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Thrombin Down-Regulates the TGF- β -Mediated Synthesis of Collagen and Fibronectin by Human Proximal Tubule Epithelial Cells Through the EPCR-Dependent Activation of PAR-1

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Abstract

Human proximal tubule (HK-2) cells are commonly used as cellular models to understand the mechanism by which inflammatory mediators cause renal injury. It has been observed that thrombin stimulates the expression of TGF- β , extracellular matrix (ECM) proteins and proinflammatory cytokines by HK-2 cells. These in vitro responses correlate well with the pathology of glomerular and tubular diseases observed in acute renal injury. HK-2 cells express PAR-1 and the thrombin activation of this receptor has been reported to up-regulate the TGF- β -mediated expression of ECM proteins, suggesting a possible pathogenic role for PAR-1 signaling by thrombin in acute renal injury. On the other hand, several recent studies have indicated that activated protein C plays a renoprotective role, thus inhibiting the inflammatory responses and attenuating renal injury, presumably by activating the same cell surface receptor. In this study, we show that HK-2 cells express endothelial protein C receptor (EPCR) and that the occupancy of this receptor by protein C switches the signaling specificity of thrombin so that the activation of PAR-1 by thrombin inhibits the TNF- α -mediated synthesis of IL-6 and IL-8 and down-regulates the TGF- β -mediated expression of ECM proteins. These results suggest a possible protective role for EPCR in acute kidney injury.

Thrombin is a plasma serine proteinase that activates platelets and cleaves fibrinogen to stop bleeding at vascular injury sites (Mann et al., 1988). Thrombin also down-regulates its own production by a feed back inhibition mechanism when it forms a complex with the endothelial cell surface receptor thrombomodulin (TM) to activate protein C to activated protein C (Esmon, 1993b). Activate protein C (APC) functions as a potent anticoagulant by proteolytically degrading factors Va and VIIIa, which are essential cofactors for thrombin generation in both extrinsic and intrinsic pathways (Walker and Fay, 1992; Dahlback and Villoutreix, 2005). In addition to these pivotal roles in the clotting cascade, thrombin has regulatory roles in inflammation, angiogenesis, apoptosis, expression of growth factors and tissue remodeling (Nierodzik et al., 1992; Esmon, 1993a; Grand et al., 1996; Huang et al.,

2001; Ruf et al., 2003; Li et al., 2004; Coughlin, 2005). The non-hemostatic cellular effects of thrombin are mediated through the activation of G-protein coupled receptors, known as protease-activated receptors (PARs), which are expressed on the surface of endothelial cells and many other cell types (Grand et al., 1996; Coughlin, 2005). Among the four members of the PAR family, PAR-1, PAR-3, and PAR-4 can be cleaved by thrombin. PAR-2, however, does not appear to be a substrate for thrombin, but it may be cleaved by factors VIIa and Xa and the non-plasma protease trypsin (Coughlin, 2005).

Cultured vascular endothelial cells express all four PARs and the traditional view is that the activation of PAR-1 by thrombin up-regulates proinflammatory responses in endothelial cells. Thus, it has been demonstrated that the thrombin activation of PAR-1 promotes the interaction of leukocytes with endothelial cells and up-regulates the expression of cell adhesion molecules through the activation of the NF- κ B pathway (Kaplanski et al., 1998; Joyce et al., 2001; Feistritzer and Riewald, 2005; McLaughlin et al., 2005). Thrombin has also been demonstrated to induce apoptosis and to disrupt the barrier permeability function of endothelial cells (Ruf et al., 2003; Flynn and Buret, 2004; Feistritzer and Riewald, 2005). However, in a series of recent studies we discovered that the occupancy of endothelial protein C receptor (EPCR) by protein C blocks all in vitro indices of the PAR-1-dependent proinflammatory activities of thrombin. Thus, similar to APC, the activation of PAR-1 by thrombin initiated potent anti-inflammatory responses in endothelial cells (Bae et al., 2007a; Bae and Rezaie, 2008, 2009). Whether the occupancy of EPCR can switch the PAR-1-dependent signaling specificity of thrombin in non-vascular cells has never been examined.

