Proinflammatory and proliferative responses of human proximal tubule cells to PAR-2 activation

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Vesey DA, Kruger WA, Poronnik P, Gobé GC, Johnson DW. Proinflammatory and proliferative responses of human proximal tubule cells to PAR-2 activation. Am J Physiol Renal Physiol 293: F1441–F1449, 2007. —Despite the abundant expression of protease-activated receptor (PAR)-2 in the kidney, its relevance to renal physiology is not well understood. A role for this receptor in inflammation and cell proliferation has recently been suggested in nonrenal tissues. The aims of this study were to demonstrate that human proximal tubule cells (PTC) express functional PAR-2 and to investigate whether its activation can mediate proinflammatory and proliferative responses in these cells. Primary human PTC were cultured under serum-free conditions with or without the inflammatory and proliferative responses in these cells. The primary human PTC were cultured under serum-free conditions with or without the PAR-2-activating peptide SLIGKV-NH₂ (up to 800 nM). Trypsinogen expression (RT-PCR), intracellular Ca²⁺ mobilization (fura-2 fluorescence), DNA synthesis (thymidine incorporation), fibronectin production (ELISA, Western blotting), and monocyte chemotactic protein (MCP)-1 secretion (ELISA) were measured. Trypsinogen expression in kidney and PTC cultures was determined by immunohistochemistry and Western blotting. In the kidney PTC were the predominant cell type expressing PAR-2. SLIGKV-NH₂, but not VKGIS-NH₂, stimulated a rapid concentration-dependent mobilization of intracellular Ca²⁺ and ERK1/2 phosphorylation and, by 24 h, increases in DNA synthesis, fibronectin secretion, and MCP-1 secretion. These delayed responses appeared to be independent of ERK1/2. Trypsin produced similar rapid but not delayed responses. Trypsinogen was weakly expressed by PTC in the kidney and in culture. In summary, PTC are the main site of PAR-2 expression in the human kidney. In PTC cultures SLIGKV-NH₂ initiates proinflammatory and proliferative responses. Trypsinogen expressed within the kidney has the potential to contribute to PAR-2 activation in certain circumstances.

Proteinase-activated receptor-2; fibronectin; monocyte chemotactic protein-1; DNA synthesis

Protease-activated receptor (PAR)-2 is the second member of a unique subfamily of G protein-coupled receptors (GPCRs) of which there are four known members (PAR-1 to -4). Unlike other GPCRs, which are activated by binding of extracellular ligands, PARs are activated by proteolytic cleavage of their NH₂-terminal extracellular domain, by certain serine proteases, to expose a new NH₂ terminus that then acts as a tethered ligand, binding intramolecularly to the second extracellular loop of the receptor (4, 11, 29). A peptide of six amino acids in length corresponding to the distinct tethered ligand domain of PAR-2 has been designed that can selectively activate PAR-2 in the absence of proteolytic cleavage (16). This peptide has been used experimentally to investigate PAR-2 function.

The cloning of PAR-2 in the mid-1990s was accompanied by the demonstration that PAR-2 mRNA is expressed in a wide variety of human tissues including notably the small intestine, colon, liver, pancreas, prostate, and kidney (5, 27). However, its role in these tissues is still poorly understood. Infusion or injection of PAR-2-activating peptides or proteases into the lungs, colon, or joints of mice induces pronounced inflammatory responses that include enhanced cytokine production, increased transcellular permeability, inflammatory cell infiltration, and swelling (6, 13, 31). These responses were greatly reduced in PAR-2-null mice. In vitro studies also indicate the involvement of PAR-2 in inflammatory responses; PAR-2 agonists can activate NF-κB in cultured cells, and PAR-2 is upregulated in cultured cells in response to treatment with inflammatory cytokines (23, 28).

