Nevertheless, signaling events associated with the regulation of the podocyte filtration barrier itself, thus with the development of proteinuria, are still poorly understood. It was shown that ANGII induces apoptosis through p38 mitogen activated protein kinase (MAPK) activation in human podocytes (Lai et al., 2004). There is also evidence that BK increases intracellular Ca^{2+} concentrations in immortalized mouse podocytes (Mundel et al., 1997) and causes contraction of human podocytes (Langham et al., 2002). However, few studies describe the signaling mechanisms underlying the regulation of podocyte permeability. A trans-epithelial permeability study using FITC-labeled bovine serum albumin (BSA) showed that angiotensin II increases podocyte monolayer permeability through the AT1R, src family kinases, and PLC activation (Macconi et al., 2006). However, no data are available to describe how kinins modulate podocyte permeability.
In this study, we sought to (1) determine the effect of BK on glomerular permeability and (2) characterize BK-induced signaling mechanisms that modulate podocyte permeability to identify potential therapeutic targets for the treatment of diabetic nephropathy and for other chronic kidney diseases. We hypothesized that BK has a beneficial effect on glomerular function by decreasing glomerular permeability. We also hypothesized that, as a G-protein receptor agonist, BK induces downstream signaling mechanisms through metalloenzyme activation and EGFR trans-activation and that a disintegrin and metalloenzyme (ADAM) 17 is a key metalloenzyme regulating these events.
METHODS

Chemicals and antibodies. All cell culture media, fetal bovine serum (FBS) and antibiotics, 4–12% and 3–8% acrylamide gels, SDS (sodium dodecyl sulfate) sample buffer, and sample reducing agent were purchased from Invitrogen (Carlsbad, CA). Bovine serum albumin (BSA), FITC (fluorescein isothiocyanate)-conjugated BSA, bradykinin, heparin, HOE140 (Icatibant) and EGF were from Sigma (Milwaukee, MI); GM6001 (N-[(2R)-2-(hydroxamidocarbonylmethyl)-4-methylpentanoyl]-L-tryptophan methylamide) and the inactive GM negative control (N-t-butoxycarbonyl-L-leucyl-L-tryptophan methylamide), AG1478 (N-(3-chlorophenyl)-6,7-dimethoxy-4-quinazolinamine hydrochloride), and protease inhibitor cocktail set III was from EMD Biosciences (La Jolla, CA). Antibodies against HB-EGF, TGFα, and amphiregulin were purchased from R&D Systems (Minneapolis, MN); epiregulin and ZO-1 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); ERK, phospho-ERK antibodies, EGFR and phospho-EGFR (Tyr1068) antibodies were from Cell Signaling Technologies (Danvers, MA). ADAM17 antibody was purchased from R&D Systems, Minneapolis, MN (AB930). GAPDH was from Sigma, (Milwaukee, MI) and α-actinin was from Santa Cruz Biotechnology (Santa Cruz, CA). Chemiluminescence detection reagent was from Thermo Scientific (Rockford, IL). Non-targeting and mouse ADAM17 silencing RNA and Dharmafect transfection reagent were purchased from Dharmacon (Lafayette, CO).

Cell culture. Immortalized murine podocytes were kindly provided by Dr. Peter Mundel (Mt. Sinai School of Medicine). Undifferentiated cells were maintained in RPMI-1640 medium containing 10 units/ml of mouse recombinant γ-interferon, 10% (v/v) FBS, penicillin (100 units/ml) and streptomycin (100 μg/ml) in tissue culture dishes coated with collagen I at 33°C in 95% air and 5% CO2. To induce differentiation cells were placed at 37°C and cultured in the same medium without γ-interferon for 10–14 days. Differentiation of the cells is routinely tested by positive synaptopodin staining. Cells between passage numbers 17–26 were used in our
studies. Immortalized human podocytes were kindly provided by Dr. Moin Saleem (Bristol University). Undifferentiated cells were maintained in RPMI-1640 medium containing 10 μg/ml insulin, 5.5 μg/ml transferrin, 5 ng/ml sodium selenite (Sigma, St. Louis, MO) and 10 % (v/v) FBS at 33 °C in 95% air and 5% CO₂. Differentiation was induced by placing the cells to 37°C for 14 days in collagen coated dishes. Cells between passage numbers 17–20 were used in our studies.