It is known that acute renal injury is associated with the activation of the clotting cascade and the initiation of inflammatory responses that culminate in the deposition of fibrin and accumulation of extracellular matrix (ECM) proteins within the interstitial spaces and basement membranes of glomerular and tubular cells (Klahr et al., 1988; Tipping et al., 1988; Kaizuka et al., 1999; Grandaliano et al., 2000; Shirato et al., 2003; Vesey et al., 2005). Recent in vitro data have indicated that thrombin, through the activation of PAR-1, contributes to the pathology of acute renal injury and that APC plays a renoprotective role, possibly in part through the activation of the same receptor on kidney cells (Xu et al., 1995; Gupta et al., 2007, 2009; Isermann et al., 2007). These studies have used primary or immortalized proximal tubule epithelial (HK-2) cells as in vitro models to conclude that thrombin can initiate proinflammatory responses in the kidney through the stimulation of TGF- β and subsequent induction of ECM proteins (MacKay et al., 1989; Olgemoller and Schleicher, 1993; Ryan et al., 1994; Laping et al., 1997; Grandaliano et al., 2000; Shirato et al., 2003; Vesey et al., 2005; Du et al., 2010). The accumulation of ECM molecules is one common pathological feature of glomerular and tubular diseases observed in end-stage renal failure caused by severe sepsis and diabetic nephropathy (Wan et al., 2003; Bonventre and Zuk, 2004; Molitoris and Sutton, 2004). We undertook this study to determine whether HK-2 cells express EPCR and, if so, whether its occupancy by protein C can regulate the PAR-1-dependent signaling specificity of thrombin.

Materials and Methods

Reagents

The cleavage-blocking monoclonal anti-PAR-1 antibody (H-111), antibody to sphingosine-1-phosphate (S1P) receptor (S1P1), and siRNA to S1P1 were purchased from Santa Cruz Biologics (Santa Cruz, CA). The function-blocking anti-EPCR antibody was purchased from Cell Sciences (Canton, MA). The anti-TM monoclonal antibody was purchased from American Diagnostica (Stamford, CT). Methyl- β -cyclodextrin (M β CD), phosphatidylinositol 3-kinase inhibitor (LY294002) and pertussis toxin (PTX) were purchased from Sigma (St. Louis, MO). The thrombin receptor agonist peptide (TRAP) was

purchased from Bachem Bioscience (Torrance, CA). APC was purchased from Haematologic Technologies, Inc. (Essex Junction, VT). TGF- β and TNF- α were purchased from R&D Systems (Minneapolis, MN). The Ser-195 to Ala substitution mutant of protein C (PC-S195A) was prepared as described (Bae et al., 2007a).

Cell culture

Immortalized human umbilical vein endothelial cells (EA.hy926) were kindly provided by Dr. C. Edgell from University of North Carolina at Chapel Hill and maintained as described (Bae et al., 2007a). Immortalized proximal tubule epithelial (HK-2) cells were obtained from ATCC (Manassas, VA) and maintained with Keratinocyte-SFM (Life Technologies, Gaithersburg, MD) supplemented with 5% fetal calf serum.

Analysis of PAR-1 and EPCR expression

The expression of PAR-1 and EPCR was assessed in cell lysates by enzyme-linked immunosorbent assays (ELISA). Cells were grown to confluence and cell lysates were prepared using a protein extraction buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1% sodium dodecyl sulfate, 0.5% sodium deoxycholate, 1% Triton X-100, 2 mM EDTA and 1 mM dithiothreitol supplemented with complete protease inhibitor cocktail (Roche Diagnostics Australia Pty Ltd., Castle Hill, NSW, Australia). Ninety-six-well, flat bottom microtiter plates were coated with the extracted protein in 20 mM carbonate-bicarbonate buffer (pH 7.4) containing 0.02% sodium azide and incubated overnight at 4 °C. The plates were rinsed three times in PBS-0.05% Tween-20 (PBST) and kept at 4 °C. Primary antibodies against EPCR or PAR-1 (diluted 1:500) were added and incubated for 1 h at room temperature. The plates were rinsed three times with PBST and incubated for 1.5 h at room temperature with the peroxidase-conjugated anti-rabbit IgG antibodies (diluted 1:2,000) in PBST. The plates were rinsed three times with PBST and incubated for 1 h at room temperature in the dark room with 200 μ l substrate solution (100 μ g/ml *o*-phenylenediamine and 0.003% H₂O₂). After stopping the reaction with 50 μ l of 8 N H₂SO₄, the absorbance was read at 490 nm.

