Protease-activated receptor stimulation activates a Ca\textsuperscript{2+}-independent phospholipase A\textsubscript{2} in bladder microvascular endothelial cells

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Protease-activated receptor stimulation activates a Ca\textsuperscript{2+}-independent phospholipase A\textsubscript{2} in bladder microvascular endothelial cells. Am J Physiol Renal Physiol 288: F714–F721, 2005. First published November 23, 2004; doi:10.1152/ajprenal.00288.2004.—Increased mast cell numbers and mast cell activation represent one of the prevalent etiologic theories for interstitial cystitis, an inflammatory condition in the bladder. This study was designed primarily to determine whether increased mast cell tryptase in the bladder wall may play a role in activating bladder endothelial cell phospholipase A\textsubscript{2} (PLA\textsubscript{2}), leading to increased inflammatory phospholipid metabolite accumulation, which may propagate the inflammatory process. We stimulated human bladder microvascular endothelial cells with thrombin or tryptase and measured the activation of PLA\textsubscript{2} and the production of multiple membrane phospholipid-derived inflammatory mediators. Thrombin and tryptase stimulation resulted in activation of a Ca\textsuperscript{2+}-independent PLA\textsubscript{2}, leading to increased release of arachidonic acid and prostacyclin and increased production of platelet-activating factor. These responses were blocked completely by pretreatment of human bladder microvascular endothelial cells with Ca\textsuperscript{2+}-independent PLA\textsubscript{2}-selective inhibitor bromoenol lactone (BEL). The combination of increased prostacyclin and platelet-activating factor in the bladder circulation may result in vasodilation and increased polymorphonuclear leukocyte adherence to the endothelium and may facilitate recruitment of polymorphonuclear leukocytes to the bladder wall of patients with interstitial cystitis.

interstitial cystitis; inflammation

INTERSTITIAL CYSTITIS (IC) is an inflammatory bladder condition with unknown etiology characterized by symptoms of urgency, frequency, nocturia, and pain (17). Two prevalent etiologic theories of IC include a defect in bladder cytoprotection by the urothelial cell glycosaminoglycans (11) and a pathophysiologic role for mast cells (20, 23). Increased bladder permeability (9, 16) and an abnormal expression of molecular markers for bladder impermeability have been reported in IC (21). Leakage of urinary constituents through the damaged epithelium may contribute to exacerbation of inflammation in the underlying muscle layers, causing a cycle of inflammation and further increased urothelial permeability. In a subset of IC patients, mast cell activation is a characteristic pathologic finding, as demonstrated by electron microscopy (24). Furthermore, mast cells contain tryptase, which has been shown to be elevated in 24-h urine samples of IC patients (2).

Inflammation involves the activation of inflammatory cells as well as the production of multiple inflammatory mediators. Protease-activated receptors (PARs), which couple to multiple intracellular signaling pathways, including activation of phospholipases (7), are involved in the inflammatory response and may play roles in anti-inflammatory and proinflammatory behavior in endothelial and epithelial cells (4, 5). The PARs represent a family of receptors that are activated by specific serine proteases and are coupled to G proteins (7). There are four members of the PAR family: PAR-1, PAR-3, and PAR-4 are activated by thrombin; PAR-2 is activated by tryptase, but not by thrombin. The PAR is coupled to multiple intracellular signaling pathways related to growth and inflammation, including activation of phospholipases and MAP kinases (7).

Activation of phospholipase A\textsubscript{2} (PLA\textsubscript{2}) results in accelerated membrane phospholipid hydrolysis and increased production of multiple biologically active metabolites (Fig. 1). In a previous study, we demonstrated that activation of PAR-1 by thrombin and PAR-2 by tryptase on human urothelial (HUR) cells resulted in increased membrane-associated, Ca\textsuperscript{2+}-independent phospholipase A\textsubscript{2} (iPLA\textsubscript{2}) activity and accelerated production of membrane phospholipid-derived inflammatory mediators, including arachidonic acid, PGE\textsubscript{2}, and platelet-activating factor (PAF) (18). This subsequent study was designed to evaluate whether a similar intracellular signaling pathway that may contribute to propagation of the inflammatory process in IC is also evident in bladder microvascular endothelial cells.