**Signaling experiments.** For ERK phosphorylation and EGFR phosphorylation studies differentiated podocytes were plated into collagen coated 12-well plates (~100,000/ well/ 1 ml) 12 days after thermoshift, serum starved for 24 h in 0.5% (w/v) bovine serum albumin-containing RPMI medium then pretreated for 1 h with the indicated inhibitors ( 10⁻⁵ M of HOE140 or buffer; 10⁻⁷ M of AG1478 or DMSO control (Methylsulfoxide); 10⁻⁵ M of GM6001 or GM negative control compound) prior to treatment with the agonist (BK, 10⁻⁸ M or EGFR, 1 ng/ml) for 7 min. Cells were then lysed in Laemli sample buffer and analyzed by Western blotting.

**Western blot analysis of podocyte samples.** Protein samples were boiled for 3 min in reducing SDS-sample buffer and separated by 4–12% or by 3-8% acrylamide gels. Resolved proteins were transferred to 0.45-μm polyvinylidene difluoride membranes (Millipore, Temecula, CA), blocked with 4% (w/v) nonfat dry milk or 3% (w/v) bovine serum albumin for 1 h, and incubated overnight at 4°C with the following antibodies: 1:1,000 ERK and phospho-ERK, 1:1000 phospho-EGFR (Tyr1068), 1:1,000 EGFR, 1:3000 α-actinin, 1:2,000 ADAM17 or 1:1000 ZO-1 antibodies and 1:1,000 GAPDH. Membranes were washed three times in phosphate-buffered saline containing 0.05% (v/v) Tween 20 (Polysorbate 20) and incubated for 1 h at room temperature in appropriate dilutions of secondary antibodies. Immunoreactive protein bands were visualized using enhanced chemiluminescence (West Dura substrate, Thermo Scientific) and recorded on Kodak BioMax XR film. Optimally exposed autoradiographs were digitally
scanned and analyzed using NIH Image software. Un-phosphorylated ERK or un-phosphorylated EGFR was used to normalize the density of the phospho-ERK and phospho-EGFR bands, respectively.

**RNA interference.** Murine podocytes were seeded into collagen-coated 12 well plates (~100,000/ well/ 1 ml) or 6 well plates (~200,000/ well/ 1 ml) 12 days after thermoshift. Cells (60-70% confluent) were transfected with 50 nM of ADAM17 siRNA or non-targeting siRNA (as control) using Dharmacon’s Dharmafect 1 transfection reagent (1.5 μl/well or 3 μl/well, respectively) as suggested by the manufacturer. Cells were analyzed for successful ADAM17 silencing 48 h after transfection. For signaling experiments cells were serum starved for 24 h in 0.5% (w/v) BSA containing RPMI medium prior to treatment with BK (10⁻⁸ M) or EGF (1 ng/ml).

**Growth factor shedding experiments.** Murine podocytes were seeded into collagen-coated 6 well plates 12 days after thermoshift as described above. After transfection with ADAM17 siRNA or with control, non-targeting siRNA cells were serum starved for 24 h then treated with bradykinin (10⁻⁸ M) for 2 h. Cell supernatants were collected and concentrated using Pierce concentrators (Thermo Scientific) with 3 kDa cut-off membranes. Concentrated supernatants were analyzed by Western blotting as described above using 1:100 of HB-EGF, amphiregulin, TGF-α and epiregulin antibodies as described above.

**Permeability assay.** Murine podocytes (~60,000/well/400 μl) were seeded on the top of HTS Transwell inserts (24-well plate, Corning Life Sciences, Lowell, MA). Cells were serum starved in 0.5% (w/v) bovine serum albumin-containing medium and incubated for 1 h with the indicated inhibitors (10⁻⁵ M of HOE140, 10⁻⁷ M of AG1478 or DMSO control; 10⁻⁵ M of GM6001 or GM negative control compound or alternatively ADAM17 silenced or control silenced cells were used) prior to treatment with bradykinin (10⁻⁸ M) for 1 h. FITC-labeled dextran or FITC-labeled bovine serum albumin was added to the upper chambers of the Transwells for 5 min at room
temperature. Fluorescence activity of the medium in the lower chambers as reflection of the podocyte monolayer permeability was measured by fluorescence plate reader (excitation: 495 nm, emission: 520 nm, Molecular Devices, Sunnyvale, CA).

