PAR2-induced inflammatory responses in human kidney tubular epithelial cells

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1Centre for Kidney Disease Research, The University of Queensland Department of Medicine at the Princess Alexandra Hospital, Queensland, Australia; 2Department of Nephrology, Princess Alexandra Hospital, Queensland, Australia; and 3Institute for Molecular Bioscience, The University of Queensland, Brisbane, Queensland, Australia

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Vesey DA, Suen JY, Seow V, Lohman RJ, Liu L, Gobe GC, Johnson DW, Fairlie DP. PAR2-induced inflammatory responses in human kidney tubular epithelial cells. Am J Physiol Renal Physiol 304: F737–F750, 2013. First published January 2, 2013; doi:10.1152/ajprenal.00540.2012.—Protease-activated receptor-2 (PAR2) is a G protein-coupled receptor abundantly expressed in the kidney. The aim of this study was to profile inflammatory gene and protein expression induced by PAR2 activation in human kidney tubular epithelial cells (HTEC). A novel PAR2 antagonist, GB88, was used to confirm agonist specificity. Intracellular Ca2+ (iCa2+) mobilization, confocal microscopy, gene expression profiling, qRTPCR, and protein expression were used to characterize PAR2 activation. PAR2 induced a pronounced increase in iCa2+ concentration that was blocked by the PAR2 antagonist. Treatment with SLIGKV-NH2 at the apical or basolateral cell surface for 5 h induced expression of a range of inflammatory genes by greater than fourfold, including IL-1β, TRAF1, IL-6, and MMP-1, as assessed by cDNA microarray and qRTTPCR analysis. Using antibody arrays, GM-CSF, ICAM-1, TNF-α, MMP-1, and MMP-10 were among the induced proteins secreted. Cytokine-specific ELISAs identified three- to sixfold increases in GM-CSF, IL-6, IL-8, and TNF-α, which were blocked by GB88 and protein kinase C inhibitors. Treatment of cells at the basolateral surface induced more potent inflammatory responses, with release of MCP-1 and fibronectin to the apical and basolateral compartments; apical treatment only increased secretion of these factors to the apical compartment. PAR2 activation at the basolateral surface dramatically reduced transepithelial electrical resistance (TEER) whereas apical treatment had no effect. There was very little leakage (<5%) of peptides across the cell monolayer (liquid chromatography-mass spectrometry). In summary, SLIGKV-NH2 induced robust proinflammatory responses in HTEC that were antagonized by GB88. These results suggest that PAR2 antagonists could be useful disease-modifying, anti-inflammatory agents in kidney disease.

protease-activated receptor-2; kidney tubule; inflammation; cytokine

INFLAMMATION IS A COMMON FEATURE in all forms of kidney disease, irrespective of the mechanism of initiation. It is an important protective response that drives wound healing and repair processes. As in other tissues, however, protracted or uncontrolled inflammation in the kidney is detrimental, promoting tissue destruction and fibrosis, which, over time, can lead to organ failure. Mitigating ongoing inflammatory responses would be of therapeutic value. Serine proteases, such as thrombin, tryptase, and trypsin, can cause inflammation by disrupting tissue architecture or by activating certain cell surface receptors, including protease-activated receptors (PARs) (31, 39). The present study investigated the role of the second member of this class of receptor, PAR2, in inflammatory responses in kidney cells.

Protease-activated receptor 2 (PAR2) is a particular class A G protein-coupled receptor that is activated mainly by trypsin-like proteases including trypsin, mast cell tryptase, and coagulation factors Xa and VIIa (3). Proteolytic cleavage of the NH2-terminal extracellular domain of PAR2 exposes a new NH2 terminus, referred to as a tethered ligand, which triggers receptor activation. Short synthetic peptides, corresponding to the human tethered ligand sequence, Ser-Leu-Ile-Gly-Lys-Val-HNH2 (SLIGKV-NH2), can also activate PAR2 in the absence of proteolysis albeit at micromolar instead of nanomolar concentrations. Such hexapeptides have been widely used experimentally as exogenous agonists to tease out roles for PAR2 in physiological and pathophysiological conditions (3, 32).

PAR2 is expressed in a wide range of human tissues, but it is especially prominent in epithelial cells at the interface between the external environment and internal milieu, such as in the gastrointestinal tract, respiratory system, and kidney tubules (6, 15, 21). Although the precise functions of PAR2 in these tissues are unclear, there is evidence for PAR2 regulation of inflammation, cell proliferation, protease sensing, and epithelial barrier function (8, 22, 23, 27, 50). In models of colitis, lung disease, and glomerulonephritis, PAR2 knockout mice (PAR2−/−) are protected to some extent from disease progression (19, 34, 42). Direct infusion of PAR2-activating peptides or serine proteases into the lung or colon leads to increased inflammatory cytokine production and increased epithelial permeability (7–9, 29, 30, 34, 42). Our recent studies have demonstrated that a novel human PAR2 antagonist, GB88, can ameliorate inflammatory disease progression in rat models of paw edema, colitis, and arthritis (29, 30, 43).