Assays for type IV collagen and fibronectin

The concentrations of type IV collagen and fibronectin in culture supernatants were determined by an ELISA using collagen IV ELISA (Echelon Biosciences, Inc., Salt Lake City, UT) and QuantiMatrix human fibronectin ELISA kits (Millipore, Billerica, MA) according to the manufacturers' protocols. HK-2 cells were cultured in six-well plates and grown to confluence. Subsequently, cells were washed with Hanks' balanced salt solution and cultured in the presence of indicated proteases for 3 days followed by measuring the expression levels of type IV collagen and fibronectin in the cell culture supernatants. The total protein concentration was measured by the Bradford protein assay using bovine serum albumin as a standard. The levels of type IV collagen and fibronectin were expressed as ng per μ g and ng per mg of total protein, respectively.

Cell-based ELISA for EPCR-binding

Cells in 96-well plates were incubated with serially diluted APC (0.5 μ M in TBS/Ca²⁺ containing 0.1% BSA starting concentration) for 1 h at 4 °C. After washing the plates three times with the same TBS buffer containing 0.05% Tween-20, the primary antibody for APC (goat anti-protein C polyclonal antibody (1:500)) was added and incubation continued for another hour. After washing the plate, the secondary antibody (rabbit anti-goat IgG) was added and incubated for 1 h. After washing the plate, ABTS (KPL, MD) was added and the colorimetric analysis was performed by measuring the absorbance at 405 nm as described (Bae et al., 2007a).

Protein C activation

The time course of human protein C (80 nM) activation by thrombin (0.2 nM) on HK-2 cells was monitored by transferring 50 μ l of the conditioned media at different time points into 150 μ l of the HEPES buffer containing 100 nM hirudin and 0.25 mM Spectrozyme PCa. The rate of APC generation was determined from the rate of chromogenic substrate hydrolysis at 405 nm.

ELISA for IL-6 and IL-8

The concentrations of IL-6 and IL-8 in cell culture supernatants were determined by ELISA (R&D Systems) using streptavidin conjugated horseradish-peroxidase detection system according to the manufacturer's protocol.

Assay for TGF- β

The concentration of TGF- β in six-well culture supernatant was measured by a sandwich ELISA for human TGF- β 1 (R&D Systems) according to the manufacturer's protocol.

Results

HK-2 cells express EPCR and PAR-1

Previous studies have indicated that immortalized human proximal tubule epithelial (HK-2) cells have well differentiated functional and histological characteristics similar to freshly isolated ones (Ryan et al., 1994). They express PAR-1 and exhibit similar cellular responses when activated by thrombin or other proinflammatory cytokines (Grandaliano et al., 2000; Vesey et al., 2005). To determine whether these cells express EPCR, the protein level of EPCR in the cell lysates of confluent HK-2 cells were analyzed by ELISA using a specific anti-EPCR antibody. As shown in Figure 1, HK-2 cells express both PAR-1 (part A) and EPCR (part B). EA.hy926 cells, which are known to express high levels of both receptors, were used for comparison of the expression levels in these assays. Further analysis indicated that protein C/APC can bind to the cell surface EPCR on HK-2 cells normally, as evidenced by similar dissociation constants of 34 nM and 31 nM for APC interaction with EPCR on EA.hy926 and HK-2 cells, respectively (Fig. 1, part C). EA.hy926 cells also express TM (Bae et al., 2007b). However, Western blot analysis of the cell lysates using a specific antibody against TM suggested that, unlike EA.hy926 cells, HK-2 cells do not express a detectable level of TM (Fig. 1, part D, inset). These results are consistent with the observation that, unlike EA.hy926 cells which effectively supported the thrombin activation of protein C, thrombin did not activate protein C on HK-2 cells (Fig. 1, part D).

