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Redistribution of calcium-independent phospholipase A_2 isoforms in IC/BPS urothelial cells

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Abstract

Background: Interstitial cystitis/bladder pain syndrome is a debilitating inflammatory bladder disease with currently unknown etiology. We determined the expression of calcium-independent phospholipase $A_2\beta$ ($iPLA_2\beta$) and $iPLA_2\gamma$ isoforms in cultured immortalized urothelial cells isolated from normal or IC/BPS patient bladders.

Materials and methods: Immunoblot analysis was used to determine the $iPLA_2$ isoform expression in immortalized urothelial cells isolated from normal and IC/BPS patients. $iPLA_2$ activity was measured using a radiometric assay with (16:0, [^{3}H]18:1) plasmenylcholine as substrate. Platelet-activating factor (PAF) accumulation was determined by labeling cells with [^{3}H] acetic acid. Adherence of polymorphonuclear leukocytes (PMN) to urothelial cells was determined by measuring myeloperoxidase (MPO) activity.

Results: We identified the two predominant mammalian $iPLA_2$ isoforms, $iPLA_2\beta$ and $iPLA_2\gamma$, in immortalized urothelial cells. IC/BPS urothelial cells demonstrated a decrease in $iPLA_2\gamma$ and an increase in $iPLA_2\beta$ immunoprotein when compared to normal cells. Selective pharmacologic inhibition demonstrated that the majority of $iPLA_2$ activity in normal urothelium represents $iPLA_2\gamma$ whereas that in IC/BPS urothelium is $iPLA_2\beta$. Greater PAF production and PMN adherence was observed in tryptase-stimulated IC/BPS urothelial cells when compared to normal urothelial cells. In both normal and IC/BPS urothelial cells, increased PAF production and PMN adherence was completely blocked when tryptase-stimulated cells were pretreated with (*S*)-bromoenol lactone (BEL) to inhibit $iPLA_2\beta$. PMN adherence was blocked when PMNs were pretreated with ginkgolide B to block the PAF-receptor (PAFR).

Conclusions: Urothelial cells derived from IC/BPS origin exhibit a redistribution of $iPLA_2$ isoforms with $iPLA_2\beta$ being predominately expressed compared to normal urothelial cells. The redistribution of $iPLA_2\beta$, increased PAF production and increased PMN adherence may represent a mechanism whereby IC/BPS patients' exhibit increased susceptibility to bladder inflammation.

Keywords: Inflammation, platelet-activating factor, PMN adherence, urothelial cells

Introduction

The urothelium that lines the inside of the bladder was thought originally to function solely as a barrier restricting movement of urinary constituents into the underlying tissue. Subsequently, the urothelial cell has been shown to be instrumental in a variety of signaling pathways involved in inflammation, differentiation and imparting selective permeability [1,2]. Alterations in the normal signaling processes of the urothelial cell have been shown to be involved in tumor growth, reduced barrier function, and bladder inflammation. Interstitial cystitis/bladder pain syndrome (IC/BPS) is a chronic inflammatory bladder disease associated with dysfunctional urothelial cell barrier function, altered signaling responses and increased mast cell activation [3,4]. Mast cells are the primary effectors of IgE-mediated immune responses, and upon activation, release a variety of preformed and newly synthesized inflammatory mediators. Tryptase stimulation of protease activated receptors (PARs) expressed on urothelial cells results in calcium-independent phospholipase A_2 ($iPLA_2$) activation and subsequent platelet activating factor (PAF) accumulation [5].

PAF synthesis is initiated via $iPLA_2$-catalyzed hydrolysis of the *sn*-2 fatty acid from membrane phospholipids resulting in the release of a lysophospholipid which can be subsequently acetylated at the *sn*-2 position to form PAF [6]. The two major isoforms of $iPLA_2$ found in mammalian cells, $iPLA_2\beta$ and $iPLA_2\gamma$, are responsible for a wide range of physiological responses.