Within the human kidney, PAR2 is especially prominent in the proximal tubule cells of the renal cortex and renal vasculature (21, 48, 49). Studies have shown PAR2 involvement in the control of renal blood flow, ion transport, inflammation, and fibrosis (5, 17, 18, 21). Primary cultures of these cortical tubular epithelial cells express high levels of functional PAR2 (49). Activation with the PAR2 agonist, SLIGKV-NH2, elicits a rapid rise in intracellular calcium and subsequent production of the proinflammatory mediator monocyte chemoattractant protein-1 (MCP-1). In the present study, we sought to investigate in more detail the proinflammatory responses in human primary kidney tubular epithelial cells (HTEC) to activation by PAR2 activating peptides, SLIGKV-NH2 and 2f-LIGRLO-NH2, using calcium mobilization assays, microarray analysis, antibody arrays, specific cytokine ELISAs, and with the aid of a new PAR2 antagonist, GB88, the only compound currently known at low micromolar concentrations to inhibit the actions of all known PAR2 agonists.
MATERIALS AND METHODS

Tubule cell isolation and cell culture. Segments of macroscopically and histologically normal renal cortex (~10 g) were obtained aseptically from the noncancerous pole of adult human kidneys removed surgically because of small renal clear cell carcinomas1. Patients were otherwise healthy. Informed consent was obtained prior to each operative procedure and the use of human renal tissue for primary cell culture was reviewed and approved by the Princess Alexandra Hospital Research Ethics Committee, Brisbane, Australia. The method for isolation and culture of human kidney tubular epithelial cells is described in detail elsewhere (49). Briefly, the cortical tissue was minced finely, washed several times, and agitated for 20 min at 37°C in Hanks’ Balanced Salt Solution (HBSS) containing collagenase type II (1 mg/ml) and calcium. Cold HBSS was added and the solution containing tubular fragments passed through a 100-μm sieve. After washing three times, the tubular fragments were resuspended in 45% Percoll in phosphate-buffered saline (PBS) and centrifuged at 20,000 g. A high-density band, previously shown to be tubule fragments, was removed and cultured in a serum-free, hormonally defined DMEM/F12 medium containing 10 ng/ml epidermal growth factor, 5 μg/ml insulin, 5 μg/ml transferrin, 50 μM hydrocortisone, 50 μM prostaglandin E1, 50 nM selenium, 5 μM triiodothyronine, penicillin (50 U/ml), and streptomycin (50 μg/ml). Cells were routinely cultured in this medium.

Peptides, enzymes, and chemicals. The PAR2 activating peptide, SLIGKV-NH2, and a nonactivating peptide, reverse sequence peptide, VKGILS-NH2, were synthesized as carboxyl-terminal amides and purified to >95% via reversed-phased high-performance liquid chromatography by either Auspep (Melbourne, Australia) or the Division of Chemistry and Structural Biology at the Institute for Molecular Bioscience, The University of Queensland. The PAR2 antagonist GB88, PAR2 peptide agonist 2f-LIGRLO-NH2, and synthetic PAR2 agonist were synthesized as previously described (4, 43). Two commercially available protein kinase C (PKC) inhibitors bisindolylmaleimide I (1 μM) and Gö6983 (5 μM) [Merck (Victoria, Australia)] were used to assess the role PKC in PAR2-induced inflammatory cytokine production.

Cell treatments. All experiments were performed on confluent passage 2 HTEC in 96- (black walled), 48-, or 12-well plates (Corning, NY). Cells on transwell filters were cultured in 12-well plates. Before experimentation, cells were made quiescent by two washes followed by incubation for 24 h in basic media (DMEM/F12 medium with antibiotics). Effects of the PAR2 activating peptide, SLIGKV-NH2,2 or control peptide, VKGILS-NH2, on cytokine production were measured by cDNA microarray analysis, inflammation antibody arrays, and cytokine-specific ELISAs (MCP-1, GM-CSF, IL-6, IL-8, TNF-α, and fibronectin). Analysis was at 5 h or 24 h posttreatment. In some experiments, treatment was either to the apical or basolateral monolayer surface. 2f-LIGRLO-NH2 (a more potent PAR2 agonist than SLIGKV-NH2), IL-1β, and transforming growth factor-β (TGF-β) were used in some experiments for comparison and confirmation. Media conditioned by HTEC were harvested and stored at -80°C until assayed.

Quantitative RT-PCR. Cells were grown to confluence and total RNA isolated using a RNeasy Mini Kit (Qiagen) according to manufacturer’s instructions. RNA was reverse-transcribed using Superscript III (Invitrogen) and an oligo(dT) primer. Relative gene expression was quantified by real-time PCR (qRTPCR) using SYBR Green PCR master mix (Applied Biosystems, Foster City, CA) on an

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1 While the tissue used in this study was taken as “normal,” it is possible that it has been influenced at a molecular level by the adjacent cancerous tissue or some characteristic of precancerous cells, despite appearing histologically normal.