Despite the initial reports of PAR-2 expression in the kidney, there have been limited studies of its cellular localization or function here. By immunohistochemistry (IHC) the PAR-2 protein has been reported in renal vasculature, epithelial, and mesangial cells (12, 17). In an isolated perfused rat kidney model, PAR-2 activation was shown to partially reverse the vasoconstrictor effects and reduction in glomerular filtration rate caused by angiotensin II by nitric oxide-dependent and -independent mechanisms (15, 17, 36). Grandaliano et al. (14) reported increased proximal tubule cell (PTC) PAR-2 mRNA and protein expression in biopsies taken from patients with IgA nephropathy and reported that PTC in culture elaborate transforming growth factor-β1 and plasminogen activator inhibitor-1 in response to PAR-2 activation. More recently, Xiong et al. (37) reported increased expression of PAR-2 by tubulointerstitial cells in a mouse model of unilateral ureteral obstruction. These reports suggest a potential role for PAR-2 in renal inflammation and fibrosis (33).

Serine proteases that could potentially activate PAR-2 in the kidney include those expressed by epithelial cells (e.g., trypsin), coagulation proteases (e.g., factor VIIa), and inflammatory cell proteases (e.g., mast cell tryptase). Tryptase is considered a potential PAR-2 agonist in the kidney because it can stimulate renal fibroblast proliferation and matrix protein production, and mast cells have been shown to accumulate in the renal cortex in various renal disease states (19, 20). However, other proteases, including epithelial proteases, remain potential PAR-2 agonists in the kidney.
We hypothesize that PAR-2 expressed in the human kidney can mediate proinflammatory and proliferative events that may be relevant to renal disease. We set out to explore this possibility with primary cultures of PTC.

**MATERIALS AND METHODS**

Renal tissue and methods. Renal tissue samples used to localize PAR-2 and trypsinogen/trypsin by IHC were collected by Princess Alexandra Hospital (Brisbane, Australia) Tissue Bank personnel, fixed in formalin, and embedded in paraffin according to standard histological procedures. These samples had been taken from the noncancerous pole of adult human kidneys removed surgically because of small renal clear cell carcinomas (n = 4), papillary cell carcinomas (n = 2), or a renal oncocytoma (n = 1). The average patient age was 57 ± 13 yr, and the male-to-female ratio was 5:2. For PTC isolation 5–10 g of renal cortex was obtained aseptically from the normal pole of adult human kidneys. Patients were otherwise healthy. Informed consent was obtained before each operative procedure, and the use of human renal tissue for primary culture was reviewed and approved by the Princess Alexandra Hospital Research Ethics Committee.

Cell culture. The method for isolation, culture, and characterization of PTC is described in detail elsewhere (7, 18, 34). Briefly, the cortical tissue was minced finely, washed several times, and agitated for 20 min at 37°C in Krebs-Henseleit buffer (KHB) containing collagenase type II (1 mg/ml), (Worthington, Freehold, NJ). Cold KHB was added, and the solution was passed through a 297-μm sieve (50 mesh) (Sigma-Aldrich, Sydney, Australia). After being washed three times, the tubular fragments were resuspended in 45% Percoll (Amersham Pharmacia Biotech, Uppsala, Sweden)-KHB and centrifuged at 20,000 g. A high-density band previously shown to be enriched in tubule fragments was removed and cultured in a serum-free, hormone-defined DMEM-F-12 containing 50 nM hydrocortisone, 5 μg/ml insulin, 5 μg/ml transferrin, 50 nM hydrocortisone, 50 μM prostaglandin E1, 50 nM selenium, and 5 μM triiodothyronine. All these supplements were obtained from Sigma-Aldrich.

Peptides, enzymes, and chemicals. The PAR-activating peptide SLIGKV-NH₂ and the control peptide VKGILS-NH₂ were synthesized with carboxy-terminal amidation and purified to >95% via high-performance liquid chromatography by Auspep (Melbourne, Australia). Bovine pancreatic trypsin (240 U/mg protein) was purchased from Worthington Biochemical, and the mitogen-activated protein/extracellular signal-regulated kinase (ERK) kinase kinase 1 (MEK) inhibitor PD-98059 was from Merck (San Diego, CA).