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We have previously determined that endothelial cell iPLA$_2$β is the predominant isoform responsible for PAF production [7]. PAF is a potent recruiter that facilitates transendothelial and transepithelial migration of circulating inflammatory cells. This action is accomplished by the interaction of endothelial or urothelial cell PAF with the inflammatory cell PAF-receptor (PAFR) resulting in tight adherence and subsequent migration through the paracellular space [8]. Increases in PAF accumulation result in enhanced inflammatory cell adherence to cultured endothelial cells [9]. This process could play a role in the increased presence of inflammatory cells in the bladder wall of patients with IC/BPS resulting in the pain and inflammation associated with the condition.

In the present study we determine the expression of iPLA$_2$ isoforms in normal and IC/BPS urothelial cells following tryptase stimulation. We further show that PAFR antagonism by ginkgolide B prevents adherence of inflammatory cells to cultured urothelial cells regardless of tryptase stimulated increases in iPLA$_2$ and subsequent increase in urothelial PAF accumulation. This work highlights two potential therapeutic targets for the management of inflammatory bladder conditions.

Materials and methods

Cell culture

Urothelial cells were isolated from normal or IC/BPS patient bladders and immortalized with a retrovirus encoding the oncoproteins E6 and E7 of human papillomavirus type 16 and selected for stable integration of the retroviral pro virus with G418. Expanded cultures were grown in EpiLife Media (Cascade Biologics, Inc. Portland, OR) with calcium (0.06mM), growth factor supplements provided by the manufacturer and penicillin (20U/ml)/streptomycin (100mg/ml) (Sigma Chemical Company, St. Louis, MO) and incubated at 37˚C, with an atmosphere of 95% O_2, 5% CO_2. Confluent monolayers were differentiated by adding 1 mM calcium and 10% fetal bovine serum (Ca/FBS). Experiments were performed after 3 days of differentiation. Samples were collected according to an Oklahoma University Health Sciences Center Institutional Review Board approved protocol following informed written consent.

Immunoblot analysis

Urothelial cells were suspended in lysis buffer containing (mmol/l) HEPES 20 (pH 7.6), sucrose 250, dithiothreitol 2, EDTA 2, EGTA 2, β-glycerophosphate 10, sodium orthovanadate 1, phenylmethylsulfonyl fluoride 2, leupeptin 20 µg/ml, aprotinin 10 µg/ml and pepstatin A 5 µg/ml. Cells were sonicated on ice and centrifuged at 20,000xg at 4°C for 20 min to remove cellular debris and nuclei. Cytosolic protein was separated by SDS/PAGE and electrophoretically transferred to PVDF membranes (Bio-Rad, Richmond, CA). The blocked PVDF membranes were incubated with primary antibodies and horseradish peroxidase-conjugated secondary antibodies. Regions of antibody binding were detected using enhanced chemiluminescence (Amersham, Arlington Heights, IL) after exposure to film (Hyperfilm, Amersham).

Measurement of iPLA$_2$ activity

Urothelial cell cultures were washed with ice-cold PBS and suspended in 1 ml PLA$_2$ assay buffer containing (mmol/l): sucrose 250, KCl 10, imidazole 10, EDTA 5, dithiothreitol (DTT) 2 with 10% glycerol, pH=7.8. The suspension was sonicated and centrifuged to remove cellular debris and nuclei. Phospholipase A$_2$ activity in the supernatant (cytosolic and membrane protein) was assessed by incubating enzyme (50 µg protein) with 100 µM (16:0, [^{3}H]18:1) plasmenylcholine substrate in buffer containing (mmol/l): Tris 10, EGTA 4, 10% glycerol, pH=7.0 at 37°C for 5 minutes in a total volume of 200 µl. Reactions were terminated by the addition of 100 µl butanol and released radiolabeled fatty acid was isolated by application of 25 µl of the butanol phase to channeled Silica Gel G plates, development in petroleum ether/diethyl ether/acetic acid (70/30/1, v/v) and subsequent quantification by liquid scintillation spectrometry.

