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Endothelial Cell Prostaglandin I_2 and Platelet-Activating Factor Production Are Markedly Attenuated in the Calcium-Independent Phospholipase $A_2\beta$ Knockout Mouse[†]

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ABSTRACT: Damage and activation of lung endothelium can lead to interstitial edema, infiltration of inflammatory cells into the interstitium and airways, and production of inflammatory metabolites, all of which propagate airway inflammation in a variety of diseases. We have previously determined that stimulation of human microvascular endothelial cells from lung (HMVEC-L) results in activation of a calcium-independent phospholipase A_2 (iPLA$_2$), and this leads to arachidonic acid release and production of prostaglandin I_2 (PGI_2) and platelet-activating factor (PAF). We stimulated lung endothelial cells isolated from iPLA$_2\beta$-knockout (KO) and wild type (WT) mice with thrombin and tryptase to determine the role of iPLA$_2\beta$ in endothelial cell membrane phospholipid hydrolysis. Thrombin or tryptase stimulation of WT lung endothelial cells resulted in increased arachidonic acid release and production of PGI_2 and PAF. Arachidonic acid release and PGI_2 production by stimulated iPLA$_2\beta$-KO endothelial cells were significantly reduced compared to WT. Measured PLA_2 activity and PGI_2 production by iPLA$_2\beta$-KO cells were suppressed by pretreatment with (*R*)-bromoenol lactone (*R*-BEL), which is a selective inhibitor of iPLA2γ. In contrast to the increase in PAF production induced by stimulation of WT endothelial cells, none was observed for KO cells, and this suggests that endothelial PAF production is entirely dependent on iPLA$_2\beta$ activity. Because inflammatory cell recruitment involves the interaction of endothelial cell PAF with PAF receptors on circulating cells, these data suggest that iPLA$_2\beta$ may be a suitable therapeutic target for the treatment of inflammatory lung diseases.

Airway inflammation is involved in the pathogenesis of several acute and chronic lung diseases that include asthma, chronic obstructive pulmonary disease, acute respiratory distress syndrome, emphysema, cystic fibrosis, pneumonia, and interstitial fibrosis. Exposure to injurious stimuli activates a variety of cells, including eosinophils, macrophages, mast cells, fibroblasts, smooth muscle cells, and endothelial cells, and this results in the release of vasoactive mediators, toxic metabolites, and cytokines that are involved in acute and chronic bronchoconstriction (*1*, *2*). Lung endothelial injury can result in interstitial edema which contributes to increased morbidity and mortality in pulmonary diseases (*3*). In addition, neutrophil infiltration facilitated by endothelial cell barrier dysfunction contributes to tissue damage in the acute phase of lung injury (*4*−*6*).

Serine proteases such as thrombin and tryptase are released in inflammatory lung diseases. Increased numbers of mast cells are frequently observed in terminal airways, bronchoalveolar lavage fluid, and sputum of asthmatic patients (*7*). Allergen inhalation activates resident mast cells that release a variety of mediators, including arachidonic acid, PAF,[1] histamine, and tryptase (*8*−*10*). Inflammatory plasma exudates contain thrombin, which can cause smooth muscle vasoconstriction and increased pulmonary microvascular endothelial permeability (*11*). Thrombin and tryptase stimulate endothelial cell protease-activated receptor (PAR)-1 and PAR-2 respectively, which results in inflammatory metabolite production (*12*). We have previously demonstrated that stimulation of human pulmonary vascular endothelial cells (HMVEC-L) with thrombin and tryptase activates calcium-independent phospholipase A_2 (iPLA$_2$), which results in increased arachidonic acid release and production of prostaglandin I_2 (PGI_2) and platelet-activating factor (PAF) (*13*). PAF induces bronchoconstriction, bronchial hyperresponsiveness, inflammatory infiltration, mucus hypersecretion, and impaired gas exchange, and this contributes to the pathogenesis of bronchial asthma (*14*, *15*). Additionally, PAF associated with endothelial cells assists in the tethering and transendothelial migration of circulating inflammatory cells, and this results in increased pulmonary microvascular permeability and sequestration of neutrophils, platelets, and fibrin (*16*−*18*).