Cell treatment. All experiments were performed on confluent passage 2 PTC cultured in 48-, 24-, or 6-well tissue culture plates (Nalge Nunc, Rochester, NY). Cells were made quiescent by two washes followed by incubation for 24 h in basic medium (DMEM-F-12 containing 5 μg/ml transferrin). The concentration-dependent effects of trypsin, the PAR-2-activating peptide SLIGKV-NH₂, or the control peptide VKGILS-NH₂ on DNA synthesis, fibronectin production, and monocyte chemotactic protein (MCP)-1 secretion were then assessed at 24 h. Media conditioned by PTC were harvested, mixed with a cocktail of protease inhibitors, and stored at −80°C until assay. For Western blotting, cells were washed twice with ice-cold PBS and harvested as described in Western blot analysis. In some cultures MEK inhibitor PD-98059 was included at 5 or 10 μM to determine whether ERK1/2 is involved in these responses.

Fibronectin determination. The fibronectin concentration in the culture supernatant was determined with a sandwich ELISA as previously described (34).

MCP-1 determination. MCP-1 was measured in conditioned culture media with a specific ELISA (R&D Systems) according to the manufacturer’s protocols.

Mitogen-activated protein kinase ERK1/2 phosphorylation. Cells were grown to confluence on six-well culture plates in defined medium. They were then washed with basic medium and incubated for 24 h. Cells were washed a further two times with basic medium at 1 h and 30 min before exposure to PAR-2 agonists for 2–20 min. Experiments were terminated by washing the cells with ice-cold PBS and addition of cold lysis buffer. Mitogen-activated protein kinase (MAPK) ERK1/2 phosphorylation was assessed by Western blotting using specific antibodies.

Measurement of cytosolic Ca²⁺. Intracellular Ca²⁺ measurements were performed with the fluorescent ratiometric Ca²⁺ dye fura-2 (fura-2 AM, Molecular Probes) on cells grown to confluence on black 96-well plates (Perkin Elmer Life and Analytical Sciences, Melbourne, Australia) as previously described (34). An automated injection of agonist was made to give overall concentrations of SLIGKV-NH₂ and VKGILS-NH₂ of 50 or 100 μM in the corresponding wells. Trypsin was used at 0.1–100 nM. The ratio of the excitation wavelengths at 340 and 380 nm represents the change in intracellular Ca²⁺ in response to the agonist. All experiments were performed on a BMG Fluorstar Optima (BMG Lab Technologies, Offenberg, Germany).

Western blot analysis. Cells were cultured in 10-cm dishes or six-well plates. After treatment for various time periods as indicated,
media and cells were harvested. Conditioned media were removed, centrifuged at 1,000 g, and stored at −80°C. In some cases the conditioned media were concentrated 20-fold with Nanosep 3K omega spin columns (Pall Life Sciences, Melbourne, Australia) before use. Cells were washed twice with ice-cold PBS, incubated at 4°C for 10 min with lysis buffer, and prepared for electrophoresis as previously described (34). Equal amounts of conditioned medium or cell protein were diluted in a reducing SDS-PAGE sample buffer, heated to 70°C for 10 min, separated on a 4 –12% NuPAGE gel (Invitrogen, Mt Waverley, Australia) and electrotransferred to a polyvinylidene difluoride membrane (Pall Life Sciences). Membranes were blocked overnight with 5% (wt/vol) skim milk powder in PBS containing 0.1% (vol/vol) Tween 20 and 1 mM sodium orthovanadate. The primary antibodies used were a fibronectin monoclonal antibody (BD, Sydney, Australia; no. 610077, 1:10,000), phospho-MAPK p42/p44 and MAPK p42/p44 monoclonal antibodies (Cell Signaling, Danvers, MA; nos. 9101 and 9102, 1:1,000), and a trypsin rabbit polyclonal antibody (Rockland, Gilbertsville, PA; 1:6,000). Anti-mouse or anti-rabbit peroxidase-conjugated antibodies (Bio-Rad Laboratories, Regents Park, Australia), were used at recommended dilutions for the secondary antibody. Detection was with ECL-plus (Amersham Pharmacia Biotech, Amersham, UK).