**ZO-1 immunostaining.** Murine podocytes were grown on 35-mm lysine-coated glass-bottom culture dishes (MatTek Corporation, Ashland, MA). After overnight serum starving they were treated with either BK (10^{-8} M) or 10% (v/v) FBS (as positive control) then fixed with 2% (w/v) paraformaldehyde, 4% (w/v) sucrose in phosphate-buffered saline for 10 min at room temperature. Subsequently, the nonspecific binding sites were blocked with 2% (v/v) fetal calf serum, 2% (w/v) BSA and 0.2% (w/v) gelatin in PBS for 1 hr. The cells were then incubated overnight at 4°C with 1:100 dilution of rabbit polyclonal anti-ZO-1 antibody, followed by incubation for 1 h at room temperature with the appropriate Alexa Fluor-conjugated secondary antibodies (1:200 dilution, Invitrogen, Carlsbad, CA). Texas Red®-X phalloidin (Invitrogen, Carlsbad, CA) at 1:40 dilution in 1% (w/v) BSA/PBS for 20 min at room temperature was used to visualize actin. Confocal microscopy was performed using a Zeiss LSM 510 Meta laser-scanning microscope (Carl Zeiss Inc., Jena, Germany) equipped with a 60X objective, using the following laser wavelengths: excitation 488 nm, emission 505–530 nm; excitation 543 nm, emission 560 nm.

**Statistical analysis.** For analyzing permeability assay data, analysis of variance (ANOVA) was used followed by Newman-Keul post hoc comparison. For Western blot image analyses Scion Image software was employed. For statistical analysis of ZO-1 immunostaining color images were converted to grayscale then percentage of white areas was calculated using Scion Image software. Analysis of variance using Prism (GraphPad, La Jolla, CA) statistics software was then performed to determine statistical significance. \( p \) values<0.05 were regarded as statistically significant.
RESULTS

BK Decreases Podocyte Monolayer Permeability through ZO-1 Rearrangement

Because ANGII was shown previously to increase mouse podocyte monolayer permeability (Macconi et al., 2006) we first tested whether BK influences podocyte function by measuring the trans-epithelial flow of fluorescent albumin from the top chamber through the podocyte layer to the basolateral compartment in a transwell assay of monolayer permeability. We found that BK treatment (10^{-10}-10^{-6} M, 1 h) dose-dependently and significantly decreased fluorescence activity by ~10–50% in the bottom chambers compared to control (Figure 1A). We also investigated the effect of BK on the permeability of a human podocyte cell line and found that 10^{-8} M BK decreased the cells permeability to 71\% \pm 3 \% (n=4) of untreated control cells.

In vivo, podocyte permeability depends on the arrangement of slit diaphragm and cytoskeletal proteins. To investigate the mechanism through which BK decreases podocyte monolayer permeability, we studied changes in the arrangement of the cell-cell contact protein zonula occludens-1 (ZO-1). This protein is part of the slit diaphragm, and ZO-1 immunostaining has been shown to become fragmented in podocytes after ANGII treatment (Macconi et al., 2006).

Figure 1B shows that subconfluent, untreated podocytes have positive ZO-1 immunostaining. However, this staining is not continuous. BK treatment (10^{-8} M) or 10\% (v/v) FBS treatment (positive control) induced comparable increases in ZO-1 staining that became almost continuous around the cells (arrowheads). Increased staining intensity is possibly due to rearrangement of ZO-1 and not because of newly synthesized protein; amount of ZO-1 protein remained the same in control, BK-, and FBS-treated cell lysates as assessed by Western blotting (Figure 1C). Analysis of podocytes at lower magnification revealed that BK treatment for 15 and 30 min increased ZO-1 immunostaining 1.6-fold (Figure 1D) and 1.75-fold (Figure 1E), respectively, similar to exposure to the positive control, 10\% (v/v) FBS.