Measurement of PAF production

Urothelial cells grown to confluence were incubated with Hanks' balanced salt solution (135 mM NaCl, 0.8 mM $MgSO_4$, 10 mM HEPES, pH 7.4, 1.2 mM $CaCl_2$, 5.4 mM KCl, 0.4 mM KH_2PO_4 and 6.6 mM glucose) containing 10 µCi of [^{3}H] acetic acid for 20 min at room temperature. Cellular lipids were extracted using the method of Bligh and Dyer [10] and the chloroform layer was dried under nitrogen. Total lipid extracts were resuspended in 9:1 $CHCl_3$:MeOH and applied to TLC plates. Plates were developed in 100:50:16:8 chloroform, methanol, acetic acid and water. The region corresponding to PAF was scraped and measured by liquid scintillation counting.

Measurement of PMN adherence

Blood (80 ml) was obtained from healthy donors and PMNs were isolated using Polymorphprep (Axis-Shield, Oslo, Norway). PMNs ($2x10^6$) were added to urothelial cells grown to confluence in 34-mm dishes. At the end of incubation, non-adherent PMNs were removed by washing the well twice with Hanks' buffer. Urothelial cells and adherent PMNs were lysed with 0.2% Triton X-100 and myeloperoxidase (MPO) content was determined by adding 400 µL of cell lysate to a tube containing 1mL of PBS, 1.2mL Hanks buffer with bovine serum albumin, 200 µL of 0.125% 3,3'-dimethoxybenzidine, and 200 µl of 0.05% H_2O_2. After samples were incubated at 37°C for 15 min, the reaction was stopped by the addition of 200 µl of NaN_3, and the absorbance of each tube was measured at 460 nm. The MPO content in $2x10^6$ PMNs was determined and used as the value for 100% adherence.

Statistical analysis

Statistical analysis was performed using Students' t test. The

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difference between groups was considered significant at a level of $p<0.05$. All assays were performed at least four times.

Results

Expression of iPLA$_2$ isoforms in normal and IC/BPS urothelial cells

We identified the two predominant mammalian iPLA$_2$ isoforms, iPLA$_2$β and iPLA$_2$γ, in cultured immortalized urothelial cells from normal and IC/BPS patients. As shown in **Figure 1**, iPLA$_2$β immunoprotein expression was significantly greater in IC/BPS-derived urothelial cells when compared to cells derived from normal bladders. In contrast, expression of iPLA$_2$γ was significantly decreased in IC/BPS urothelial cells (**Figure 1**). In the representative blots shown in **Figure 1**, the presence of a lower molecular weight band is detected in the normal group however this band is present in both normal and IC/BPS derived urothelial cells following longer exposure time.

To determine if the redistribution of iPLA$_2$ isoform immunoprotein resulted in alterations in iPLA$_2$ activity, we measured activity using (16:0, [^{3}H]18:1) plasmenylcholine in the absence of calcium (**Figure 2**). To determine the relative contribution of iPLA$_2$β and iPLA$_2$γ to total iPLA$_2$ activity, we pretreated urothelial cell membrane fractions with bromoenol lactone (BEL) prior to incubation with the substrate. Racemic BEL inhibits both iPLA$_2$γ and iPLA$_2$β, whereas (*R*)-BEL and (*S*)-BEL inhibit iPLA$_2$γ and iPLA$_2$β respectively, with an approximate 10-fold selectivity [11]. Treatment of immortalized urothelial cells