Cell growth. PTC damage was assessed with a cytotoxicity detection kit (Roche, Dee Why, Australia), which measures lactate dehydrogenase release into the culture medium. Manufacturer’s protocols were followed. Tritiated thymidine (no. TRA120, Amersham Pharmacia Biotech) incorporation into cellular DNA, an index of DNA synthesis, was measured after washing and precipitation of cells with trichloroacetic acid, as previously described (34). Cell precipitates were dissolved in 0.3 M NaOH containing 1% sodium dodecyl sulfate and taken for liquid scintillation counting in a beta counter. Results were corrected for cellular protein content.

Detection of PAR-2 with RT-PCR. PTC were grown to confluence as described under Cell culture. Total RNA was extracted from the cells with the RNeasy Mini Kit (Qiagen, Doncaster, Australia) according to the manufacturer’s protocols. During the isolation the RNA was subjected to DNase digestion. Two micrograms of RNA was converted into cDNA with Expand reverse transcriptase (Roche) with standard methodologies. The primer sequences and thermal cycling temperatures for PAR-2 have been published previously (3, 34). PCR products were separated on 1.8% (wt/vol) agarose gel containing

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Fig. 2. Reverse transcription-polymerase chain reaction (RT-PCR) analysis of PAR-2 expression by human PTC. Total RNA was isolated from confluent passage 2 human PTC, reverse-transcribed, and amplified with PAR-2-specific primers. PCR products were separated on 1.8% agarose gels containing ethidium bromide and photographed. PTC 1 and PTC 2 (582 bp); RNA was isolated from PTC cultures from 2 different patients. Neg C, negative control. Markers, φX174 DNA/HaeIII markers.

Fig. 3. Intracellular Ca2+ mobilization in primary human PTC cultures by trypsin (A and B) and a PAR-2-activating peptide, SLIGKV-NH2 (C). Cells were grown to confluence and loaded with fura-2 AM. They were then exposed to trypsin (0.1–100 nM), PAR-2-activating peptide SLIGKV-NH2 (50 or 100 μM), or control peptide VKGILS-NH2 (100 μM), and intracellular Ca2+ fluorescence was measured. Results are expressed as the change from basal level of the fluorescence ratio (340/380 nm). Each trace is an average response from cells in 4 different wells and is representative of 2 different experiments.
Fig. 4. Extracellular signal-regulated kinase (ERK)1/2 phosphorylation in primary cultures of human PTC by PAR-2-activating peptide SLIGKV-NH2 and trypsin. Cells were grown to confluence in 6-well plates, washed twice with fresh medium, and incubated for 24 h in nonsupplemented DMEM-F-12. Cells were further washed twice, 1 h and 0.5 h before treatment with fresh warm medium. Cells were treated as indicated, harvested in lysis buffer, and prepared for Western blotting.

A: concentration-dependent increase in ERK phosphorylation in response to SLIGKV-NH2. 
B: phosphorylation of ERK in response to SLIGKV-NH2 and VKGILS-NH2. 
C: concentration-dependent increase in ERK phosphorylation in response to trypsin. 
D: time-dependent increase in ERK phosphorylation in response to SLIGKV-NH2. Con, control.