Thrombin stimulates the secretion of type IV collagen and fibronectin

Thrombin is known to promote the expression of ECM proteins including type IV collagen, laminin, heparan sulfate proteoglycan and fibronectin by tubular epithelial and mesangial kidney cells (Kaizuka et al., 1999; Shirato et al., 2003; Bonventre and Zuk, 2004; Molitoris and Sutton, 2004). The accumulation of ECM proteins is thought to contribute to the pathophysiology of renal dysfunction (Bonventre and Zuk, 2004; Molitoris and Sutton, 2004). Consistent with previous results, thrombin promoted the synthesis of both type IV collagen and fibronectin by HK-2 cells in a concentration and time dependent manner (Fig. 2). The thrombin-mediated expression of both type IV collagen and fibronectin by HK-2 cells reached optimal levels with 25 nM thrombin. The type IV collagen level was only slightly elevated after 1–2 days, however, after 3 days of culture, thrombin dramatically increased the expression level of this protein (Fig. 2, part A). A similar result was observed with fibronectin. In this case, however, a significant enhancement in the expression level of the protein was observed after 2 days of stimulation of the cells with thrombin (Fig. 2, part

C). Based on these results, a thrombin concentration of 25 nM for 3 days was used to stimulate HK-2 cells in all experiments described below.

The occupancy of EPCR by its ligand inhibits the thrombin-induced synthesis of ECM proteins

We have recently demonstrated that the occupancy of EPCR by protein C switches the PAR-1-dependent signaling specificity of thrombin in endothelial cells (Bae et al., 2007a; Bae and Rezaie, 2008, 2009). Thus, the thrombin cleavage of PAR-1, in PC-S195A-pretreated endothelial cells, initiates cytoprotective cellular responses similar to those observed with APC (Mosnier et al., 2007). To determine whether the occupancy of EPCR in HK-2 cells by its ligand can switch the signaling specificity of thrombin, cells were initially pretreated with PC-S195A and then stimulated with thrombin as described above. Interestingly, we discovered that PC-S195A inhibits the thrombin-mediated up-regulation of the expression of both type IV collagen and fibronectin by HK-2 cells (Fig. 3, parts A and C). The ECM stimulatory effect of thrombin is mediated through the activation of PAR-1 since the PAR-1 agonist peptide (TRAP) also stimulated the synthesis of both proteins (Fig. 3, parts A and C), and the pre-incubation of HK-2 cells with PC-S195A similarly inhibited the stimulatory effect of TRAP (Fig. 3, parts A and C). Further evidence for the EPCR- and PAR-1 dependent protective effect of thrombin in HK-2 cells is provided by the observation that function-blocking antibodies to both receptors eliminated the ECM inhibitory effect of thrombin in the presence of PC-S195A (Fig. 3, parts B and D).

PI3-kinase-mediated activation of S1P1 modulates the EPCR and PAR-1-dependent effect of thrombin in lipid-rafts

The EPCR and PAR-1 dependent protective cellular effect of both thrombin and APC is mediated through the activation of PI3-kinase (Finigan et al., 2005; Bae et al., 2009). To determine whether the activation of the PI3-kinase pathway is also involved in the EPCR and PAR-1 dependent ECM inhibitory effect of thrombin, HK-2 cells were preincubated with specific PI3-kinase inhibitor, LY294002. As presented in Figure 3 (parts B and D), the inhibitor effectively suppressed the ECM modulatory effect of thrombin in the PC-S195A-pretreated HK-2 cells, suggesting that this effect is mediated through the protease activating the PI3-kinase pathway by an EPCR and PAR-1 dependent mechanism, as has been observed with APC (Finigan et al., 2005; Bae et al., 2009). Further studies in the presence of the cholesterol depleting drug, M β CD, and the specific inhibitor of the Gi-protein-coupled receptor signaling, pertussis toxin (PTX), suggested that, similar to APC (Bae et al., 2007a), the EPCR and PAR-1 dependent protective effect of thrombin is mediated through the Gi-protein in the lipid-rafts of HK-2 cells (Fig. 3, parts B and D). Furthermore, similar to the protective effect of APC, a receptor crosstalk with the Gi-protein coupled receptor, S1P1, was required for the protective effect of thrombin since the S1P1-specific siRNA eliminated the ECM inhibitory effect of thrombin in the PC-S195A-pretreated HK-2 cells (Fig. 3, parts B and D). In agreement with these results, exogenously added S1P also inhibited the thrombin-mediated expression of both type IV collagen and fibronectin by HK-2 cells (data not shown).