METHODS

Culture of human bladder microvascular endothelial cells. Human bladder microvascular endothelial cells (HBMEC) were obtained from Cambrex Bioscience Walkersville (Walkersville, MD). The cells are from the bladder microvasculature of a 48-yr-old woman and were found to be human immunodeficiency virus and hepatitis B and C virus negative by PCR analysis. The original isolation of HBMEC cells displayed the characteristic cobblestone appearance of an endothelial cell culture (Fig. 2). After subculture, the cells were grown to confluence in EGM-2MV medium (Cambrex Bioscience Walkersville) and achieved a monolayer of flattened, closely apposed endothelial cells in 4–5 days.

Stimulation of HBMEC. Confluent endothelial cells were washed with a HEPES buffer of the following composition (in mmol/l): 133.5 NaCl, 4.8 KCl, 1.2 MgCl\textsubscript{2}, 1.2 KH\textsubscript{2}PO\textsubscript{4}, 10 HEPES, 10 glucose, and 1.2 CaCl\textsubscript{2} (pH 7.4). All stimulations were carried out in the tissue culture well with the endothelial cells as a confluent monolayer. Thrombin (95 IU/mg protein; Sigma), tryptase (1,200 IU/mg protein; Promega, Madison, WI), SFLRIN (Invitrogen, Carlsbad, CA), or SLIGKV (Invitrogen) was dissolved in HEPES buffer at a stock concentration 100 times the final concentration. Where appropriate, a stock solution of bromoenol lactone (BEL) in DMSO was diluted with HEPES buffer and added before stimulation by thrombin or tryptase.

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**Immunofluorescence microscopy for cadherin-5, factor VIII, and ZO-1.** To determine localization of cadherin-5, cells were stained using anti-cadherin-5 MAb (Transduction Laboratories, Lexington, KY) and anti-factor VIII rabbit IgG (Zymed Laboratories, South San Francisco, CA), respectively, and then fluorescent secondary antibodies. Briefly, cells were fixed with ice-cold methanol for 15 min, washed, and permeabilized for 2 min with 0.5% Triton X-100 in (in mM) 10 piperazine ethane sulfonic acid, 50 NaCl, 300 sucrose, and 3 MgCl2 (pH 6.8). After incubation in blocking solution (1% albumin ± normal goat serum in PBS) with primary antibodies, cultures were washed and treated with the appropriate fluorescent label: Alexa Fluor 488 goat anti-mouse IgG or Alexa Fluor 568 goat anti-rabbit IgG (Molecular Probes, Eugene, OR). Prolong Gold antifade reagent with 4,6-diamidino-2-phenylindole (Molecular Probes) was used for mounting. Images were viewed using a LOMO PLC fluorescent microscope with attached Sony 3CCD camera, saved as TIFF files, and processed using Image Pro Plus software (MediaCybernetics, Silver Spring, MD).

**RT-PCR analysis of PAR.** Total RNA was isolated from HBMEC using the Versagen Cell Kit (Genta Systems, Minneapolis, MN), and first-strand cDNA was primed with random hexamers and synthesized using the ThermoScript RT-PCR System (Invitrogen). An 888-bp fragment of PAR-1 was amplified and subjected to automated thin-layer chromatography and quantified by liquid scintillation spectrometry. Activity was standardized to protein concentration as described previously (13).

**Arachidonic acid release.** HBMEC were grown to confluence in 100-mm dishes. The surrounding buffer was removed at the end of the stimulation period and immediately replaced with ice-cold buffer containing 250 mM sucrose, 10 mM KCl, 10 mM imidazole, 5 mM EDTA, and 2 mM dithiothreitol with 10% glycerol (pH 7.8, PLA2 assay buffer). HBMEC suspended in PLA2 assay buffer were sonicated on ice six times for 10 s. PLA2 activity in the cell sonicate was assessed by incubating enzyme with 100 μM (16:0; [3H] 18:1) plasmenylcholine substrate (specific activity ~150 dpm/pmol) in assay buffer containing 100 mM Tris (pH 7.0) and 10% glycerol with 4 mM EGTA at 37°C for 5 min in a total volume of 200 μL, as described previously (6). Reactions were terminated by the addition of 100 μL of butanol, and released radiolabeled fatty acid was isolated by thin-layer chromatography and quantified by liquid scintillation spectrometry. Activity was standardized to protein concentration as described previously (13).