2 In initial experiments only SLIGKV-NH2 was available for use. In later experiments we had the option to use 2f-LIGRLO-NH2, which is more potent and apparently more selective. In all experiments SLIGKV-NH2 and 2f-LIGRLO-NH2 produced similar responses, but the latter was about 50-fold more potent.
Applied Biosystems Prism 7000 sequence detector. Amplification cycle proceeded as follows: 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 50°C for 1 min. cDNA levels at the linear phase of amplification were compared with hypoxanthine guanine phosphoribosyl transferase (HPRT) levels, and expressed as a relative expression of HPRT. The sequence of the primers used in this study were HPRT, forward primer 5'-TCA GGC AGT ATA ATC CAA AGA TGG T-3' and reverse primer 5'-AGT CTG GCT TAT ACT CAA CAC TTC G-3'; PAR2, forward primer 5'-GGT TTT GCC AAG TAA CGG C-3' and reverse primer 5'-AGT CTC GTG CAC TCT CGC CTC CAG-3'; IL-6, forward primer 5'-ACC ACC GTA AGG CGC TTG TGG AGA AG-3' and reverse primer 5'-GGT CTT GGC AAA ACT GCA CCT TCA CAC-3'; CCL2, forward primer 5'-AGC AAC ATG ACA GAG AGG-3' and reverse primer 5'-GGA ATT GAT TGC ATC TGG CTG AGC-3'; F3, forward primer 5'-CCG GCG CTT CAG GCA CTA CAA A-3' and

Fig. 2. PAR2 activation on the surface of HTEC. A: inhibition of SLIGKV-NH2-induced iCa2+ release by 1 and 10 μM GB88. Data points are means ± SE from triplicate measurements. *P < 0.005 vs. control, #P < 0.005 vs. SLIGKV-NH2 alone. B and C: confocal imaging of tight junction protein 1 (ZO-1) and PAR2 in HTEC grown on polyester transwell membranes (maximum intensity projection with a ×63 objective). Mouse anti-ZO-1 with Texas Red-labeled secondary antibody and rabbit anti-PAR2 with Alexa fluor 488-labeled secondary antibody. At left the PAR2 antibody was preincubated with the control peptide antigen (1 μg/μl) prior to the primary antibody incubation. The ZO-1 antibody was omitted. C: an orthogonal projection of confocal z stacks.
Table 1. Expression of genes in response to basolateral and apical treatment of human tubular epithelial cells.

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<th>Gene Symbol</th>
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<td>Transmembrane prostate androgen-induced protein</td>
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kines secreted by HTEC treated with SLIGKV-NH₂. In some experiments, cells were treated apically or basolaterally with SLIGKV-NH₂ (100 μM) with or without GB88 for 24 h, and conditioned medium was collected from the apical and basolateral compartments and stored at −80°C until assayed. Conditioned cell medium (0.5–1 ml) was used in each array. GM-CSF, MCP-1, IL-8, IL-6, and tumor necrosis factor-α (TNF-α) were measured using a commercial ELISA kit (RnD Systems, Minneapolis, MN) according to the manufacturer’s instructions.

Fig. 3. qRT-PCR analysis of SLIGKV-NH₂-induced inflammatory gene expression. HTEC were grown to confluence [transepithelial electrical resistance (TEER) > 600 Ω·cm²] on transwell inserts and then treated with or without SLIGKV-NH₂ (100 μM) from the apical or basolateral aspect for 5 h. RNA was extracted and analyzed by qRT-PCR using specific primers for IL-8, IL-1ß, IL-6, TNFα, CCL2, CSF2, IL-11, tissue factor F3, MMP-1, and TNF receptor-associated factor-1 (TRAF-1). Data represent the average of 3 independent experiments using cells from 3 separate donors. Bars: white, no treatment (control); black, basolateral (Baso) treatment; gray, apical treatment. **P < 0.05.
instructions. Matrix metalloproteinase (MMP) secretion by cells was also measured using a MMP antibody array (AAH-MMP-1, RayBio-tech, Norcross, GA) and fibronectin concentrations were measured using a specific fibronectin ELISA (Dako, Noble Park, Australia) as previously described (49).

**cDNA microarray experiments.** Microarray analysis was performed on RNA extracted from primary cultures of HTEC (grown on transwell inserts), following treatment for 5 h with SLIGKV-NH$_2$ (100 μM) or control peptide VKGILS-NH$_2$ (100 μM) at either the apical or basolateral surface. Total RNA was extracted using a Qiagen RNeasy mini kit (Qiagen, Hilden, Germany), and its concentration, purity, and integrity measured using a NanoDrop spectrophotometer and Agilent 2100 Bioanalyzer (Agilent Laboratories, Palo Alto, CA). Biotinylated cRNA was prepared from 200 ng of total RNA using Ambion Illumina RNA amplification kit and hybridized to a Sentrix Human-6 Expression BeadChips (>46,000 gene transcript targets, Illumina, San Diego, CA) using the manufacturer’s hybridization solution. All reagents and protocols for washing, detection, and scanning were used according to the BeadStation 5003 system protocols (Illumina, San Diego, CA).

Genes were considered differentially expressed between agonist-treated, (apical and basolateral), and control peptide treated if they displayed a minimum fourfold change in expression. Additionally, to avoid genes with close to background levels of expression, only a raw signal of >15 times the control sample was considered.