The occupancy of EPCR inhibits the TNF- α -mediated expression of IL-6 and IL-8 by HK-2 cells

It is known that the expression of proinflammatory cytokines is up-regulated in the injured kidney and that APC exhibits a protective effect through the inhibition of the expression of these molecules in part by the EPCR-dependent activation of PAR-1 (Gupta et al., 2007, 2009; Isermann et al., 2007; Mosnier et al., 2007). In agreement with these results, TNF- α up-regulated the expression of IL-6 and IL-8 in HK-2 cells and neither thrombin nor the PAR-1 agonist peptide altered this response (Fig. 4). To determine whether the occupancy of

EPCR by protein C can confer a PAR-1-dependent protective activity for thrombin, the PC-S195A-pretreated HK-2 cells were incubated with thrombin prior to stimulation by TNF- α . As shown in Figure 4, when EPCR was occupied, the activation of PAR-1 by thrombin inhibited the TNF- α -mediated up-regulation of both IL-6 (part A) and IL-8 (part B) in HK-2 cells. The same results were obtained with the PAR-1 agonist peptide (Fig. 4), suggesting that it is the EPCR occupancy and not the type of protease (thrombin or APC) cleaving the receptor that determines the PAR-1-dependent signaling specificity (proinflammatory or anti-inflammatory) in this cellular system.

The occupancy of EPCR by protein C inhibits the thrombin-mediated expression of TGF- β

TGF- β plays a pivotal role in the pathophysiology of inflammatory renal diseases (MacKay et al., 1989; Border, 1994; Shirato et al., 2003; Vesey et al., 2005). Previous studies have demonstrated that thrombin stimulates type IV collagen and fibronectin by endothelial and epithelial cells of the injured kidney through a TGF- β -dependent mechanism (Shirato et al., 2003; Vesey et al., 2005). To determine whether the occupancy of EPCR by protein C inhibits the thrombin-mediated induction of TGF- β , HK-2 cells were pretreated with PC-S195A before stimulation with thrombin. The time course and concentration-dependence of the TGF- β induction by thrombin in the absence and presence of PC-S195A is presented in Figure 5. Thrombin augmented the secretion of TGF- β by HK-2 cells after 1 day of stimulation and further augmentation was observed following extended incubation (Fig. 5, part A). Thus, the time course and optimal concentration of thrombin required to effectively induce both TGF- β (Fig. 5, parts A and B) and ECM proteins (Fig. 2) were essentially identical. Interestingly, similar to results presented above, the pre-incubation of HK-2 cells with PC-S195A potentially inhibited the thrombin-mediated expression of TGF- β (Fig. 5, part C). Similar to the inhibition of expression of ECM molecules, the inhibition of expression of TGF- β by thrombin occurred by an EPCR, PAR-1 and PI3-kinase dependent mechanism through the Gi-protein-coupled receptor signaling in the lipid-rafts of HK-2 cells as evidenced by the function-blocking antibodies specific to either receptor, LY294002, M β CD and PTX all abrogating the protective effect of thrombin in the PC-S195A-treated cells (Fig. 5, part D). APC also inhibited the expression of TGF- β , suggesting that when EPCR is occupied by its ligand the activation of PAR-1 by either APC or thrombin similarly down-regulates the TGF- β pathway in HK-2 cells (Fig. 5, part C). In agreement with these results, a specific anti-TGF- β antibody abrogated the ECM stimulatory effect of thrombin in HK-2 cells (Fig. 6, parts A and B). Furthermore, the incubation of HK-2 cells with purified TGF- β also up-regulated the expression of both type IV collagen and fibronectin (Fig. 6, parts C and D). However, prior treatment of HK-2 cells with thrombin plus PC-S195A or APC abrogated the up-regulation of ECM proteins by TGF- β (Fig. 6, parts C and D). Taken together, these results support the hypothesis that the EPCR-dependent protective effect of thrombin is mediated through the inhibition of the TGF- β pathway.