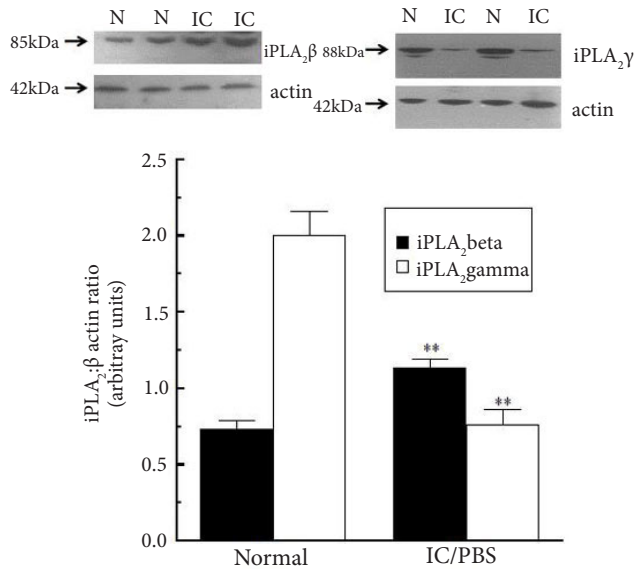

Figure 1. Immunoprotein expression of iPLA$_2$ isoforms (iPLA$_2$β and iPLA$_2$γ) in normal (N) and IC/BPS (IC) urothelial cells. Representative immunoblots are shown in the top panels for iPLA$_2$β and iPLA$_2$γ. Densitometric analysis is shown in the bottom panel and shows data normalized to actin. Data shown in the bottom panel are for 3 different experiments using 4 normal and 4 IC/BPS isolations. **$p<0.01$ when comparing normal and IC/BPS cells.

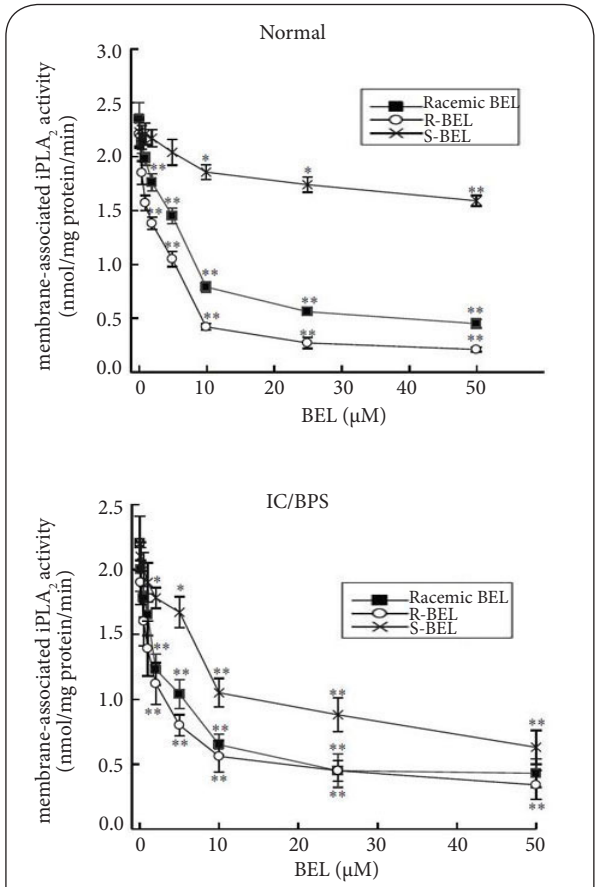

Figure 2. iPLA$_2$ activity in immortalized normal (upper panel) and IC/BPS (lower panel) urothelial cells. Data shown are for 3 different experiments using 4 normal and 4 IC/BPS isolations. *$p<0.05$, **$p<0.01$ when compared to activity in the absence of inhibitor.

isolated from normal bladders demonstrated PLA2 activity in the absence of calcium that was inhibited by pretreating with racemic BEL or (*R*)-BEL in a concentration dependent manner (**Figure 2**, upper panel). iPLA$_2$ activity in normal urothelial cells was relatively resistant to pretreatment with (*S*)-BEL, suggesting that the majority of iPLA$_2$ activity could be contributed to iPLA$_2$γ (**Figure 2**, upper panel). In contrast, urothelial cells isolated from IC/BPS patients demonstrated a much greater inhibition of iPLA$_2$ activity with (*S*)-BEL pretreatment (**Figure 2**, lower panel) than that observed at corresponding concentrations in normal urothelial cells (**Figure 2**, upper panel). Inhibition of iPLA$_2$ activity in IC/BPS urothelial cells was comparable when using both racemic BEL and (*R*)-BEL, suggesting a lesser role for iPLA$_2$γ in these cells (**Figure 2**, lower panel). Taken together, our data indicate a redistribution of iPLA$_2$β and iPLA$_2$γ in IC/PBS urothelial cells demonstrated by immunoblot analysis and measurement of iPLA$_2$ activity.