Three classes of phospholipase A_2 coexist in mammalian cells, secretory (sPLA$_2$), cytosolic (cPLA$_2$), and iPLA$_2$ (for review, see refs *19*−*22*). The enzymes within each class have been further divided into groups and subgroups based on their amino acid sequences (*23*). Secretory PLA_2 isoforms require the presence of millimolar concentrations of calcium for phospholipid hydrolysis, demonstrate no preference for the *sn*-2 fatty acid, and are proposed to play a role in inflammatory conditions such as rheumatoid arthritis and ulcerative colitis. Cytosolic PLA_2 is expressed constitutively in most cells, demonstrates a preference for arachidonylated phospholipids, and has been demonstrated

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[1]Abbreviations: BEL, bromoenol lactone; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol tetraacetic acid; HMVEC-L, human microvascular endothelial cells-lung; iPLA$_2$, calcium-independent phospholipase A_2; KO, knockout; PAF, platelet-activating factor; PAR-1, protease-activated receptor-1; PAR-2, protease-activated receptor-2; PLA_2, phospholipase A_2; PGI_2, prostaglandin I_2; WT, wild type.

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to play a critical role in agonist-induced eicosanoid production in several cells and tissues. However, in several previous studies, we have demonstrated that the majority of endothelial cell PLA_2 activity is $iPLA_2$ and that agonist stimulation results in $iPLA_2$ activation, accelerated membrane phospholipid hydrolysis, and the subsequent production of PGI_2 and PAF (*13*, *24*−*27*). Most $iPLA_2$ activity in mammalian cells resides in the Group VIA and VIB enzymes designated $iPLA_2\beta$ and $iPLA_2\gamma$ (*28*−*30*). Homology between $iPLA_2\beta$ and $iPLA_2\gamma$ includes an ATP binding motif, a consensus serine lipase catalytic center, and a region of nine amino acids of currently unknown functional significance (*31*). These two enzymes exhibit differential sensitivity to inhibition by enantiomers of the suicide substrate designated bromoenol lactone (BEL). Racemic BEL inhibits $iPLA_2$ activity at concentrations over 1000-fold lower than those required to inhibit $cPLA_2$ and $sPLA_2$ enzymes (*32*). In addition, (*S*)-BEL inhibits $iPLA_2\beta$ preferentially over $iPLA_2\gamma$, and the converse is true for (*R*)-BEL (*33*). BEL also inhibits phosphatidate phosphohydrolase which converts phosphatidic acid to diacylglycerol (*34*), and hydrolysis of BEL by $iPLA_2$ generates a diffusible bromomethyl keto acid product that can alkylate thiol groups and that might inhibit neighboring enzymes such as those with active cysteine residues (*35*). Such "off target" effects complicate the interpretation of studies in which BEL is used as a pharmacologic inhibitor of $iPLA_2$ and have motivated studies of genetic manipulations of $iPLA_2$ enzymes to elucidate their roles in biological processes (*36*−*46*).

Mice that do not express $iPLA_2\beta$ have been generated by homologous recombination (*36*), and these $iPLA_2\beta$-KO mice have been used to identify roles for $iPLA_2\beta$ in insulin secretion and glucose homeostasis (*41*, *44*), in macrophage functions (*37*, *39*, *40*), and in vascular myocyte biology (*38*, *42*, *46*). Here, we have used $iPLA_2\beta$-KO mice to study the role of this enzyme in production of the phospholipid-derived inflammatory mediators arachidonic acid, PGI_2, and PAF by isolated pulmonary endothelial cells upon stimulation with thrombin and tryptase.

EXPERIMENTAL PROCEDURES

$iPLA_2\beta$ Knockout Mice. The generation of mice deficient in $iPLA_2\beta$ has been described previously (*36*). Mice were housed in a pathogen-free facility and studies were conducted under protocols approved by Saint Louis University Animal Care and Use Committee.

Endothelial Cells. Human microvascular endothelial cells-lung (HMVEC-L) were obtained from Lonza (Walkersville, MD). HMVEC-L were grown to confluence in EGM-2MV media (Lonza) and incubated at 37 °C, with an atmosphere of 95% O_2, 5% CO_2. Cells were passaged using subculture pack (Lonza) in a 1:3 ratio. Cells from passage 3−4 were used for experiments.