Discussion

Acute renal injury is associated with the activation of the clotting cascade and initiation of inflammatory responses that, if not regulated properly, can lead to renal failure. The pathophysiology of the disease involves microvascular thrombosis, injury to endothelial cells, disruption of endothelial barrier function, apoptosis, infiltration of activated leukocytes, and elaboration of proinflammatory cytokines (Wan et al., 2003; Bonventre and Zuk, 2004; Molitoris and Sutton, 2004). These inflammatory events can rapidly extend to other kidney tissues including proximal tubule cells, thereby causing renal dysfunction/failure (Molitoris and Sutton, 2004). Recent in vivo data (both in humans and in animal models) have demonstrated that APC, whether generated endogenously or administered therapeutically, in addition to controlling microvascular thrombosis via the down-regulation

of thrombin generation, also exerts potent cytoprotective activities that can inhibit inflammatory responses mentioned above, thus reversing the course of the disease and improving renal function (Bernard et al., 2001; Taylor and Kinasewitz, 2004; Gupta et al., 2007, 2009). The anti-inflammatory activity of APC is thought to be responsible for its beneficial therapeutic effect in reducing the mortality rate in adult severe sepsis syndrome (Bernard et al., 2001; Mosnier et al., 2007). The mechanism by which APC exerts its cytoprotective activities is not fully understood, however, an increasing body of evidence suggests that the activation of PAR-1 by APC in complex with EPCR accounts, at least in part, for the ability of APC to exert a protective effect and to suppress the inflammatory responses (Ruf et al., 2003; Kerschen et al., 2007; Mosnier et al., 2007). This hypothesis has not been fully established since thrombin can also activate the same receptor (PAR-1) with greater than three orders of magnitude higher catalytic efficiency to initiate proinflammatory responses (Coughlin, 2005; Ludeman et al., 2005). It is not known how the activation of PAR-1 by two different proteases can elicit paradoxical cellular effects, though the concept that thrombin elicits a proinflammatory response through the activation of PAR-1 is a conclusion that is primarily based on cellular models using in vitro assay systems (Kaizuka et al., 1999; Grandaliano et al., 2000; Feistritzer and Riewald, 2005; McLaughlin et al., 2005; Vesey et al., 2005).

In a series of recent studies we provided some insight into the nature of this problem and demonstrated that the occupancy of EPCR by protein C in cultured endothelial cells can switch the PAR-1-dependent signaling specificity of thrombin, thus the activation of PAR-1 by thrombin elicited only cytoprotective responses similar to those observed with APC if cells were pretreated with a sub-physiological concentration of the zymogen protein C (10 nM) immediately prior to their stimulation by thrombin (Bae et al., 2007a; Bae and Rezaie, 2008, 2009). These observations raised the possibility that, contrary to the traditional view, the thrombin activation of PAR-1 may actually play a protective role in vascular endothelial cells expressing EPCR under normal physiological conditions. Noting that primary or immortalized proximal tubule cells have been used as cellular models to infer a PAR-1-dependent proinflammatory role for thrombin in acute kidney injury (Grandaliano et al., 2000; Shirato et al., 2003; Vesey et al., 2005), we decided to investigate this question further and monitor the PAR-1-dependent responses of thrombin in HK-2 cells under conditions where EPCR is occupied by its ligand protein C. We first demonstrated that HK-2 cells express high levels of functional EPCR on their surface. Interestingly, we discovered that, similar to vascular endothelial cells, thrombin elicits a PAR-1-dependent cytoprotective response in HK-2 cells when EPCR is occupied by protein C, suggesting that the previous in vitro cellular data, which have all been conducted in the absence of protein C, may not accurately reflect the true PAR-1-dependent physiological function of thrombin in kidneys. It was interesting to note that HK-2 cells express high levels of EPCR, but they do not express TM to a detectable level. It follows therefore the leakage of plasma and activated leukocytes during an injury and/or inflammation from circulation to proximal tubule, which is known to express high levels of tissue factor (Nestoridi et al., 2005), can activate the clotting cascade to generate thrombin, but thrombin cannot activate protein C on tubule cells since they do not appear to express TM. Thus, based on these cellular data, which have been conducted for the first time in a more physiologically relevant manner, we hypothesize that the occupancy of EPCR on proximal tubule cells would alter the PAR-1-dependent signaling specificity of thrombin so that the activation of the receptor would down-regulate the inflammatory responses in injured kidney cells. Based on this model, we predict an important renoprotective function for EPCR in regulating the activity of thrombin, thus ensuring that following activation of the clotting cascade in the injured kidney, the protease does not amplify the inflammatory responses uncontrollably to cause severe ischemia and apoptosis of tubular epithelial cells. Further studies are required to determine whether tubular epithelial cells can themselves express low levels of protein C to protect kidneys