BK2Rs Transactivate the EGFR in Podocytes
BK is known to act through G-protein coupled receptors (GPCRs) that couple to $G_{q/11}$ and $G_i$ family G proteins. In addition, some of its effects result from inter-receptor crosstalk between the BK2R and the EGFR (Gooz et al., 2006; Mukhin et al., 2006). In murine inner medullary collecting duct (mIMCD)-3 cells, BK stimulates ERK1/2 activity by transactivating the EGFR (Mukhin et al., 2006). To characterize the signal transduction events behind the permeability changes, we first determined whether BK regulated EGFR and whether EGFR transactivation contributed to downstream BK signaling in podocytes. Figures 2A and 2B show concomitant activation of ERK (~3-fold) and EGFR (~2-fold) upon $10^{-8}$ M BK treatment. Pre-treating the cells with the EGFR tyrosine kinase inhibitor AG1478 ($10^{-7}$ M) almost completely blocked BK-induced EGFR and ERK activation. As expected, AG1478 inhibited EGF-induced phosphorylation of the EGFR and ERK, suggesting that most of the BK-induced ERK activation is mediated through transactivation of the EGFR in podocytes.

In mature podocytes, BK stimulates $Ca^{2+}$ influx through BK2Rs (Ardaillou et al., 1996; Mundel et al., 1997). We employed the BK2R antagonist HOE140 to determine whether ERK activation is also mediated by the BK2R. Figure 2C shows that 1 h pretreatment with the BK2R antagonist HOE140 ($10^{-5}$ M) blocked $10^{-8}$ M BK-induced ERK phosphorylation in our cells. As control, we used 1 ng/ml EGF treatment. As expected, HOE140 treatment had no effect on direct EGF-induced ERK activation.

**EGFR Transactivation is ADAM Metalloenzyme-Dependent**

Matrix metalloproteinases (MMPs) are $Zn^{2+}$-dependent endopeptidases. Because MMPs including the ADAMs have been shown to mediate crosstalk between GPCRs and receptor tyrosine kinases (RTKs) by promoting the shedding of endogenous EGFR ligands (Gooz et al., 2006; Mukhin et al., 2006), we investigated the possible involvement of these enzymes in BK-induced EGFR transactivation in podocytes. We found that metalloenzyme inhibition using the broad-range MMP-inhibitor GM6001 ($10^{-5}$ M) completely blocked $10^{-8}$ M BK-induced ERK
(Figure 3A) and EGFR phosphorylation (Figure 3B). At the same time, metalloenzyme inhibition did not affect EGF-induced ERK activation and EGFR phosphorylation.

We have previously shown that ADAM17 contributes to GPCR and tyrosine kinase receptor crosstalk (Gooz et al., 2006), and ADAM17 was already identified as a potential new target in chronic kidney disease (Lautrette et al., 2005). To identify the metalloenzyme participating in BK2R-EGFR crosstalk, we transfected podocytes with small interfering RNA (siRNA, Dharmacon) to downregulate ADAM17 expression. As a negative control, we used non-targeting siRNA transfection. Figure 4A shows that ADAM17 silencing completely blocked BK-induced ERK phosphorylation but did not affect EGF-induced ERK activation (middle blot). Successful ADAM17 silencing was confirmed by Western blot (Figure 4A, top blot) in each condition. To identify specific growth factors shed by ADAM17 in response to BK, we analyzed the concentrated supernatants of podocytes by Western blotting. We found that BK treatment significantly (~1.6-fold) induced epiregulin (ER) shedding of non-targeting siRNA transfected cells (Figure 4B). ADAM17 silencing significantly attenuated BK-induced ER shedding indicating that ADAM17 has a significant role in BK-stimulated ER processing. The fact that we were unable to see shedding of HB-EGF, amphiregulin, or TGF-α (data not shown) could be due to the low abundance of these proteins in the conditioned culture medium.