Endothelial cells were isolated from mouse lung by collagenase digestion. The diced lung tissue was incubated in 1 mg/mL collagenase for 1 h @ 37 °C and the digested tissue was passed through a cell strainer. A single cell suspension was obtained by incubating in trypsin-EDTA for 10 min. Endothelial cells were isolated by incubating with murine immunoglobulins to block Fc receptors and then incubating with rat antimouse CD31, rat antimouse CD105, and biotinylated isolectin B4. Cells were washed, incubated with rat antimouse Ig, and streptavidin-conjugated microbeads and separated using an AutoMACs cell separator. The eluted cells were washed, resuspended in EGM-2MV cell culture medium (Lonza), and plated in 25 cm^2 culture flasks. Nonadherent cells were removed the next day, and cells were grown to confluence and passaged at a 1−3 dilution. Cells from passage 3−4 were used for experiments.

Immunofluorescence Microscopy for Factor VIII in Mouse Endothelial Cells. To determine purity of mouse endothelial cell isolations, 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 $MgCl_2$ (pH 6.8). After incubation in blocking solution (1% albumin and normal goat serum in PBS) with rabbit antifactor VIII antibody (AbCam, Cambridge, MA), cultures were washed and treated with Alexa Fluor 568 goat antirabbit 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).

Prostaglandin I_2 Release. Endothelial cells were grown to confluence in 16 mm tissue culture dishes. Cells were washed twice with Hank's balanced salt solution (HBSS) containing (in mmol/L) 135 NaCl, 0.8 $MgSO_4$, 10 HEPES (pH = 7.6), 1.2 $CaCl_2$, 5.4 KCl, 0.4 KH_2PO_4, 0.3 Na_2HPO_4, and 6.6 glucose. After washing, 0.5 mL of HBSS with 0.36% bovine serum albumin was added to each culture well. Endothelial cells were stimulated with the appropriate human recombinant skin β-tryptase (Promega, Madison, WI) and thrombin (Sigma Chemical Co., St. Louis, MO) concentrations. The surrounding buffer was removed after selected time intervals and PGI_2 release was measured immediately using an immunoassay kit (R&D Systems, Minneapolis, MN).

Arachidonic Acid Release. Endothelial cells were grown to confluence in 35 mm tissue culture dishes. Arachidonic acid release was determined by measuring [^{3}H] arachidonic acid released into the surrounding medium from endothelial cells prelabeled with 1 μCi of [^{3}H] arachidonic acid (specific activity 100 Ci/mmol; Perkin-Elmer Life Sciences, Boston, MA) per culture dish for 18 h. Cells were washed three times with HEPES buffer containing (in mmol/L) 133.5 NaCl, 4.8 KCl, 1.2 $CaCl_2$, 1.2 $MgCl_2$, 1.2 KH_2PO_4, 10 HEPES (pH 7.4), 10 glucose, and 0.36% bovine serum albumin and incubated at 37 °C for 15 min before experimental conditions. At the end of the stimulation period, the surrounding medium was transferred to a scintillation vial and the remaining cells were lysed in 10% sodium dodecyl sulfate and the lysate was then transferred to a separate vial. Radioactivity in the medium and cells was quantified by liquid scintillation spectrometry. Arachidonic acid mobilized from cellular phospholipids was expressed as the percentage of total incorporated radioactivity.

Phospholipase A_2 Activity Measurement. Cells were grown to confluence in 100 mm culture dishes. At the end of each stimulation period, media was removed and immediately replaced with ice cold buffer containing (mmol/L): 250 sucrose, 10 KCl, 10 imidazole, 5 EDTA, 2 dithiothreitol, with 10% glycerol (pH = 7.8). The cells were removed from the tissue culture plate by scraping and the suspension was sonicated on ice for six bursts of 10 s. PLA_2 activity in the lysates was assessed by incubating the cellular protein with 100 μM 1-palmitoyl-2-oleoyl plasmenylcholine [oleoyl-9,10-^{3}H] or 1-palmitoyl-2-oleoyl phosphatidylcholine [oleoyl-9,10-^{3}H] substrate (specific activity

approximately 150 dpm/pmol) in assay buffer containing 10 mM Tris, 10% glycerol with 4 mM EGTA or 1 mM calcium, pH = 7.0 at 37 °C for 5 min in a total volume of 200 μL. Reactions were initiated by adding the radiolabeled phospholipid substrate as a concentrated stock solution in ethanol. Reactions were terminated by the addition of 100 μL of butanol. The radiolabeled fatty acid released in the above reaction was isolated by application of 25 μL of the butanol phase to channeled Silica Gel G plates and then developed in petroleum ether/diethyl ether/acetic acid (70/30/1,v/v/v). Results were quantified by liquid scintillation spectrometry and normalized for protein content in each sample.