PAF production in normal and IC/BPS urothelial cells

To determine if the redistribution of iPLA$_2$ isoforms in IC/BPS urothelial cells resulted in a difference in PAF production,

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we used a radiometric assay to quantify PAF accumulation in cultured urothelial cells. When normal uro thelial cells were stimulated with tryptase, we observed a significant increase in PAF production compared to unstimulated controls (**Figure 3**). The observed increase in PAF production in response to tryptase was even greater in urothelial cells derived from IC/BPS patients (**Figure 3**). Since PAF production is initiated by iPLA$_2$ activation, we pretreated urothelial cells with BEL prior to tryptase stimulation. As shown in **Figure 3**, pretreatment of both normal and IC/BPS derived urothelial cells with BEL prior to tryptase stimulation blocked PAF production completely.

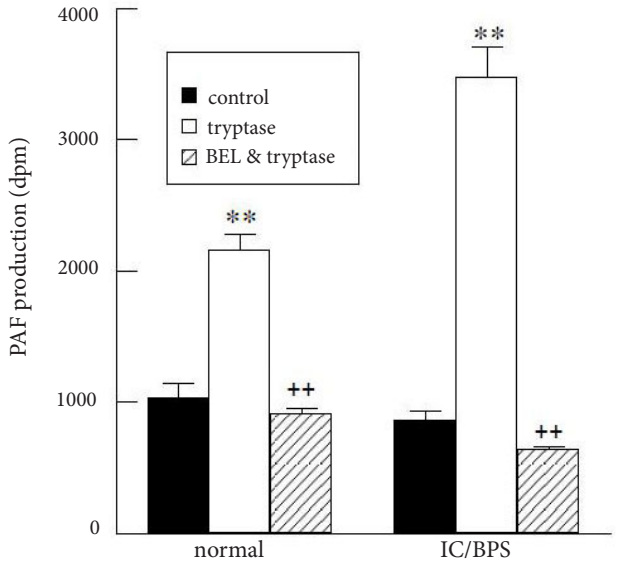

Figure 3. PAF production in immortalized urothelial cells isolated from normal and IC/BPS patients. Data shown are for 3 different experiments using 4 normal and 4 IC/BPS isolations. **p<0.01 when compared to unstimulated controls. $^{++}$p<0.01 when comparing data in the presence or absence of inhibitor.

PMN adherence to normal and IC/BPS urothelial cells

PAF facilitates transepithelial migration via interaction with the PAF-receptor (PAFR) expressed on the inflammatory cell surface. Modulation of this PAF-PAFR interaction could serve as a beneficial means of managing bladder inflammation. Corresponding to the increased accumulation of PAF in urothelial cells, we observed a significant increase in PMN adherence to tryptase-stimulated urothelial cells that was greater in IC/BPS-derived urothelial cells when compared to normal (**Figure 4**) PMN adherence to both normal and IC-BPS-derived urothelial cells was blocked by pretreating the urothelial cells with BEL, further demonstrating the role of iPLA$_2$ and PAF in modulating inflammation (**Figure 4**, gray bars). Pretreatment of PMN with ginkgolide B to block the PAFR also inhibited cell adherence to tryptase-stimulated urothelial cells (**Figure 4**, hatched bars).