The BK-induced ZO-1 Redistribution and Permeability Change is Dependent on EGFR Activation

In the next set of experiments, we characterized how EGFR trans-activation by BK contributes to the observed permeability changes. To test whether EGFR activity is necessary for BK-induced ZO-1 rearrangement, we pre-treated subconfluent podocytes with the EGFR kinase inhibitor AG1478 before BK treatment. As shown in Figure 5A, EGFR inhibition significantly inhibited (~50%) BK-induced redistribution of ZO-1 immunostaining (AG+BK). At the same time,
AG1478 did not significantly affect ZO-1 staining. We next tested whether EGFR activation has a role in BK-induced trans-epithelial permeability changes. Podocytes seeded on Transwell inserts were pre-treated with AG1478 before being stimulated with BK. As shown in Figure 5B, BK-induced decrease in podocyte permeability was completely blocked by AG1478. Because we identified ER as the EGFR ligand important for BK-induced EGFR activation in podocytes, we next investigated whether direct application of ER affects permeability. Using recombinant ER to test whether ER treatment is sufficient and necessary to induce a permeability change in podocytes, we found that ER (10 ng/ml) induced a significant (45%) decrease in podocyte monolayer permeability, similar to BK treatment (Figure 5B, insert).

To confirm the involvement of the BK2R in BK-induced permeability changes, podocyte on Transwell membrane were pre-treated with HOE140 before being stimulated with BK. Figure 5C shows that BK2R inhibition significantly inhibited permeability decrease by BK.

**Metalloenzyme Inactivation Changes Podocyte Permeability**

To test the effect of metalloenzyme/ADAM17 inhibition on podocyte monolayer permeability first we pre-treated cells on Transwell membrane with the broad-spectrum MMP-inhibitor GM6001 or its control compound before treated with BK. In subsequent experiments we treated cells transfected with ADAM17 silencing RNA or control RNA (Csi) with BK. Pretreatment with GM6001 had no significant effect on permeability, but completely inhibited BK-induced permeability changes (Figure 5D). Similar results were obtained in cells treated with ADAM17 specific siRNA. ADAM17 silencing resulted in a modest but significant reduction in basal podocyte permeability in unstimulated cells and a complete loss of BK responsiveness (Figure 5E).
DISCUSSION

We report here for the first time that stimulation of the BK2R leads to rearrangement of the tight junction protein ZO-1 with a subsequent decrease in podocyte monolayer albumin permeability. Furthermore, we demonstrate that BK treatment induces a cascade of signals resulting in trans-activation of EGFR through ADAM17 activation and ER shedding, and we provide evidence that these signaling events contribute to the observed permeability change.

Changes in podocyte permeability leading to albuminuria are often the first clinical sign of renal disease. Broadening of podocyte foot processes and decreases in the number of slit pores are often observed in diabetic nephropathy (Mifsud et al., 2001). Hypertrophy and degeneration of the filtration barrier can even lead to urinary excretion of podocytes and thus to podocyte loss (Nakamura et al., 2000). Although there is evidence that podocytes can be replaced, to some extent, by stem cells (Prodromidi et al., 2006), the most important goal is to preserve podocytes and their function in the kidney glomerulus.

ACEIs are the most important, therapeutic tool to preserve kidney function and delay the progression of renal failure (Nakamura et al., 2000). Previous work has demonstrated that, in addition to changing kidney hemodynamics by lowering intracapillary hypertension, they also modulate the intrarenal renin-angiotensin system (RAS), and by increasing intrarenal BK, attenuate macrophage-mediated mesangial cell scarring (Pawluczyk et al., 2004). There is increasing evidence of their effects on podocytes: ACEI treatment has been shown to preserve podocyte architecture (Mifsud et al., 2001) and to prevent podocyte loss in diabetic animal models (Gross et al., 2003). Affecting the redistribution of ZO-1 appears to be one mechanism whereby ACEIs affect the progression of proteinuria (Macconi et al., 2000). In Munich Wistar Frömter (MWF) rats, redistribution of ZO-1 protein in the glomerulus correlates with the age-dependent onset of proteinuria (Macconi et al., 2000). In mouse podocyte cultures ZO-1
immunostaining becomes more scattered in ANGII treated cells (Macconi et al., 2000); ACEI preserved the distribution of ZO-1 in both models. In each case, the effect was on ZO-1 distribution, not expression; the total amount of ZO-1 did not change throughout these investigations (Macconi et al., 2000). Our observations agree with these previous studies: in our experiments BK increased ZO-1 immunostaining between neighboring cells’ processes, neither did we observe changes in ZO-1 expression. Thus, ZO-1 distribution-redistribution has an important role in regulating glomerular permeability, and opposite to the previously observed effect of ANGII (Macconi et al., 2000), BK can decrease permeability by tightening cell connections between podocytes.

