Protease-activated receptor stimulation activates a Ca\(^{2+}\)-independent phospholipase A\(_2\) in bladder microvascular endothelial cells

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Submitted 5 August 2004; accepted in final form 17 November 2004

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 pathophysiological 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 pathological 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\(_2\) (PLA\(_2\)) 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\(^{2+}\)-independent phospholipase A\(_2\) (iPLA\(_2\)) activity and accelerated production of membrane phospholipid-derived inflammatory mediators, including arachidonic acid, PGE\(_2\), 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\(_2\), 1.2 KH\(_2\)PO\(_4\), 10 HEPES, 10 glucose, and 1.2 CaCl\(_2\) (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), SFLLRN (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 concentration 100 times the final concentration. Where appropriate, a stock solution of bromoenol lactone (BEL) in DMSO was diluted concentration 100 times the final concentration.

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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 secondary anti-mouse IgG tetramethylrhodamine isothiocyanate conjugate (Sigma), based on our previously described method (19). Factor VIII and ZO-1 were visualized in HBMEC cultures using antibodies, mouse anti-factor VIII, and rabbit anti-ZO-1 (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 Versagene 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 sequencing to verify identity using the primers 5'-GTCTGTGCGGCCTGTTGCTG-3' (forward) and 5'-GGGACTTCTTGTGCGGTTGGCAACTG-3' (reverse). Similarly, a 320-bp fragment of PAR-2 was amplified and sequenced using the primers 5'-GGCGATCTGGCCTGTGCT-3' (forward) and 5'-GGCGAGGAATGAGATGGCTGCG-3' (reverse).

Immunoblot analysis of PAR. HBMEC were suspended in lysis buffer containing 20 mM HEPES (pH 7.6), 250 mM sucrose, 2 mM dithiothreitol, 2 mM EDTA, 2 mM EGTA, 10 mM β-glycerophosphate, 1 mM sodium orthovanadate, 2 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, 10 μg/ml aprotinin, 5 μg/ml pepstatin A, and 1% Triton X-100. Cells were sonicated on ice for six bursts of 10 s and centrifuged at 14,000 g at 4°C for 10 min to remove cellular debris and nuclei. HBMEC protein was mixed with an equal volume of SDS sample buffer and heated at 95°C for 5 min before it was loaded onto a 10% polyacrylamide gel. Protein was separated by SDS-PAGE at 200 V for 35 min and electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Richmond, CA) at 100 V for 1 h. Nonspecific sites were blocked by incubation of the membranes with Tris buffer solution containing 0.05% (vol/vol) Tween 20 (TBST) and 5% (wt/vol) nonfat milk for 1 h at room temperature. The blocked PVDF membrane was incubated with primary antibodies to PAR-1 (1:2,500 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) or PAR-2 (1:100 dilution; Santa Cruz Biotechnology) for 1 h at room temperature. Unbound antibodies were removed with three washes with TBST solution, and membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies (1:5,000 dilution) before experimental conditions. At the end of the stimulation period, membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies (1:50,000 dilution; Amersham, Arlington Heights, IL). After six washes with TBST, regions of antibody binding were detected using enhanced chemiluminescence (Pierce Laboratories, Rockford, IL) and exposure to film (Hyperfilm, Amersham).

PLA2 activity. 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).

Arachidonic acid release. HBMEC were grown to confluence in 35-mm tissue culture dishes. Arachidonic acid release was determined by measuring [3H]arachidonic acid released into the surrounding medium from HBMEC prelabeled with 3 μCi of [3H]arachidonic acid (specific activity 100 Ci/mmol; Perkin-Elmer Life Sciences, Boston, MA) per culture dish for 18 h. This resulted in incorporation of >90% of the arachidonic acid into the sn-2 position of the membrane phospholipids. HBMEC were washed three times with HEPES buffer containing 133.5 mM NaCl, 4.8 mM KCl, 1.2 mM CaCl2, 1.2 mM MgCl2, 1.2 mM KH2PO4, 10 mM HEPES (pH 7.4), 10 mM glucose, and 3.6% bovine serum albumin and incubated at 37°C for 15 min before experimental conditions. At the end of the stimulation period, the surrounding medium, which represented the amount of radiolabeled arachidonic acid released from HBMEC, was transferred to a scintillation vial. The amount of radiolabeled arachidonic acid in the cells was measured by lysis of the cells in 10% SDS and transferred to

![Fig. 1. 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+-dependent 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.](http://ajprenal.physiology.org/)}
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, phospholipids 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).

PAF production. Confluent HBMEC were grown in 35-mm-diameter tissue culture dishes and 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 immunocytochemical 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. 3C). 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.
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 lyso[18:1]plasmylethanolamine (12). To determine whether PAF production in HBMEC is dependent on iPLA2 activation, we measured PAF production after thrombin or tryptase stimulation (Fig. 6). 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. control. PAF2 activity was significantly (P < 0.01) lower in HBMEC pretreated with BEL than in corresponding samples with no BEL pretreatment.
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