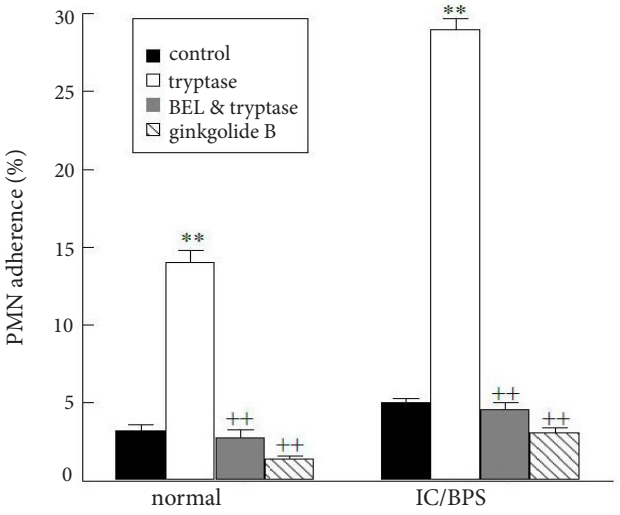

Figure 4. Polymorphonuclear leukocyte (PMN) adherence to normal and IC/BPS urothelial cells. Data shown are for 3 different experiments using 4 normal and 4 IC/BPS isolations. **p<0.01 when compared to untreated controls. $^{++}$p<0.01 when comparing data in the presence or absence of inhibitor.

Discussion

The urothelium that lines the inside of the bladder provides a barrier that prevents leakage of urinary constituents into the underlying tissue that can lead to pain and inflammation. Disruption of the barrier function can occur during times of inflammation, infection or mechanical damage and result in activation of resident mast cells and can directly depolarized nerve and muscle leading to pain and further inflammation [**2**,**12**]. Mast cells release a variety of inflammatory mediators upon degranulation, including tryptase. Tryptase has been detected in the urine of patients with IC/BPS indicating mast cell activation [**13**]. Previous studies have shown that tryptase stimulation of human urothelial cells, through PAR-2 cleavage, results in the activation of iPLA$_2$ and subsequently PAF production [**5**]. Platelet activating factor (PAF) is a potent inflammatory mediator whose formation is facilitated by iPLA$_2$ mediated hydrolysis of the *sn-2* fatty acid from membrane phospholipids resulting a free fatty acid and a lysophospholipid, which can be acetylated subsequently to produce PAF [**6**]. The majority of iPLA$_2$ activity in mammalian cells is contributed by iPLA$_2$β and iPLA$_2$γ [**14**]. In this study, we show that there is a redistribution of iPLA$_2$ isoforms in urothelial cells derived from IC/BPS patients, with a decrease in iPLA$_2$γ and increased iPLA$_2$β. We have demonstrated previously that pharmacological inhibition or genetic ablation of iPLA$_2$β results in attenuated PAF production and significantly reduced inflammatory cell adherence to endothelial cells [**7**]. Similarly, in this study, the increased iPLA$_2$β immunoprotein expression and contribution to total iPLA$_2$ activity observed in IC/BPS-derived urothelial cells is associated with increased PAF production and PMN adherence. PAF is known to be involved in many cellular processes, including

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angiogenesis, tumor growth, platelet aggregation and inflammation. A significant increase in PAF production in tryptase-stimulated urothelial cells provides evidence that this response could be involved in bladder inflammation mediated by mast cell activation, such as occurs in IC/BPS patients. A redistribution of $iPLA_2$ isoforms could have a profound impact on membrane phospholipid derived inflammatory mediators resulting in altered signaling responses. In IC/BPS derived urothelial cells, the increase in $iPLA_2\beta$ activity could render the bladders from these patients more susceptible to inflammation via increased PAF production and inflammatory cell retention. As $iPLA_2\beta$ is known to be the predominant isoform responsible for PAF production and the role of PAF in inflammation is well established, the work detailed in the paper provides evidence of altered inflammatory signaling in IC/BPS patients that could predispose to increased susceptibility to bladder inflammation. Increased PAF accumulation on urothelial cells could propagate the inflammatory response by facilitating the urothelial cell PAF-inflammatory cell PAFR interaction, retaining the inflammatory cells in the bladder wall, instead of allowing for clearance into the urine, furthering perpetuating and prolonging the inflammatory response. PAF released from activated urothelial cells could directly activate residing mast cells [15], act in an autocrine manner to promote further urothelial cell PAF production or stimulate endothelial cell PAFR-mediated $iPLA_2$ activation.