**PAR activation in bladder endothelial cells.** PLA2 catalyzes hydrolysis of membrane phospholipids at the sn-2 position, resulting in stoichiometric production of a free fatty acid and a lysophospholipid. In endothelial cells, activation of protease-activated receptors (PAR) leads to increased Ca2+ independent PLA2 (iPLA2) activity, accelerated membrane phospholipid hydrolysis, and production of multiple biologically active phospholipid metabolites, including lysoplasmenylcholine, platelet-activating factor (PAF), arachidonic acid, and PGI2.
a scintillation vial. Radioactivity in surrounding medium and cells was quantified by liquid scintillation spectrometry. Arachidonic acid mobilized from cellular phospholipids was expressed as percentage of total incorporated radioactivity.

**PGE₂ and PGI₂ release.** HBMEC cells were grown to confluence in 15-mm-diameter cell culture wells. Cells were washed twice with Hanks’ balanced salt solution as described above and incubated with 50 μCi of [³H]acetic acid (specific activity 2.5 Ci/mmol; Perkin-Elmer Life Sciences) for 20 min. After stimulation for the selected time interval, lipids were extracted from the cells by the method of Bligh and Dyer (1). The chloroform layer was concentrated by evaporation under N₂, applied to a silica gel G thin-layer chromatography plate, and developed in chloroform-methanol-acetic acid-water (50:25:8:4 vol/vol). The region corresponding to PAF was scraped, and radioactivity was quantified with liquid scintillation spectrometry. Loss of PAF during extraction and chromatography was corrected by addition of a known amount of [¹⁴C]PAF as an internal standard, as described previously (14).

**RESULTS**

After reaching confluence, cultures of HBMEC were initially characterized by immunocytological studies with use of antibodies to two molecules that are specific for endothelial cells: the cell adhesion molecule cadherin-5 and factor VIII (Fig. 3, A and B). Cells stained positive for both endothelial cell markers: cadherin-5 was present at junctions between adjacent cells showing cells of polygonal morphology, and factor VIII staining was detected in the cytoplasm. To identify the percentage of cells containing factor VIII in our confluent cultures, we simultaneously stained for factor VIII, ZO-1, and cell nuclei (Fig. 3 C). ZO-1 is a peripheral membrane, tight junction-associated protein found on epithelial and endothelial cell membranes. Immunocytochemical staining for ZO-1 enabled us to delineate individual cell margins within the monolayer. At passage 5, 165 stained cells were counted, with 96% showing positive staining for factor VIII. Taken together, these results confirmed that our cell isolation (Cambrex Bioscience Walkersville) was primarily endothelial.

Having characterized the growth and properties of the endothelial cells isolated from the bladder, we verified by immunoblot analysis that HBMEC possessed PAR-1 and PAR-2 receptors (Fig. 4). Anti-PAR-1 or anti-PAR-2 antibodies were used to demonstrate the presence of an immunoreactive band at ~55 kDa in cell lysates prepared from the HBMEC (Fig. 4). Subsequently, we measured HBMEC PLA₂ activity after incubation with thrombin and tryptase (Fig. 5). We measured PLA₂ activity using (16:0, [³H]18:1) plasmenylcholine substrate in the absence of Ca²⁺ (4 mM EGTA); thus the activity measured represents iPLA₂. After stimulation with 0.1 IU/ml thrombin, iPLA₂ was activated maximally after 1 min and was significantly increased until after 5 min. In contrast, tryptase-stimulated iPLA₂ activity was maximal at 5 min and returned to baseline after 10 min. Thus there is a slight delay in iPLA₂.
activation with tryptase compared with thrombin stimulation. This may reflect a difference in coupling of the enzyme to PAR-1 and PAR-2 in HBMEC. Pretreatment of HBMEC with BEL (2 μM for 10 min), a selective inhibitor of iPLA₂ (25), inhibited the increase in iPLA₂ activity in response to thrombin or tryptase (Fig. 5).

Activation of iPLA₂ results in increased membrane phospholipid hydrolysis, leading to increased production of multiple phospholipid metabolites that may play a role in inflammation (Fig. 1). To determine whether thrombin- or tryptase-stimulated iPLA₂ activity resulted in a net accumulation of membrane phospholipid metabolites, we carried out subsequent studies to measure arachidonic acid and eicosanoid release from HBMEC monolayers. Arachidonic acid release from HBMEC monolayers stimulated with thrombin or tryptase demonstrated a significant increase after 2 min (Fig. 6). The increase in arachidonic acid release from tryptase-stimulated HBMEC was delayed compared with that from thrombin-stimulated cells, mirroring the increase in the iPLA₂ activity time course for each (Fig. 5). The increase in arachidonic acid release from HBMEC stimulated with thrombin or tryptase was completely inhibited by pretreatment with BEL, further supporting the involvement of iPLA₂ (Fig. 6).