Measurement of PAF Production. Endothelial cells grown in 35-mm culture dishes were washed twice with Hanks' balanced salt solution containing (in mM) 135 NaCl, 0.8 $MgSO_4$, 10 HEPES (pH 7.4), 1.2 $CaCl_2$, 5.4 KCl, 0.4 KH_2PO_4, 0.3 Na_2HPO_4, and 6.6 glucose. Cells were incubated with 10 μCi [^{3}H] acetic acid/well for 20 min. After stimulation with thrombin or tryptase, lipids were extracted from the cells using the method of Bligh and Dyer (*47*). The chloroform layer was concentrated by evaporation under nitrogen, resuspended in 9:1 $CHCl_3$/ CH_3OH, applied to a silica gel 60 TLC plate, and developed in chloroform−methanol−acetic acid−water (50:25:8:4 vol/vol/vol/vol). The region corresponding to [^{3}H]PAF was scraped, and radioactivity was quantified by liquid scintillation spectrometry. Loss of PAF during extraction and chromatography was corrected by adding a known amount of [^{14}C] PAF as an internal standard.

Adherence of RAW 264.7 Cells to Endothelial Cell Monolayers. Murine monocyte/macrophage RAW 264.7 cells were labeled with calcein-AM (4 μg/mL, Alexis Biochemicals, Lausen, Switzerland) for 45 min at 37 °C. After washing three times, 2×10^6 cells were layered onto confluent endothelial cell monolayers. Medium and unbound cells were removed and discarded. Adherent RAW 264.7 and endothelial cells were washed with Dulbecco's phosphate buffered saline and lysed with 1 mL of 0.2% Triton. Samples were sonicated (550 Sonic Dismembrator, Fisher Scientific, Pittsburgh, PA) for 10 s. The amount of calcein-AM fluorescence was measured using a Synergy 2 microplate reader (Biotek, Winooski, VT) at an excitation wavelength of 485 nm and emission wavelength of 530 nm. The percent of RAW cell adherence was calculated from the amount of calcein-AM fluorescence measured in 2×10^6 cells.

RESULTS

In previous studies, we have demonstrated that stimulation of HMVEC-L with thrombin and tryptase activates iPLA$_2$ resulting in arachidonic acid release and production of PGI_2 and PAF. These responses were inhibited by pretreatment with racemic BEL, and we have now examined the effects of BEL enantiomers (Figure 1). Stimulation of HMVEC-L with thrombin or tryptase resulted in a significant increase in PAF production (Figure 1, black bars). Pretreating HMVEC-L with 5 μM (*R*)-BEL resulted in no significant inhibition of thrombin- or tryptase-stimulated PAF production (Figure 1, open bars). In contrast, pretreatment with 5 μM (*S*)-BEL resulted in complete inhibition of thrombin- or tryptase-stimulated PAF production (Figure 1, gray bars), suggesting that iPLA$_2\beta$ activity is required for these responses and that stimulation of HMVEC-L with thrombin or tryptase results in activation of iPLA$_2\beta$.

Similarly, stimulation of HMVEC-L with thrombin or tryptase resulted in a significant increase in prostaglandin I_2 (PGI_2)