from injury by thrombin or proinflammatory cytokines. It is noteworthy that a significantly higher level of EPCR expression has been observed in the kidney after polymicrobial-induced sepsis in rats (Gupta et al., 2007).

A common pathophysiological feature of acute inflammatory renal injury is the excessive accumulation of ECM proteins in the basement membrane of glomerular and proximal tubular cells mediated by the fibrotic growth factor TGF- β (MacKay et al., 1989; Shirato et al., 2003; Bonventre and Zuk, 2004). It is thought that thrombin plays a role in propagating this cellular response based on the observation that thrombin induces the expression of TGF- β in HK-2 cells (MacKay et al., 1989; Shirato et al., 2003; Bonventre and Zuk, 2004). It is also known that TGF- β induces the expression of plasminogen activator inhibitor 1 which can inhibit plasmin generation, thus up-regulating fibrin deposition and inhibiting the turnover of the ECM proteins, thereby causing tissue fibrosis within the injured kidney (Grandaliano et al., 2001). As demonstrated above, thrombin inhibited the TGF- β -mediated expression of both type IV collagen and fibronectin by HK-2 cells if cells were pretreated with PC-S195A, supporting the hypothesis that the occupancy of EPCR by its ligand can confer a PAR-1-dependent renoprotective role for thrombin in acute renal injury. Further support for this hypothesis is provided by the observation that thrombin also inhibited the TNF- α -mediated expression of IL-6 and IL-8 in the PC-S195A-treated HK-2 cells. Thus, similar to vascular endothelial cells, thrombin can elicit PAR-1-dependent protective responses in proximal tubule cells if EPCR is occupied by its natural ligand protein C.

Previous studies have indicated that the EPCR- and PAR-1-dependent anti-inflammatory effect of both APC and thrombin on vascular endothelial cells is mediated through the PI3-kinase-dependent activation of the AKT survival pathway (Finigan et al., 2005; Bae et al., 2009). The observation that the specific cell permeable PI3-kinase inhibitor (LY-294002) inhibited the protective function of thrombin in HK-2 cells suggests that this pathway is also involved in this system. Furthermore, it is known that the PAR-1-dependent protective effect of APC is mediated through a receptor crosstalk with the Gi-protein coupled S1P receptor-1 (S1P1) in the lipid-rafts of endothelial cells (Feistritzer and Riewald, 2005; Bae et al., 2007b; Bae and Rezaie, 2008). HK-2 cells also express S1P1 (Iwata et al., 1995). The observations that both M β CD and siRNA for S1P1 inhibited the EPCR and PAR-1 dependent protective effect of thrombin in HK-2 cells suggest that similar mechanisms are involved in this system. In conclusion, our data suggests that EPCR has a cytoprotective role in proximal tubule cells if the receptor is bound by its ligand, protein C.

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Fig. 1.

Expression of EPCR and PAR-1 by HK-2 cells. Protein expression of both EPCR (A) and PAR-1 (B) in cell lysates (100 μg total protein) of HK-2 and EA.hy926 cells were monitored by ELISA as described under Experimental Procedures Section. BSA was used as a negative control. All results are shown as means ±SD of five different experiments. * $P < 0.01$ as compared to BSA. C: The interaction of APC with the cell surface EPCR on either HK-2 (●) or EA.hy926 (○) cells were monitored by a cell-based ELISA as described under Experimental Procedures Section. D: The time course of activation of human protein C (80 nM) by thrombin (0.2 nM) on HK-2 (●) or EA.hy926 (○) cells were monitored by a chromogenic substrate assay as described under Experimental Procedures Section. (Inset) Western blot analysis of TM expression by EA.hy926 and HK-2 cells.