These data represent a potential signaling pathway that is up regulated in IC/BPS derived urothelial cells that could be targeted for management of bladder inflammation and potentially as a means of preventing recurrence of symptoms (**Figure 5**). Mast cell accumulation and activation has been accepted as a hallmark characteristic in IC/BPS [3]. The observation that tryptase results in accumulation of urothelial cell PAF, which is potentiated in IC/BPS derived urothelial cells, suggests that the PAF-PAFR interaction would be an important event in bladder inflammation, particularly in the scenario of mast cell degranulation and IC/BPS. Our studies provide two potential therapeutic targets for the management of IC/BPS symptoms: inhibition of urothelial cell $iPLA_2\beta$ activity or blocking the PAFR on bladder wall resident inflammatory cells. To date there is no cure or universal management strategy for IC/BPS and treatment options are aimed at symptom management (**Figure 5**). Due to the ambiguity of IC/BPS pathogenesis, development of treatment options has been very difficult. *Gingko biloba* has been used to manage symptoms of several diseases including memory loss, circulatory diseases and asthma [8,16,17]. Ginkgolide B is an extract of the *Gingko biloba* plant, that accumulates unchanged in the urine [18], making it an exciting potential therapeutic approach for managing bladder inflammation. Our data suggest that targeting the inflammatory cell PAFR with a PAFR antagonist such as *Ginkgo biloba* may alleviate the symptoms of IC/BPS, particularly in acute inflammatory flare-ups, by managing the recruitment and retention of inflammatory cells in the bladder wall.

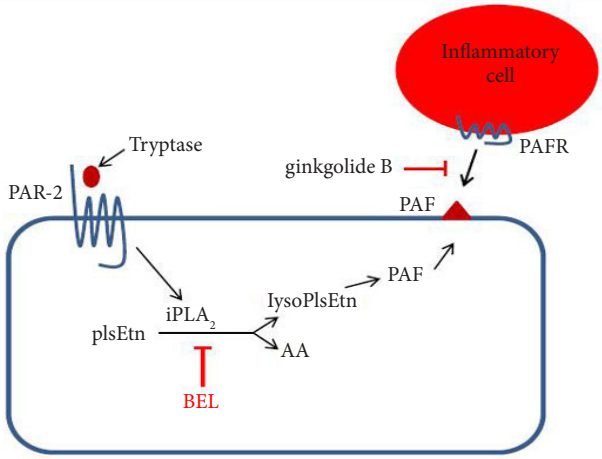

Figure 5. Proposed mechanism for enhanced inflammatory cell adherence to the urothelium. Tryptase stimulation of protease-activated receptor-2 (PAR-2) on the urothelial cell surface results in $iPLA_2$ activation, hydrolysis of plasmenyl ethanolamine (PlsEtn) and release of arachidonic acid (AA) and lysoplasmenylethanolamine (lysoPlsEtn). lysoPlsEtn is acetylated to produce platelet-activating factor (PAF) that is expressed on the urothelial cell surface. Interaction of PAF with the PAF receptor (PAFR) on inflammatory cells results in enhanced adherence to the urothelium. Adherence may be modulated by blocking urothelial cell PAF production with bromoenol lactone (BEL) or by blocking the inflammatory cell PAF receptor with ginkgolide B.

Conclusion

The data shown above demonstrate that IC/BPS is associated with an increase in $iPLA_2\beta$ activity, increased PAF production and PMN adherence. A redistribution of $iPLA_2$ isoforms in IC/BPS may represent a novel therapeutic target to manage IC/BPS. These data show that targeting of urothelial $iPLA_2\beta$ or PAFR antagonism could be beneficial therapeutic targets for the management of bladder inflammation.

List of abbreviations

IC/BPS: Interstitial cystitis/bladder pain syndrome
$iPLA_2$: calcium-independent phospholipase A_2
PAF: Platelet activating factor
PAFR: Platelet activating factor receptor
PMN: Polymorphonuclear leukocytes
BEL: Bromoenol lactone
PAR: Protease activated receptor

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Authors' contributions	JOM	JM
Research concept and design	--	✓
Collection and/or assembly of data	✓	--
Data analysis and interpretation	✓	✓
Writing the article	✓	✓
Critical revision of the article	--	✓
Final approval of article	--	✓

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