Paradoxically, BK is well-known to increase paracellular permeability: it was shown to induce leakage in postcapillary venules of rat mesentery (Shigematsu et al., 2002) involving activation of protein kinase C and cytochrome P450 epoxygenase. In contrast, BK induced activation of eNOS in cardiac capillary endothelial cells through transactivation of the vascular endothelial growth factor receptor (VEGFR) (Thuringer et al., 2002). In this cell model, EGFR kinase inhibitor had no effect on BK-induced eNOS production.

However, there is evidence that direct, ligand-induced activation of the EGFR and ERK can decrease permeability in epithelial cells. In Madin-Darby canine kidney epithelial cells, HB-EGF treatment increased trans-epithelial resistance and decreased permeability (Singh et al., 2007). Similarly, in primary canine gastric epithelial cells, EGF increased transepithelial resistance and decreased paracellular permeability of the cells (Chen et al., 2001). There is also evidence that EGF protects from oxidative stress-induced intestinal barrier damage (Banan et al., 2002). Interestingly, there are data to show that EGFR activation increases permeability. Several ErbB family inhibitors, including Herceptin have been shown to reduce vascular permeability in a mouse model of human breast cancer (Izumi et al., 2002) and EGFR inhibition was shown to
decrease vascular permeability in vivo in a lung cancer model (Wu et al., 2007). A novel finding of our studies is our evidence that EGFR activation contributes to BK-induced permeability decrease in podocytes through ADAM17–mediated ER shedding. The fact that EGFR activation can also increase permeability as shown by others in other cell models suggests that mechanisms other than EGFR activation may also be involved. The bidirectional effect of EGFR activation on permeability can be analogous to the effect of EGFR activation on proliferation and apoptosis—depending on the cellular context EGFR can induce either cell division or cell death (Jackson et al., 2003). Therefore direct activation of the EGFR by growth factors, oxidative stress or, in our case, inter-receptor crosstalk can have different effects.

ADAM17 previously was implicated in EGFR activation and permeability changes in cells other than podocytes. ADAM17 inhibition was effective in a mouse ischemia reperfusion model assessed by decreases in vascular permeability (Souza et al., 2007). Inhibiting ADAM17 impaired TNF-α mediated increases in broncho-alveolar permeability in a rat model of ischemia/reperfusion (Georgieva et al., 2007). Furthermore, oxidative stress-induced hyperpermeability was blocked by inhibitors of EGFR, ADAM17, TGFα and ERK1/2 in Caco-2 human colonic epithelial cells (Forsyth et al., 2007). In the present study we identified ER as a growth factor that is shed by ADAM17 upon BK treatment in podocytes and we showed that ER is necessary and sufficient for the permeability changes seen during BK treatment. ER was already shown to participate in kidney development (Kim et al., 2007); to promote proximal tubular epithelial cell migration by EGFR activation (Zhuang et al., 2007), and to participate in kidney mesangial cell proliferation, as were HB-EGF and amphiregulin (Mishra et al., 2002). Cell-specific differences in ADAM17-induced permeability changes might be explained by the example of Caco-2 cells: ADAM17 cleaves TGFα (Forsyth et al., 2007) and in our podocytes the enzyme sheds ER. We showed previously that different EGFR ligands initiate various signaling
events in the same cell (Baldys et al., 2009); therefore, it is possible that different growth factors induce unique responses depending on the cell type. ADAM17-shedded growth factors or cytokines can cell-specifically activate not only the EGFR but other RTKs by which they subsequently influence net cellular response. Our data showing that ADAM17 silencing decreases podocyte permeability and that in ADAM17-silenced cells BK is no longer capable of changing permeability confirm that ADAM17 has a crucial and complex role in podocyte permeability regulation and suggest that BK possibly regulates other signaling molecules besides EGFR through ADAM17. Our laboratory is currently investigating these other BK-specific signaling events in podocytes.