To determine whether arachidonic acid liberated from membrane phospholipids is further metabolized, resulting in release of prostaglandins from HBMEC monolayers, we measured PGI₂ and PGE₂ production in response to thrombin or tryptase stimulation. Results showed a concentration- and time-dependent increase in PGI₂ release (Fig. 7) but
little change in PGE2 release (Fig. 8). Pretreatment with BEL completely inhibited PGI2 release in response to thrombin and tryptase stimulation but had little effect on PGE2 production (Fig. 9).

To determine whether the tryptase- and thrombin-stimulated increases in prostaglandin production were mediated via proteolytic cleavage of the PAR-1 and PAR-2 receptors, respectively, we incubated HBMEC with increasing concentrations of the peptides SFLLRN (which represents the tethered ligand sequence for PAR-1) or SLIGKV (which represents the tethered ligand sequence for PAR-2). Incubation with either peptide resulted in a concentration-dependent (1–100 μM) and time-dependent (up to 60 min) increase in PGI2. Maximal increases in PGI2 release were observed with 100 μM SFLLRN or SLIGKV and were significant after 5 min of incubation (data not shown). The increases were similar to those measured with thrombin or tryptase and support our hypothesis that these agents proteolytically cleave PAR on the HBMEC surface.

As shown in Fig. 1, PLA2-catalyzed release of arachidonic acid from membrane phospholipids is accompanied by the stoichiometric production of lysophospholipids. In a previous study, we demonstrated that PAF production in thrombin-stimulated human coronary artery endothelial cells is dependent on activation of iPLA2 and the increased production of lysoosphomyl ethanolamine (12). To determine whether PAF production in HBMEC is dependent on iPLA2 activation, we measured PAF production after thrombin or tryptase stimulation. The results are shown in Fig. 6. PAF release was significantly increased (P < 0.01) in HBMEC treated with thrombin or tryptase as compared to untreated controls. Pretreatment with BEL reduced PAF production (Fig. 6) and supported our hypothesis that these agents proteolytically cleave PAR on the HBMEC surface.

Fig. 4. Immunoblot analysis of PARs in HBMEC lysate. Proteins were transferred to polyvinylidene difluoride membranes. Membranes were probed with anti-PAR-1 (1:2,500 dilution) or anti-PAR-2 (1:100 dilution) antibodies and incubated with horseradish peroxidase linked secondary antibodies (1:50,000 dilution). Immunoblots were detected with enhanced chemiluminescence and exposure to film for 5 min. Major immunoreactive band at 55 kDa corresponds to apparent molecular weight of PAR-1 and PAR-2 as described by the antibody manufacturer (Santa Cruz Biotechnology) and agrees with our previous studies in human urothelial cells (18).

Fig. 5. Changes in HBMEC PLA2 activity in response to incubation with thrombin (0.1 IU/ml) or tryptase (20 ng/ml). PLA2 activity was measured using (16:0 and [3H]18:1) plasmenylcholine substrate in the absence of Ca2+ (4 mM EGTA). Pretreatment with 2 μM bromoenol lactone (BEL) inhibited thrombin- or tryptase-stimulated iPLA2 activity. Values are means ± SE of independent results from 4 separate cell cultures. *P < 0.05; **P < 0.01 vs. control. PLA2 activity was significantly (P < 0.01) lower in HBMEC pretreated with BEL than in corresponding samples with no BEL pretreatment.

Fig. 6. Arachidonic acid release as percentage of total incorporated radioactivity from HBMEC stimulated with thrombin (0.1 IU/ml) or tryptase (20 ng/ml). Increase in arachidonic acid release in response to thrombin and tryptase was completely inhibited by pretreatment for 10 min with 2 μM BEL. Values are means ± SE of measurements of 6 cell cultures. *P < 0.05; **P < 0.01 vs. unstimulated controls.
bin and tryptase stimulation. Stimulation with either agent resulted in a significant increase in PAF production that was completely inhibited by BEL pretreatment (Fig. 10). In addition, a 3.1-fold increase in PAF production was observed when HBMEC were stimulated for 10 min with 100 μM SFLLRN \( (P < 0.01, n = 4) \) and a 2.6-fold increase was observed with 10 min of stimulation with 100 μM SLIGKV \( (P < 0.01, n = 4) \).