**Liquid chromatography (LC)-mass spectrometry (MS).** After 24-h treatment, medium was collected from both the basolateral and apical compartments of the HTEC monolayers and stored at −80°C until analyzed. For analysis, 100-μl samples were diluted 1:2 in acetonitrile, vortexed, sonicated, and centrifuged (13 K rpm, 5 min). Standard curves of 2f-LIGLRO-NH$_2$ and SLIGKV were constructed using the same method, where stock concentrations were diluted 1:2 in acetoni

![Fig. 4. Human inflammation antibody array of inflammatory factors secreted by HTEC treated for 24 h with PAR2 agonist SLIGKV-NH$_2$. HTEC were grown to confluence in serum-free defined medium, washed twice with buffer, and transferred to basic medium for 24 h, before treatment with PAR2 agonist SLIGKV-NH$_2$ (100 μM). Medium was analyzed in an inflammation antibody. The experiment was repeated 3 times with cells isolated from 3 different patients. A: representative array. B: densitometric analysis of array. Arrows indicate proteins, CMCSF and TNF-α, which showed the greatest changes in expression following SLIGKV-NH$_2$ treatment.](http://ajprenal.physiology.org/doi/10.1152/ajprenal.00540.2012)
nitrile (final concentration 0.12, 1.2, and 12 μM). All samples were analyzed by LCMS/MS (ABSCIEX 4000 QTRAP Triple Quadrupole, Linear Ion-Trap LC/MS/MS mass spectrometer). Chromatography was carried out on a C4 column (Phenomenex, 5 μm, 2.1 × 50 mm) using a linear gradient (5–80% buffer B in 8 min, flow rate 0.35 ml/min). Buffer A was 0.1% formic acid (aq), and buffer B was 90/10 acetonitrile/0.1% formic acid (aq). Retention times were 6.39 and 6.89 min for SLIGKV and 2-furoyl-LIGRLO, respectively, in positive-ion mode.

Statistical analysis. All studies were performed in triplicate from HTEC cultures obtained from at least three separate human donors unless otherwise indicated. Each experiment contained internal controls originating from the same culture preparation. For the purposes of analysis, each experimental result was expressed as a change from the control value, which was regarded as 100%, and analyzed independently. Results are expressed as means ± SE. Statistical comparisons between two groups were made using unpaired t-tests. Multiple group comparisons were made by ANOVA. GraphPad Prism version 5.03 was used to construct graphs and statistical analysis. P values < 0.05 were considered significant. Antibody array densitometric analysis was made using ImageJ 1.45s.

RESULTS

HTEC express high levels of PAR2 mRNA and functional PAR2 protein at their cell surface. We have previously measured PAR2 mRNA expression in 22 established human cell lines by qRT-PCR and found that HT29 colon carcinoma cells and HEK293 human embryonic kidney cells expressed relatively high levels of PAR2 transcript (43). Here we examined kidney HTECs established from normal adult human kidney cortex, which had been cultured under serum-free conditions (passage 2). PAR2 mRNA was threefold more highly expressed in HT29 or HEK293 cells (Fig. 1A, n = 7). An intracellular calcium (iCa2+) mobilization assay was used to measure iCa2+ flux in these three cell types in response to the PAR2 agonist, 2f-LIGRLO-NH2, to gauge whether the cells differentially expressed PAR2 functional protein on the cell surface. The signal intensity for each of the three cell types was very similar (Fig. 1B), suggesting comparable cell surface expression of PAR2 despite different intracellular levels of PAR2 mRNA. The PAR2 peptide agonist 2f-LIGRLO-NH2 (EC50 1.5 ± 0.5 μM) and the synthetic PAR2 nonpeptide agonist GB110 (EC50 1.7 ± 0.8 μM) induced a rapid concentration-dependent increase in intracellular calcium (Fig. 1C). Trypsin-induced responses were approximately two log units more potent than for these synthetic agonists in this particular cell type (EC50 30 ± 23 nM, Fig. 1C). The PAR2 antagonist GB88 effectively inhibited iCa2+ release induced by trypsin, SLIGKV-NH2, GB110, and 2f-LIGRLO-NH2 (Fig. 1D and Fig. 2A) with a similar IC50 (10 μM) in each case in this cell type.

HTEC form monolayers that express PAR2. HTEC grown on polyester transwell membrane inserts formed tight cobblestone monolayers that expressed tight junction protein, ZO-1, and developed TEER. Recent studies (27) have indicated that there is differential signaling via PAR2 at apical and basolateral cell membranes, so we wished to investigate possible differential inflammatory responses to PAR2 activation at these two different cell surfaces. Experiments using cells grown in this format were performed once cells reached a TEER of >600 Ω·cm². Confocal microscopy revealed that whereas ZO-1 was exclusively localized to the cell membrane, prominent punctate PAR2 staining was present in the cytoplasm and to a lesser extent at the cell membrane (Fig. 2B). By construction of Z stacks, PAR2 appeared to be present both apically and basolaterally with no preferential polarized localization (Fig. 2C).