Taken together, we provide evidence in this report that BK is directly capable of decreasing podocyte permeability by rearranging ZO-1 distribution and tightening cell-cell contacts. Based on our results, we propose a model for BK-induced permeability decreases in podocytes in which the metalloenzyme ADAM17 plays a central role: BK2R engagement induces ADAM17 activation which contributes to ER shedding and subsequent activation of the EGFR, which in turn regulates permeability of the podocyte monolayer by rearranging ZO-1 protein (Figure 6). Further investigations are needed to identify the signaling events leading from EGFR activation to ZO-1 rearrangements and to learn how other slit diaphragm proteins are affected by BK. However, the present study support the hypothesis that BK can have antiproteinuric effect and provide strong evidence that the beneficial effect of ACEIs may arise from their amplification of kinin signals as well as inhibition of the angiotensin (AT1) receptor in vivo. Furthermore, these data provide evidence of the importance of ADAM17 and EGFR in permeability regulation and identify them as potential novel therapeutic targets in diabetic nephropathy and other chronic kidney diseases.
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FOOTNOTES

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LEGENDS FOR FIGURES

Figure 1. **BK decreases podocyte monolayer permeability through ZO-1 rearrangement.** A) Trans-epithelial albumin permeability of podocyte monolayer was measured using fluorescent BSA after incubating the cells for 1 h with various concentrations of BK (10^{-10}–10^{-6} M) or 10% (v/v) FBS (insert). Data are means ± SE of four independent experiments, *p < 0.05 versus control. B) ZO-1 re-distribution (green, highlighted with arrowheads) is shown upon 10^{-8} M BK treatment and 10% (v/v) fetal bovine serum treatment (FBS, as positive control) without and with bright field image overlay (lower part of images, 60X). C) Immunoblot (WB) analysis show equal amount of ZO-1 expression in control, 10^{-8} M BK and 10% (v/v) FBS treated cell lysates. GAPDH immunoblot was used as loading control. (D&E) ZO-1 re-distribution was induced in podocytes by treatment with 10^{-8} M BK or and visualized by immunostaining (green, 40X) after 15 min (D) and 30 min (E) treatment. Texas Red®-X phalloidin was used to visualize actin (red). Quantified ZO-1 immunostaining intensity is shown in bar graphs next to the images; *p < 0.05; **p < 0.01 versus corresponding controls: C 15 and C 30, respectively.

Figure 2. **BK induces extracellular signal regulated kinase phosphorylation (pERK) through the EGFR and BK2 receptor activation.** Podocytes were preincubated with AG1478 (EGFR tyrosine kinase inhibitor) for 1 h then stimulated with 10^{-8} M BK or 1 ng/ml EGF (as positive control) for 7 min. Cell lysates were immunoblotted against A) phosphorylated ERK (pERK) and total ERK (tERK), and against B) phosphorylated EGFR (pEGFR) and total EGFR (tEGFR). C) Podocytes were preincubated for 1 h with either control buffer (C) or with 10^{-5} M of HOE140 (BK2R antagonist) and stimulated for 7 min with BK (10^{-8} M) or EGF (1 ng/ml). Cell lysates were immunoblotted against phosphorylated ERK (pERK) and total ERK (tERK). Results are mean ± SE (n = 3) **p < 0.01; ***p < 0.001 versus control; ##p < 0.01 versus BK alone, $$p < 0.01 versus EGF alone. Quantified pERK and pEGFR immunostainings are shown in bar graphs; tERK and tEGFR were used as loading controls.
Figure 3. **EGFR transactivation is metalloenzyme dependent.** Podocytes were preincubated for 30 min with $10^{-5}$ M GM6001 (broad-spectrum MMP inhibitor) or with its inactive control compound GM C and treated for 7 min with BK ($10^{-8}$ M) or EGF (1 ng/ml). Cell lysates were immunoblotted against A) pERK and tERK and B) against phosphoTyr-1068 EGFR (pEGFR) and tEGFR. Results are mean ± SE ($n = 3$), **$p < 0.01$; ***$p < 0.001$ versus GM control; $^\#p < 0.05$ versus BK GM control; ### $p < 0.001$ versus BK GM control. Quantified pERK and pEGFR immunostainings are shown in bar graphs; tERK and tEGFR were used as loading controls.