Fig. 5. Effect of trypsin, PAR-2-activating peptide SLIGKV-NH2, and control peptide VKGILS-NH2 on human PTC DNA synthesis (A), monocyte chemotactic protein (MCP)-1 secretion (B), and cell protein levels (C). Confluent passage 2 human PTC were treated with or without trypsin (1–100 nM), SLIGKV-NH2 (up to 800 μM), or VKGILS-NH2 (200 μM) for 24 h and assayed for the above responses as detailed in MATERIALS AND METHODS. Data points are means ± SE from at least 3 independent experiments, each performed in triplicate *P < 0.05.
1 μg/ml ethidium bromide in 1× Tris-borate-EDTA buffer and viewed and photographed under ultraviolet light. A HaeIII digest of φX174 was used as a marker. The 528-bp PAR-2 PCR products were column purified (Qiagen, Doncaster, Australia) and subsequently sequenced in both directions. Sequencing of the PCR-amplified products was performed by the Australian Genome Research Facility (University of Queensland, Brisbane, Australia). nBLAST analysis was performed to verify the identity of the sequences (accession no. NM_005242).

IHC staining of renal tissue for PAR-2 and trypsinogen/trypsin. IHC was performed on 4-μm-sectioned paraffin-embedded tissue with routine histological procedures. The primary antibodies used were a mouse anti-human PAR-2 (SAM-11, Santa Cruz; 1:50) and a rabbit anti-human trypsin (MAB1482, Chemicon, Boronia, Australia; 1:500 for kidney sections, 1:1,000 for pancreas positive control section). The PAR-2 antibody binds to amino acids 37–50 at the NH2 terminal of the receptor. Dako Envision+ (Dako, Botany, Australia) and 3,3′-diaminobenzidine hydrochloride were used.

Statistical analysis. All studies were performed in triplicate from PTC cultures obtained from three separate donors. 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 unless otherwise stated. Statistical comparisons between two groups were made with unpaired t-tests. Multiple-group comparisons were made by analysis of variance (ANOVA). The SPSS software package (version 11.5) was used. P values < 0.05 were considered significant.

RESULTS

Human kidney PTC express PAR-2 protein. Although there was variability in the staining intensity between samples, they all showed cytoplasmic PAR-2 staining of PTC. A representative image is shown in Fig. 1A. In Fig. 1B the primary PAR-2 antibody has been omitted. In some sections there was also staining of the distal and collecting tubules. The glomerulus was negative, apart from a few cells near or in the Bowman capsule in some sections. Vascular smooth muscle cells within some renal blood vessel walls stained strongly.

Primary cultures of human PTC express PAR-2. By conventional RT-PCR, primary cultures of human PTC expressed PAR-2. Figure 2 shows a strong PCR product of 582 bp for cells isolated from the kidneys of two donors. When sequenced, these products corresponded exactly with the published PAR-2 sequences.

Intracellular cytosolic Ca2+ is rapidly mobilized in response to PAR-2 activation. Both trypsin and SLIGKV-NH2, but not VKGILS-NH2 (up to 200 μM), simulated rapid concentration-dependent increases in cytosolic Ca2+ (Fig. 3). Significant increases in intracellular Ca2+ were first observed at 1 nM trypsin. The EC50 for trypsin was 4.6 ± 1.5 nM. With 10 nM trypsin, the peak response was attained ~40 s after its addition.

ERK1/2 is activated by PAR-2 agonists in PTC. The ability of SLIGKV-NH2 and trypsin to activate ERK1/2 in cultured
PTC was investigated. In initial studies background ERK1/2 phosphorylation was found to be high, which obscured attempts to measure phosphorylated ERK1/2 levels in response to PAR-2 agonists. Subsequently we found that this background could be reduced by washing the cells with fresh medium two times, 1 h and again 0.5 h before experiments were initiated. Both SLIGKV-NH$_2$ (25–100 µM) and trypsin (2.5–10 nM) stimulated a rapid phosphorylation of ERK1/2 (Fig. 4, A–C). ERK1/2 was phosphorylated within 2 min of SLIGKV-NH$_2$ addition (Fig. 4D).