Inflammatory gene expression induced by SLIGKV-NH2. To profile inflammatory gene expression induced in HTEC by PAR2 activation, a cDNA microarray screening approach was initially used. Cells were treated with SLIGKV-NH2 (100 μM) on their
apical or basolateral surface for 5 h, and RNA was extracted, amplified, labeled, and examined using a Sentrix Human-6 Expression BeadChip. For this study, only selected genes that showed an increased transcript expression in response to treatment were reported. Genes were only considered to exhibit differential expression when the raw signal for treated cells was 15 times greater than background and exhibited a ≥4-fold change in expression compared with control cells treated with the non-PAR2-activating control peptide VKGILS-NH2.

A total of 36 genes showed increased expression when cells were treated from the basolateral side, whereas only 11 were induced ≥4-fold when treated from the apical side. None of the genes expressed by apical treatment was different from those expressed in response to basolateral treatment (Table 1). The data indicated that for many genes there was greater induction when treatment was from the basolateral surface. The raw signal was greatest for the genes IL-6, IL-8, and CCL-20. When cells were treated with SLIGKV-NH2 at the basolateral surface the expression of at least 10 genes associated with proinflammatory signaling were significantly induced. This included genes in the TNF gene family (TNF receptor-associated factor 1 (TRAF1) and TNF alpha-induced protein 3 (TNFAIP3)), the IL gene family (IL-8, IL-6, IL-1α, IL-1β, IL-11 and IL-25A), and IL-1 receptor associated protein genes, namely IL-1 receptor-associated kinase 2 (IRAK2) and IL-1 type 2 receptor (IL-1R2). Other similarly upregulated inflammatory genes included the chemokine CCL20, tissue factor (F3), colony stimulating factor 2 (CSF2), pentraxin 3 (PTX3), and intercellular adhesion molecule 1 (ICAM-1). The cyclooxygenase-2 (COX-2) prostanoid synthase gene PTGS2 was not upregulated following PAR2 activation, as has been reported recently for HEK293 cells and other cells (16, 33, 44). A number of growth factors, including platelet-derived growth factor subunit B (PDGFβ), heparin binding epidermal growth factor (HBEGF), epiregulin (EREG), bone morphogenic protein 2 (BMP2), leukemia inhibitory factor (LIF), and angiopoietin-like-4b, (ANGPTL4), were upregulated. Protease genes, including MMP-1 and -10, serine protease 22, PRSS22, and protease inhibitor SERPINB9, were induced. Genes for other MMPs were not induced by SLIGKV-NH2 at 5 h.

RTPCR analysis of upregulated inflammatory genes. Using qRTPCR the expression of 10 of the key inflammatory genes upregulated in the microarray experiment was more closely examined (Fig. 3). All showed a significant upregulation of mRNA in response to SLIGKV-NH2 treatment (3- to 26-fold), which was in a similar induction range to that observed in the microarray experiment. However, whereas the microarray analysis showed

A. P P N N 1 2 3 4

CONTROL SLIGKV-NH2

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<tr>
<td>9</td>
<td>10</td>
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P: Positive control
N: Negative control
MMP1
MMP2
MMP3
MMP8
MMP9
MMP10
MMP13
TIMP1
TIMP3
TIMP4

Fig. 6. Human MMP antibody array of conditioned medium from HTEC treated for 24 h with or without PAR2 agonists SLIGKV-NH2. HTEC were treated with PAR2 agonist SLIGKV-NH2 (100 μM) for 24 h and MMP secretion into the conditioned medium measured using an MMP antibody array. The experiment was repeated 3 times with cells isolated from 3 different patients. A: representative array. B: densitometric analysis.
significantly more induction following basolateral than apical treatment, the qRT-PCR analysis showed that only genes for TNF-α and TRAF1 were significantly different. The gene encoding MCP-1, CCL2, was included in the qRT-PCR analysis as it had been previously shown, at the protein level, to be induced by SLIGKV-NH₂ (49).

**SLIGKV-NH₂ induces secretion of inflammatory cytokines.** A membrane-based human inflammation antibody array was used to determine whether any of the inflammatory genes highlighted in the microarray and qRT-PCR experiments were translated into secreted proteins. A previous study had demonstrated that SLIGKV-NH₂ markedly induces secretion of MCP-1 by proximal tubule cell cultures (49). Figure 4 shows enhanced secretion of a range of inflammatory proteins in response to SLIGKV-NH₂, the most prominent differentially secreted cytokines being ICAM-1, GM-CSF, and TNF-α. Other secreted cytokines and chemokines detected included IL-6, IL-8, MCP-1, regulated on activation, normal T cell expressed and secreted (RANTES), tissue inhibitor of metalloproteinase 2 (TIMP-2), and macrophage inflammatory protein-1 β (MIP-1β), but using this antibody array, there did not appear to be enhanced secretion by treated cells. The mRNA levels of most of these proteins increased within 5 h (Table 1). Although the expression of IL-1α and IL-1β mRNA was markedly induced by SLIGKV-NH₂ (Table 1), no secreted IL-1 was detectable using this antibody array (positions 5 and 6, Fig. 4). Of the three CSF proteins present on the array, CSF1, -2, and -3, at positions 1, 2, and 11, only CSF2 (GM-CSF) appeared to be significantly induced by SLIGKV-NH₂.