Taken together, these data demonstrate that thrombin and tryptase stimulation of HBMEC activates iPLA₂, resulting in the production of several biologically active phospholipid metabolites that may play an active role in the inflammatory process in conditions such as IC.

**DISCUSSION**

The bladder is a hollow sphere, with the wall of the sphere comprising the serosa, muscularis, submucosa, muscularis mucosa, and lamina propria \( (10) \). Embedded within these structures is a circulatory system, sensory and motor neurons, and an immune system. The bladder is protected from its contents by a layer of epithelial cells (the urothelium) and an adhering glycosaminoglycan layer. Thrombin and tryptase activate PAR-1 and PAR-2, respectively. In IC, extracellular tryptase from bladder mast cells, as well as increased enzyme levels in 24-h urine samples, have been documented \( (2, 22) \). Furthermore, thrombin may be present in the plasma exudates during inflammation. Thus thrombin and tryptase may be present in the IC bladder and activate cell PARs. We previously demonstrated that short intervals of thrombin and tryptase stimulation of HUR results in increased iPLA₂ activity, resulting in increased production of PAF, arachidonic acid, and PGE₂. The dual production of PAF and PGE₂ is interesting, because it indicates that activation of PARs on the HUR surface can result in biologically active phospholipid metabolites that could play an inflammatory (PAF) or a protective (PGE₂) role in the epithelial cells.

Stimulation of endothelial cells in the bladder wall by thrombin or tryptase may result in a similar pattern of phospholipid metabolite production and may play a role in the inflammatory process in IC. For example, increased endothelial PAF production may lead to an increase in polymorphonuclear leukocyte (PMN) infiltration into the bladder wall. In patients with IC, PMNs have been detected.
in 94% of urine specimens (8), suggesting PMN recruitment during the inflammatory process. Thus we performed this study to determine whether short intervals of thrombin or tryptase stimulation of HBMEC resulted in activation of iPLA2 and an increase in inflammatory phospholipid metabolite production. The data presented here demonstrate that thrombin and tryptase stimulation of HBMEC activates iPLA2, resulting in increased production of several biologically active phospholipid metabolites that may play a role in inflammation.

The time course of activation of iPLA2, release of arachidonic acid, and production of PGI2 was slightly delayed when HBMEC were stimulated with tryptase compared with thrombin. This may suggest a different intracellular coupling between iPLA2 and PAR-1 or PAR-2 or may represent a delay in activation of PAR-2 compared with PAR-1. Although we measured significant increases in PGI2 production (Fig. 7), little change in PGE2 production was observed with thrombin or tryptase (Fig. 8). In contrast, in our studies in HUR (18) and in coronary artery endothelial cells (data not shown), PGE2 production increased significantly with thrombin or tryptase. PGE2 has generally inhibitory effects on inflammation-induced cellular injury and downregulates the cytokine response of inflammatory cells (3). Thus it is possible that endothelial cells in the bladder may have less capacity to play an anti-inflammatory role than endothelial cells in other vascular beds and possibly make the bladder more susceptible than other organs to inflammatory conditions.

As recently reviewed by Meyer and McHowat (15), PAF is a biologically active phospholipid metabolite that serves as an intercellular and intracellular messenger and is involved in the development and progression of several physiological and pathological processes. After production, PAF remains cell-associated and assists in the tethering and migration of circulating cells through the endothelium. Other inflammatory actions of PAF include interaction with the production and effect of inflammatory cytokines, contribution to increased expression of other lipid mediators, and increased vascular permeability (3). The production of PAF in HBMEC may play an active role in inflammation in the recruitment and migration of PMNs into the bladder wall. This role is under investigation in our laboratory.

In conclusion, this study indicates that tryptase and thrombin stimulation of HBMEC results in an increase in iPLA2-catalyzed membrane phospholipid hydrolysis and accumulation of inflammatory mediators, including arachidonic acid, PAF, and PGI2. Our previously reported results in HUR demonstrated that activation of PAR-1 or PAR-2 resulted in increased membrane-associated iPLA2 activity and increased arachidonic acid, prostaglandin E2, and PAF production (19). We have now extended these studies in HBMEC and demonstrate that tryptase and thrombin stimulation of PAR-1 and PAR-2 may be acting through the same intracellular signaling pathways and may play a role in the propagation of the inflammatory response at urothelial and microvascular sites in disease processes, such as IC. Further studies are needed to determine differences in individual prostaglandin production and net balances of these
metabolites in a cytoprotective or inflammatory role involved in the pathophysiology of the disease process.

REFERENCES