Fig. 2.

Effect of thrombin on expression of the type IV collagen and fibronectin. A: Time course of secretion of type IV collagen (IV Col) by HK-2 cells was monitored in the absence (○) or presence of 25 nM thrombin (●) by an ELISA as described under Experimental Procedures Section. B: The same as (A) except the concentration-dependence of thrombin-mediated secretion of type IV collagen was monitored. C,D: The same as above except that the thrombin-mediated secretion of fibronectin (FN) was monitored. All results are means $\pm$ SD of three different experiments. * $P < 0.01$ as compared to 1 day culture (parts A and C) or 0 nM thrombin (parts B and D).

Fig. 3.

Effect of PC-S195A on the PAR-1-dependent thrombin-mediated expression of type IV collagen and fibronectin. A: HK-2 cells were pre-incubated with PC-S195A (50 nM) or APC (20 nM) for 30 min prior to stimulation by thrombin (25 nM) or TRAP (100 μ M) for 3 days. The expression of type IV collagen was measured by an ELISA as described under Experimental Procedures Section. B: The same as (A) except that HK-2 cells were pre-incubated with function-blocking antibodies to PAR-1 or EPCR (25 μ g/ml for 30 min), M β CD (10 mM for 1 h), LY294002 (10 μ M for 1 h), PTX (100 ng/ml for 16 h) or transfection with non-specific (NS) or specific siRNA (10 nM) for S1P1 before treating cells with PC-S195A and stimulation by thrombin. C,D: The same as above except that the thrombin-mediated secretion of fibronectin was monitored. All results in parts A and C are means $\pm$ SD of three different experiments. * P < 0.01 as compared to thrombin (Th).

Fig. 4.

EPCR occupancy inhibits the TNF- α -mediated expression of cytokines by HK-2 cells. Confluent HK-2 cells were treated with TNF- α (5 ng/ml) with or without prior incubation of cells with PC-S195A (50 nM), APC (20 nM), thrombin (25 nM) and TRAP (100 μ M). The protein levels of IL-6 (A) or IL-8 (B) were determined by an ELISA as described under Experimental Procedures Section. * $P < 0.01$ as compared to TNF- α .

Fig. 5. EPCR occupancy inhibits the thrombin-mediated expression of TGF- β by HK-2 cells. A: Time course of the expression of TGF- β by HK-2 cells in the absence (○) or presence (●) of 25 nM thrombin was measured. B: The same as (A) except that the concentration-dependence of thrombin-mediated secretion of TGF- β was monitored. C: Thrombin (25 nM)-mediated expression of TGF- β was monitored in HK-2 cells pretreated with PC-S195A (50 nM) or APC (20 nM). D: The same as (C) except that HK-2 cells were pre-incubated with function-blocking antibodies to PAR-1 or EPCR (25 μ g/ml for 30 min), M β CD (10 mM for 1 h), LY294002 (10 μ M for 1 h), PTX (100 ng/ml for 16 h) before treating cells with PC-S195A and stimulation by thrombin. All results are means $\pm$ SD of three different experiments with * P < 0.01 as compared to 1 day (A), no thrombin (B), or plus thrombin (C).

Fig. 6.

Thrombin up-regulation of ECM proteins through TGF- β and its inhibition by PC-S195A. HK-2 cells were pre-incubated with a control IgG or an antibody specific for TGF- β (25 $\mu\text{g}/\text{ml}$ for 30 min) before stimulation with thrombin (25 nM) and analysis of collagen type IV (A) or fibronectin (B) by an ELISA as described under Experimental Procedures Section. C: The protein level of collagen type IV in response to TGF- β (5 ng/ml) was measured with or without pre-treating cells with PC-S195A (50 nM), thrombin (25 nM) and APC (20 nM). D: The same as (C) except that the protein level of fibronectin (FN) was monitored. All results are means $\pm$ SD of three different experiments. * $P < 0.01$ in all parts.