To confirm these results, the cytokines were measured using specific ELISAs. IL-6, IL-8, GM-CSF, and TNF-α were all significantly enhanced by SLIGKV-NH₂ (Fig. 5, A–D). Using the samples in the array described above, the ELISA indicated a >10-fold difference in the levels IL-6 and IL-8 between controls and treatment. Release of each of the four cytokines measured was antagonized by the PAR2 antagonist, GB88 (Fig. 5, A–D). As previously described, SLIGKV-NH₂ induced secretion of significant amounts of fibronectin and MCP-1, which were also antagonized by GB88 (not shown).

**Induction of matrix metalloproteinases (MMP) by SLIGKV-NH₂.** A membrane-based human MMP antibody array analysis of medium from HTEC treated with SLIGKV-NH₂ for 24 h demonstrated that secretion of MMP-1 and -10, but not MMP-2, -3, -8, -9, and -13, was upregulated by 4 and >60-fold, respectively (Fig. 6). This correlated with observations from the microarray and qRT-PCR experiments. Tissue inhibitors of metalloproteinases (TIMPs)-1 and -3 were strongly expressed by HTEC, but it is unclear from this array whether levels were affected by treatment.

**Secretion of TNF-α and GM-CSF by HTEC is PKC dependent.** To investigate whether PAR2-induced cytokine secretion involved signaling via PKC, HTEC cells were grown to confluence in 48-well tissue culture plates and then pretreated for 1 h with PKC inhibitors bisindolylmaleimide 1 (1 μM) or Gö6983 (5 μM) prior to 24 h exposure to 2f-LIGRLO-NH₂. Conditioned medium was taken for analysis of GM-CSF and TNF-α. Both of these inhibitors almost completely blocked the secretion of these cytokines (>95% for TNF-α and >90% for GM-CSF, Fig. 7). The concentrations of inhibitors used were similar to those used previously and were nontoxic (MTT assay and lactate dehydrogenase assay; data not shown) (28).

**Differential secretion of cytokines in response to apical or basolateral treatment.** Using cells grown to confluence on transwell inserts, the secretion of MCP-1 and fibronectin was examined in response to treatment with SLIGKV-NH₂ at the basolateral or apical compartments. These responses were compared with those elicited by TGF-1β (5 ng/ml), a known inducer of fibronectin production by HTEC, and by IL-1β, a known inducer of both fibronectin and MCP-1 (11, 47, 49). Under control conditions, significantly more fibronectin and MCP-1 were secreted into the apical compartment (3:1). As expected, treatment of the cells with SLIGKV-NH₂ from the basolateral or apical surface increased secretion of both fibronectin and MCP-1. However, whereas apical treatment with SLIGKV-NH₂ only increased secretion of these molecules from the apical surface, basolateral treatment appeared to increase secretion from both membrane aspects. TGF-1β only increased secretion of fibronectin from the surface to which it was added. IL-1β (1 ng/ml), on the other hand, appeared to induce secretion into both compartments irrespective of the treatment surface (Fig. 8).

**SLIGKV-NH₂ reduces transepithelial resistance (TEER) only when cells are treated from the basolateral surface.** Basolateral PAR2 activation using SLIGKV-NH₂ and 2f-LIGRLO-NH₂ has previously been shown to compromise the integrity of lung and intestinal epithelial cell monolayers (27, 50). Altered HTEC monolayer integrity was thus considered as a possible explanation for enhanced levels of fibronectin and MCP-1 in...
the apical compartment following basolateral treatment. To test this idea, the TEER across the HTEC monolayers was measured at various times following treatment. Basolateral treatment of HTEC with SLIGKV-NH₂ induced a concentration- and time-dependent loss of TEER. At 100 μM SLIGKV-NH₂, TEER was decreased by 70% within 1 h, and it remained so for the remainder of the experiment (24 h). At 24 h, 25 and 12.5 μM SLIGKV-NH₂ decreased TEER by approximately 40% and 25%, respectively. When cells were treated with 100 μM SLIGKV-NH₂ from the apical surface there was no alteration in TEER (Fig. 9). IL-1β (1 ng/ml) added either to the basolateral or apical compartment greatly reduced TEER by >70% within 1 h. TGF-β (1 ng/ml) added at the apical or basolateral compartment failed to influence TEER. GB88 at 10 μM failed to antagonize the SLIGKV-NH₂-induced reduction in TEER. In fact GB88 alone reduced TEER by >50% over the course of the experiment. The PKC inhibitor Bis-1 failed to reduce the SLIGKV-NH₂-induced reduction in TEER. Alone it had no action on TEER.

**PAR2 activating peptides fail to cross epithelial monolayers.** One possibility to account for the enhanced secretion of MCP-1 and fibronectin when cells were treated from the basolateral surface is that the activating peptides are able to pass through the epithelial monolayer when the TEER is reduced and then also activate apical PAR2. Using LC-MS we were unable to detect significant passage of the peptides (<5%) from one side of the cell layer in either direction even after 24 h of basolateral treatment (Fig. 10).