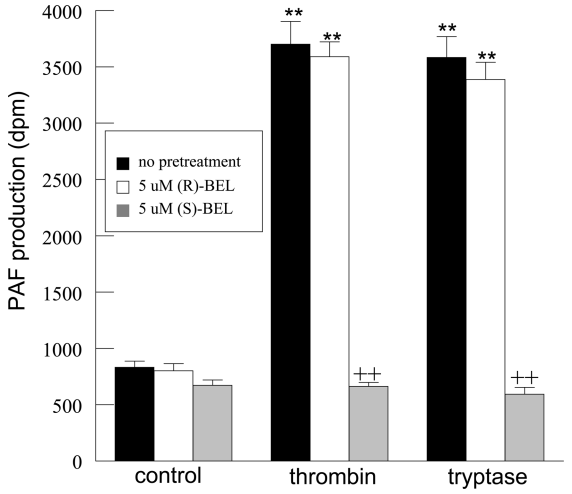

FIGURE 1: Effect of pretreatment with (*R*)-bromoenol lactone (5 μM, 10 min, (*R*)-BEL) or (*S*)-BEL (5 μM, 10 min) on platelet-activating factor (PAF) production in human pulmonary microvascular endothelial cells stimulated with thrombin (1 IU/mL, 10 min) or tryptase (20 ng/mL, 10 min). Data are expressed as mean + SEM for six separate cell cultures. $**p < 0.01$ when compared to control values. $++p < 0.01$ when comparing PLA_2 inhibitor-treated values with corresponding stimulated data.

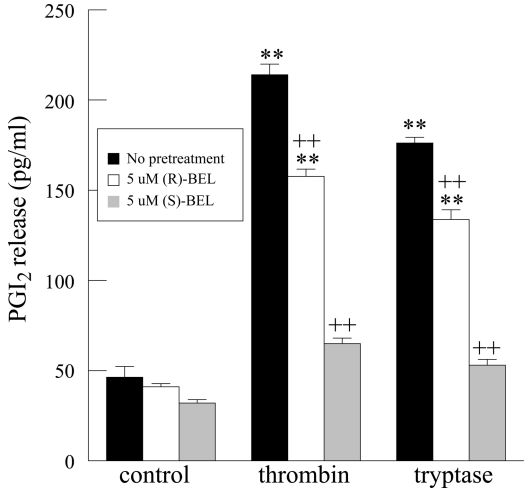

FIGURE 2: Effect of pretreatment with (*R*)-bromoenol lactone (5 μM, 10 min, (*R*)-BEL) or (*S*)-BEL (5 μM, 10 min) on prostaglandin I2 (PGI_2) release from human pulmonary microvascular endothelial cells stimulated with thrombin (1 IU/mL, 10 min) or tryptase (20 ng/mL, 10 min). Data are expressed as mean + SEM for four separate cell cultures. $**p < 0.01$ when compared to control values. $++p < 0.01$ when comparing PLA_2 inhibitor-treated values with corresponding stimulated data.

release (Figure 2, black bars). Pretreating HMVEC-L with 5 μM (*R*)-BEL resulted in no significant inhibition of thrombin- or tryptase-stimulated PGI_2 production and release (Figure 2, open bars). In contrast, pretreatment with 5 μM (*S*)-BEL resulted in complete inhibition of thrombin- or tryptase-stimulated PGI_2 production (Figure 2, gray bars), suggesting that iPLA$_2\beta$ activity is required for PGI_2 production and release and that stimulation of HMVEC-L with thrombin or tryptase results in activation of iPLA$_2\beta$.

We next isolated endothelial cells from the lungs of WT and iPLA$_2\beta$-KO mice by selecting cells that expressed CD31 and CD105, and the isolated cells were grown to confluence. Confluent monolayers were stained for factor VIII and found to consist of >80% endothelial cells (Figure 3). Phospholipase A_2