PAR-2 activation stimulates DNA synthesis in human PTC. Trypsin stimulated a small increase in DNA synthesis. Significant ($P < 0.05$) increases were only detected at 12.5 and 25 nM trypsin. The peak response of 133% of control levels was observed at 25 nM (Fig. 5A). Concentrations above 50 nM caused significant cell detachment. SLIGKV-NH$_2$ enhanced DNA synthesis in PTC in a concentration-dependent manner (Fig. 5A). Significant responses were only observed at concentrations of $\geq$100 µM. The maximal response of 300% of control was at 400 µM. The control peptide VKGILS-NH$_2$ had no effect on DNA synthesis in these cells. Cell viability (not shown) or protein levels were not significantly affected by these peptides over the 24-h test period (Fig. 5C). The MEK inhibitor PD-98059 at concentrations of 5 and 10 µM significantly reduced the DNA synthetic response induced by SLIGKV-NH$_2$. At these concentrations there was also a significant reduction in DNA synthesis in untreated cells (see Fig. 7A).

PAR-2 activation stimulates MCP-1 secretion in PTC. SLIGKV-NH$_2$ was found to enhance MCP-1 production by human PTC in a concentration-dependent manner (Fig. 5B). A significant increase in MCP-1 secretion in response to SLIGKV-NH$_2$ was first seen at 50 µM. At a concentration of 400 µM MCP-1 concentrations were 356 ± 70% of control values. MCP-1 secretion in the medium of untreated control cells was $\sim$10 ng·mg cell protein$^{-1}$·day$^{-1}$. Trypsin, on the other hand, did not significantly increase MCP-1 secretion. The MEK inhibitor PD-98059 did not significantly reduce MCP-1 secretion by PTC treated with SLIGKV-NH$_2$ (see Fig. 7C).

PAR-2 activation stimulates fibronectin secretion by human PTC. Figure 6A shows the effect of trypsin and SLIGKV-NH$_2$ on fibronectin secretion by PTC. SLIGKV-NH$_2$ increased fibronectin secretion as measured by ELISA. Significant effects of SLIGKV-NH$_2$ were first observed at 50 µM and increased in a concentration-dependent fashion up to 800 µM. PTC cultures secreted between 125 and 250 ng fibronectin/mg cell protein per 24 h. The MEK inhibitor PD-098059, at 5 and 10 µM, significantly reduced basal and SLIGKV-NH$_2$-induced fibronectin secretion (Fig. 7B). Trypsin also appeared to significantly increase fibronectin secretion by PTC. At 10 nM trypsin, fibronectin levels were twice those found in control cultures.

Western blotting was also used to examine fibronectin production by PTC. In control cultures cell-associated and secreted fibronectin routinely appeared as a single band with an apparent molecular mass of 260 kDa (Fig. 6, B–D). SLIGKV-NH$_2$ treatment was observed to significantly enhance secreted fibronectin and, to a small extent, cell-associated fibronectin (Fig. 6, B–D). Treatment of the cells with trypsin >2 nM, however, resulted in cleavage of the 260-kDa band in the cell culture medium and the appearance of multiple lower-molec-
ular-mass bands at 194, 138, 71, 42.7, and 34.9 kDa (Fig. 6B). There were marginal increases in cell-associated fibronectin levels in response to trypsin treatment. There was no evidence of fibronectin cleavage products in cell lysate from these cells.