**DISCUSSION**

PAR2 was originally cloned in 1994 and was shown to be widely expressed by human tissues, notably the skin, pancreas, liver, gut, and kidneys (6, 15, 35). Epithelial cells lining the gut, where trypsin and other PAR2-activating proteases are present at relatively high concentrations, express especially high levels of PAR2 at both the apical and basolateral membranes. PAR2 here is known to have important roles in ion transport, barrier protection, and inflammatory responses (12, 26, 30, 32, 36, 49, 50), but its physiological importance in kidney, where serine proteases are not normally present in appreciable concentrations, is unknown (48). This study has been directed toward examining inflammatory responses elicited by PAR2 activation in tubular epithelial cells derived from the cortex of human kidneys.

HTEC cells express relatively high levels of PAR2 mRNA, threefold higher than in HEK293 and HT29 cells, which were among the highest PAR2 expressing cells in a recent study of 22 human cell lines commonly used in PAR2 studies (32). Confocal microscopy demonstrated that PAR2 protein was located both at the cell surface and within the cytoplasm, with no apparent preference for apical or basolateral membrane expression. This was consistent with comparable iCa²⁺ release induced by PAR2 agonist 2f-LIGRLO-NH₂ in all three cell types, and indicates that HTEC have significantly greater cytosolic reserves of PAR2 mRNA on hand for translation to protein and rapid mobilization to the cell surface as needed. This may be a consequence of a need for the kidney to maintain high surveillance and sensitivity to the presence and action of proteases that can potentially degrade proteins leading to tissue damage and renal dysfunction. In line with this protective measure, a number of studies have shown upregulation of PAR2 by inflammatory mediators such as IL-1, TNF-α, and lipopolysaccharide (LPS) and, interestingly, also PAR2 agonists (38, 44). Although not reported here, because it was below the fourfold induction cut-off, SLIGKV-NH₂ induced a 1.5-fold increase in PAR2 mRNA expression.
HTEC respond to a number of functional modulators of PAR2, such as the endogenous agonist trypsin, the synthetic hexapeptide agonist 2f-LIGRLO-NH₂, the synthetic nonpeptide agonist GB110, and the nonpeptide antagonist GB88, in a similar manner to many other cell types (43). However, slightly higher concentrations of these agonists seem to be required to elicit comparable agonist and antagonist effects in HTEC than other cell types (43). The iCa²⁺ response of HTEC to trypsin showed considerable variability (EC₅₀ 30 ± 23 nM, n = 7), in contrast to that seen with the synthetic agonists where the EC₅₀ was relatively tight (1.6 ± 0.7 μM, n = 7). This variability may relate to nonspecific receptor activation by trypsin contributing some Ca²⁺ release, or to PAR2 polymorphisms, or to different glycosylated PAR2 isoforms, which can alter or mask the protease cleavage/activation site (13, 14). The PAR2 antagonist used in these studies, GB88, was effective at blocking iCa²⁺ mobilization induced by all three agonist types used in these studies on HTEC cells.

Using cDNA microarray analysis and qRT-PCR we profiled the proinflammatory genes induced in HTEC within 5 h by activation of PAR2 with the human PAR2 tethered ligand sequence SLIGKV-NH₂, and also assessed protein expression after 24 h using cytokine protein arrays and individual ELISAs. Other studies have consistently shown that activation of PAR2 induces expression of proinflammatory proteins, such as COX-2, TNF-α, IL-8, and IL-6 in a variety of cell types including lung, intestinal, and gastric epithelia and blood monocytes (24, 37, 44). A recent report by Suen et al. compared the expression of genes induced by the PAR2 agonists, 2f-LIGRLO-NH₂ and trypsin, in the human embryonic kidney cell line HEK239 (44). Proinflammatory genes, COX-2, IL-8, and TNF-9, were among those induced by both agonists and could be therefore attributed specifically to PAR2 induction. In the present study, gene profiling of HTEC exposed basolaterally to SLIGKV-NH₂ for 5 h induced 36 genes by fourfold or more, many of which have previously been associated with proinflammatory responses. Treatment at the apical surface induced a similar array of genes but the induction level was much reduced. Ten of the genes examined by qRT-PCR supported the finding of enhanced inflammatory gene expression following PAR2 activation. However, only the genes for TRAF1 and TNF-α were more consistently, strongly, and differentially induced by treatment at different cell surfaces. These findings are consistent with other studies, where basolateral PAR2 activation elicited a more pronounced response (27, 46). Using antibody cytokine arrays it was shown that a number of proinflammatory genes expressed after PAR2 activation are then converted to protein and secreted. The most

![Fig. 9. Basolateral but not apical treatment of HTEC with SLIGKV-NH₂ causes loss of TEER (A) and is not blocked by a PAR2 antagonist, GB88 (B). Cells were grown to confluence on membrane transwells. The TEER before treatment was ∼600 Ω·cm². They were then treated either on the apical or basolateral surface with SLIGKV-NH₂ at the indicated concentrations, and 24 h after treatment, the TEER was again measured. Data presented are means ± SE for representative experiments (n = 4). Measurements were made with a Milli-cell-ERS (Millipore). Each measurement was the mean of 3 separate measurements. *P < 0.05.](http://ajprenal.physiology.org/content/early/2012/08/28/ajprenal.00540.2012)

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prominent of these proteins were the known proinflammatory agents GM-CSF, TNF-α, MMP-1, and MMP-10. The production of IL-6, IL-8, GM-CSF, and TNF-α was suppressed by GB88, consistent with their PAR2-dependent expression.