Figure 4. **Silencing ADAM17 expression inhibits BK-induced ERK phosphorylation and shedding of the EGFR ligand epiregulin.** Silencing RNAs against ADAM17 enzyme (ADAM17si) or non-targeting siRNA (Csi) were transfected into podocytes. A) Csi and ADAM17si cells were treated 48 h after transfection with BK ($10^{-8}$ M) or EGF (1 ng/ml) for 7 min. The same cell lysates were immunoblotted concomitantly against ADAM17 (upper panel) and against pERK and tERK. Results are presented as mean ± SE ($n = 3$), **$p < 0.01$; ***$p < 0.001$ versus Csi control; $^\#p < 0.01$ versus Csi BK. B) Csi and ADAM17si cells were treated with $10^{-8}$ M BK for 2 h and shedded epiregulin (ER) was assessed by Western blotting from concentrated supernatant of the cells. Results are presented as mean ± SE ($n = 3$), *$p < 0.05$ versus Csi control; $^\#p < 0.05$ versus Csi BK. Quantified pERK, pEGFR and ER immunostainings are shown in bar graphs; tERK and tEGFR were used as loading controls.

Figure 5. **BK induces ZO-1 rearrangement and decreases podocyte monolayer permeability through EGFR activation.** A) Podocyte monolayer was pretreated for 1 h with $10^{-7}$ M AG1478 or DMSO as Control and stimulated for 1 h with BK ($10^{-8}$ M). ZO-1 distribution was visualized by immunostaining (green, 60X). Texas Red®-X phalloidin was used to visualize actin. Quantified ZO-1 immunostaining intensity is shown in bar graphs next to the images. Results are means ± SE of three independent experiments, **$p < 0.05$ versus DMSO-C (control),
#p<0.05 versus DMSO-BK. B-E) Transepithelial albumin permeability of podocyte monolayer was measured using fluorescence BSA after cells were preincubated for 1 h with 10^{-7} M of AG1478 (EGFR inhibitor) or DMSO control and stimulated for 1 h with 10^{-8} M BK (B). Effect of recombinant ER on the transepithelial albumin permeability of podocyte monolayer was measured after cells were preincubated with 10 ng/ml of epiregulin for 1 h (B insert). Effect of BK2R inhibition was studied using the BK2R inhibitor HOE140 (10^{-5} M) for 1 h before stimulating the cells with for 1 h with 10^{-8} M BK (C). Effect of metalloenzyme inhibition on BK-induced transepithelial albumin permeability was measured (D&E). Cells were preincubated for 30 min with 10^{-5} M GM6001 (broad-spectrum MMP inhibitor) or with its inactive control compound GM C and treated for 1 h with 10^{-8} M BK (D). Control silenced and (Csi) and ADAM17 silenced (ADAMsi) cells were treated 48 h after transfection for 1 h with 10^{-8} M BK and permeability of podocyte monolayer was measured using fluorescence BSA (E). Data are means ± SE of three independent experiments, *p <0.05, NS=non significant change versus untreated control.

Figure 6. **Model of podocyte permeability regulation by BK.** BK induces activation of ADAM17 which sheds the EGFR ligand ER. Activated EGFR induces downstream signaling events and ZO-1 rearrangement which leads to decreased paracellular permeability.
Figure 1

A

Permeability (fold increase)

Bradykinin [log M]

C -10 -9 -8 -7 -6

* * * *

B

Control BK FBS

C

Control BK FBS

WB: ZO-1

WB: GAPDH

D

C 15 BK 15 FBS 15

ZO-1 intensity (fold increase)

E

C 30 BK 30 FBS 30

ZO-1 intensity (fold increase)
Figure 4

A

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<thead>
<tr>
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ADAM17 | [Image of ADAM17 proteins]

pERK | [Image of pERK proteins]

tERK | [Image of tERK proteins]

B

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Epiregulin | [Image of Epiregulin proteins]
Figure 5

A

Control  BK
AG1478  AG1478+BK

B

Permeability (fold increase)

C

Permeability (fold increase)

D

Permeability (fold increase)

E

Permeability (fold increase)