_Trypsinogen is expressed by the human kidney._ Pancreatic trypsin is a potent activator of PAR-2 and is likely to be the physiologically relevant agonist of PAR-2 in the gastrointestinal tract (2). In other tissues where PAR-2 is highly expressed, including the prostate, lung, skin, and kidney, the physiologically relevant PAR-2 agonists are not known (27). Recently, a number of studies have shown the expression of various trypsinogen isoforms in extrapancreatic cancerous and normal tissues (10, 22, 25). To determine whether trypsin could be a potential agonist of PAR-2 in the kidney, we examined the expression of trypsinogen/trypsin in renal tissue. By IHC, trypsinogen/trypsin was shown to be produced in the kidney predominantly by distal tubules and collecting duct cells. There was weaker staining in other tubular elements, including PTC (Fig. 8A). Slides in which the specific trypsinogen/trypsin antibody was omitted were negative (Fig. 8C). By Western blotting, trypsinogen was shown to be produced by PTC in culture. The specific band seen by Western blotting was at ~30 kDa (Fig. 9A). A very small amount appeared to be secreted into the medium. HT29 (colon), PC-3 (prostate), and THP-1 also expressed trypsinogen, but HT29 was the only cell line tested that secreted significant amounts (Fig. 9B).

**DISCUSSION**

In this study, we have shown that PAR-2 is expressed at a functional level by human PTC in culture. This reflects the expression of this receptor by these cells in vivo. Activation of PAR-2 in these cultures with a specific activating peptide, SLIGKV-NH₂, stimulates a rapid mobilization of intracellular Ca²⁺ and phosphorylation of signaling molecule MAPK ERK 1/2. This was followed within 24 h by increases in DNA synthesis and production of fibronectin and MCP-1. Trypsinogen was shown to be expressed in the human kidney by

![Fig. 8. Immunohistochemical localization of trypsinogen in normal kidney tissue. A: kidney, B: kidney negative control (staining in absence of primary antibody). C: positive control, small intestine. D: positive control, pancreas.](http://aprenal.physiology.org/)

![Fig. 9. Western blot analysis of trypsinogen expression by proximal tubule cell cultures. Trypsinogen protein expression was determined by Western blot of PTC lysate (30 μg) using a specific trypsin polyclonal antibody. The trypsinogen band was at ~30 kDa. A: lanes 1–4, PTC lysate from 4 different PTC cultures; lane 5, trypsin. B: trypsinogen protein expression by various cell types and that released into conditioned media. Lane 1, trypsin (control); lanes 2 and 3, PTC lysate and conditioned medium; lanes 4 and 5, HT29 (colon) lysate and medium; lanes 6 and 7, PC-3 (prostate) cell lysate and conditioned medium; lane 8, THP-1 (macrophage) cell lysate.](http://aprenal.physiology.org/)
paradoxically distal/collection duct cells, but also by PTC and by cultured PTC. Treatment of PTC with trypsin, while initiating similar rapid cellular responses to SLIGKV-NH2 (Ca2+ mobilization and ERK1/2 phosphorylation), did not stimulate or only slightly stimulated delayed responses. The relevance of PAR-2 expression in the kidney to renal physiology and pathophysiology is not known. The finding that PTC are the predominant site of kidney PAR-2 expression and that its levels are enhanced in certain renal diseases suggests potential roles for this receptor here (12, 14, 37). The major finding in this study further support potential proinflammatory and proliferative roles for PAR-2 in the renal tubulointerstitium. Specifically, activation of PAR-2 by SLIGKV-NH2 was shown to induce robust DNA, fibronectin, and MCP-1 synthetic responses in PTC cultures, suggesting possible roles for PAR-2 in tubular regeneration and repair, tubulointerstitial fibrosis (TIF), and mediation of inflammation in the kidney, respectively. The PTC proliferative response induced by PAR-2 activation indicates tubule cell activation, which could promote interstitial inflammation. TIF is the strongest predictor of progression to end-stage renal failure in all forms of chronic kidney disease and is characterized by increased interstitial production and accumulation of matrix proteins, such as fibronectin and collagen type I (26). A number of reports have linked PAR-2 expression to increased renal fibrosis (14, 37). Similarly, MCP-1 is a potent chemotactic agent for monocytes and macrophages and has been shown to be an important promoter of tubulointerstitial inflammation and fibrosis in renal diseases such as diabetic nephropathy (8, 30). It has also been shown to directly activate tubule cells, leading to enhanced proinflammatory cytokine secretion and adhesion protein production by NF-κB- and AP-1-dependent mechanisms (35).