Like other GPCRs, PAR2 activation is known to elicit biological responses via activation of various G proteins of which the G_{q/11} subunit is known to be central. G_{q/11} activates phospholipase C, which in turn cleaves phosphatidylinositol 4,5-bisphosphate (PIP2) to produce diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3). These mediators then lead to activation of PKC and 
\[ \text{iCa}^{2+} \] release. To determine if PKC is involved in PAR2-mediated cytokine production, two commercial PKC inhibitors were used to block PKC activity. Without causing toxicity, the secretion of GM-CSF and TNF-α were almost completely abolished, suggesting the importance of PKC in PAR2-mediated GM-CSF and TNF-α secretion. Recent studies have also shown the important involvement of PKC in PAR2-induced inflammation (2, 10).

The reduction in TEER following basolateral, but not apical treatment of polarized HTEC with PAR2 agonists SLIGKV-NH_2 and 2f-LIGRLO-NH_2, may indicate loss of epithelial monolayer integrity, such that proteins and peptides (including the PAR2 agonists) can pass from one side of the membrane to the other. The apparent increase in fibronectin and MCP-1 secretion from the apical cell surface following basolateral treatment may also reflect this. We found no evidence that either SLIGKV-NH_2 or 2f-LIGRLO-NH_2 could pass through the epithelial monolayer. The fact that GB88 failed to reduce the SLIGKV-NH_2-induced reduction in TEER may be related to the fact that GB88 is a PAR2 pathway-specific antagonist (43). It specifically blocks G_{q/11} mediated intracellular signaling pathways that lead to 
\[ \text{iCa}^{2+} \] release, but fails to antagonize ERK1/2 activation. The inability of Bis-1 to block the SLIGKV-NH_2 induced reduction in TEER suggests that PAR2-induced cytokine production and loss of TEER are mediated by different PAR2 signaling pathways.

This finding may be important. Inflammatory responses in kidney tubules may be mediated by serine proteases arising from sources within the organ parenchyma (i.e., the basolateral side), and not the lumen of the tubular organ (i.e., external to the tissue). This would be consistent with chronic inflammatory conditions in the kidney being driven by endogenous proteases (such as tryptase produced by mast cells), and not those present in lumen (apical portion) of the tubular tissue, such as those that could be produced by bacteria, viruses, or other pathogens. This finding may help guide drug treatment regimes for kidney diseases, whereby drugs which target PAR2 basolaterally may be more effective than those which target apical PAR2. This viewpoint parallels current thinking about PAR2 activation and PAR2 antagonist treatment in the colon (27).

A large number of serine proteases (>30) have now been shown to activate PAR2 in vitro (1). The clotting factors X and VII, when activated, are able to form PAR2-activating complexes with tissue factor (TF-FXa-FVIIa). Clotting disorders that affect the kidney, such as thrombotic microangiopathy, could lead to basolateral PAR2 activation via this mechanism. Mast cells, which produce PAR2-activating proteases chymase and tryptase, have been shown to accumulate in the diseased kidney and promote inflammation and fibrosis and circulating tryptase levels increase with renal dysfunction (20, 40, 41, 45). Trypsinogen has also been shown to be produced by kidney tubule cells (25). Its function in the kidney, however, is unclear. Its activation following injury or infection may well lead to PAR2 activation.

In summary, there is increasing recognition of the roles of proteolytic enzymes in cell signaling and inflammation. A key receptor for their actions is PAR2, which is expressed on the surface of a range of human cell types. This study shows that PAR2 expression is considerably higher in HTEC than in many of the cell types previously recognized as among the most important in PAR2 research, raising questions about the roles for PAR2, and its activating proteases, in the kidney. Here we have profiled effects of PAR2 agonists and antagonists in

![Graph showing the permeability of SLIGKV-NH_2 and 2f-LIGRLO-NH_2](http://ajprenal.physiology.org/fig/10.png)
HTEC and discovered that proinflammatory gene and protein expression is mediated through PAR2 and can be downregulated by a PAR2 antagonist, GB88. Moreover, treatment of HTEC with SLIGKV-NH₂ at the basolateral cell surface was more effective than at the apical cell surface, and induced more potent responses in expression of inflammatory genes and inflammatory proteins. These responses were substantially blocked by the PAR2 antagonist GB88 and by commercially available PKC inhibitors. These findings indicate that PAR2 is an important mediator of inflammatory responses in the kidney, and suggests that PAR2 antagonists could have promising anti-inflammatory activity in chronic kidney diseases.

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DISCLOSURES

J. Y. Suen and D. P. Fairlie are inventors on a patent AU20109033378 covering PAR2 agonists and antagonists that is owned by The University of Queensland.

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