Another important observation in this study was that trypsin, unlike SLIGKV-NH2, only had limited effects on PTC DNA synthesis, MCP-1 secretion, and fibronectin secretion. One possible explanation for this finding is that PTC might produce protease inhibitors that could limit the activity of trypsin at the cell surface. Second, trypsin could cleave other proteins secreted or associated with PTC, and this could curb the PAR-2-mediated responses. Third, the delayed responses mediated by SLIGKV-NH2 could be by a mechanism other than by PAR-2. This could perhaps involve cross-reactivity with other receptors (1). As a final consideration, it is known that the posttranslational modifications of PAR-2 can change the ability of proteases to activate PAR-2. For example, PAR-2 can exist in various glycosylation states, and it has been shown that this can alter the ability of trypase to activate it (9).

In the present study, as measured by ELISA, trypsin appeared to increase fibronectin secretion. However, by Western blotting trypsin, at even low concentrations, was found to cause significant fibronectin degradation. The presence of fibronectin fragments within conditioned media could account for the apparent elevation of fibronectin production by cells treated with trypsin as measured by ELISA. The fact that trypsin is able to cleave fibronectin, even at low concentrations, is an important observation because it is frequently used experimentally in excess of 5 nM, and sometimes at much higher concentrations to activate PAR-2 both in vivo and in vitro (14, 24). Clearly, trypsin may have other actions on cells through cleavage of non-PAR-2 cell surface or secreted proteins. Although we have shown in this investigation that trypsinogen is expressed locally within the kidney, activation of PTC PAR-2 by this protease in vivo may not lead to increased accumulation of interstitial fibronectin. Other proteases that are produced in the kidney may well elevate fibronectin production via a PAR-2-dependent mechanism without leading to fibronectin degradation. For example, the PAR-1-activating protease thrombin has previously been shown not to cleave fibronectin produced by PTC (34). The identification of PAR-2-activating proteases expressed and activated within the kidney could lead to novel therapeutic strategies for treatment of kidney disease.

It has previously been shown in a number of other cell types that PAR-2 activation can stimulate the ERK1/2 MAPK pathway (21, 32). This in turn can mediate a range of responses including, notably, cell proliferation. Because in this study SLIGKV-NH2 was able to strongly induce ERK1/2 phosphorylation, the possibility that this pathway was involved in mediating the delayed responses of DNA synthesis, fibronectin secretion, and MCP-1 secretion was investigated. The MEK inhibitor PD-98059 was shown to significantly reduce DNA synthesis and fibronectin secretion both under control conditions and in cells treated with SLIGKV-NH2. This suggests the involvement of the ERK1/2 MAPK pathway in the basal level of DNA synthesis and fibronectin secretion by cultured PTC, not necessarily after PAR-2 activation. The secretion of MCP-1 in response to SLIGKV-NH2 did not appear to involve activation of the ERK1/2 MAPK pathway.

A potential limitation of our study was the use of normal human kidney tissue obtained from the uninvolved poles of adult human kidneys removed surgically because of small benign or malignant renal tumors. Although the tissue was histologically confirmed as normal and isolated PTC were passaged twice in defined medium before experimentation, the possibility of remote “paraneoplastic” effects of the tumor on remaining normal kidney tissue could not be entirely excluded. In summary, PTC appear to be the predominant site of PAR-2 expression in the human kidney and, when established in culture, maintain functional levels of this receptor. While SLIGKV-NH2 and trypsin rapidly enhanced Ca2+ mobilization and ERK1/2 phosphorylation, only the peptide notably increased delayed responses of DNA synthesis, MCP-1, and fibronectin secretion. Because trypsinogen is expressed in the human kidney, trypsin could potentially act as an agonist for PAR-2 under some circumstances. Further studies are warranted to elucidate the role of PAR-2 and its mechanism of activation in tubule cells.

REFERENCES