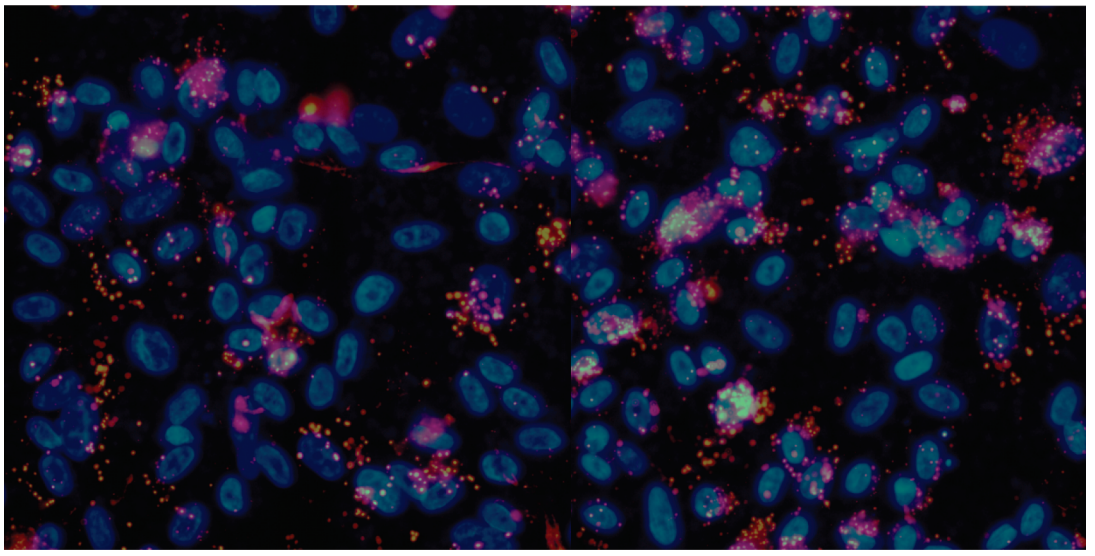

FIGURE 3: Lung endothelial cell cultures isolated from wild type (left panel) and knockout (right panel) mice. Cultures were stained with rabbit antifactor VIII antibody followed by goat antirabbit Alexa Fluor 568 (red) and with DAPI (blue) to localize cell nuclei.

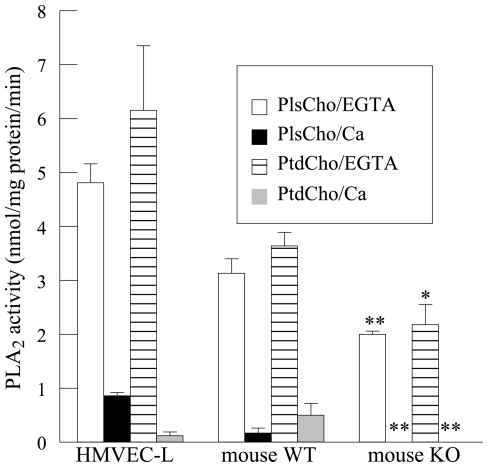

FIGURE 4: Phospholipase A_2 (PLA_2) activity in human pulmonary vascular endothelial cells (HMVEC-L) and endothelial cells isolated from the lungs of wild type (WT) and $iPLA_2\beta$ knockout (KO) mice. Activity was measured using 100 μM 1-palmitoyl-2-oleoyl plasmenylcholine [oleoyl-9,10-^{3}H] (PlsCho) or phosphatidylcholine (PtdCho) substrate in the presence (1 mM Ca) or absence (4 mM EGTA) of calcium. Results represent mean + SEM of six separate experiments.

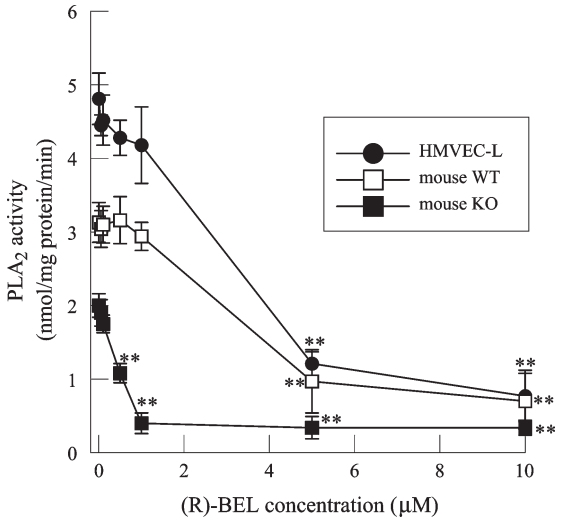

FIGURE 5: Calcium-independent phospholipase A_2 ($iPLA_2$) activity in human pulmonary vascular endothelial cells (HMVEC-L) and endothelial cells isolated from the lungs of wild type (WT) and $iPLA_2\beta$ knockout (KO) mice. Protein was incubated with indicated concentrations of (*R*)-bromoenol lactone (BEL) for 10 min prior to activity assay measurements. Activity was measured using 100 μM 1-palmitoyl-2-oleoyl plasmenylcholine [oleoyl-9,10-^{3}H] in the presence of 4 mM EGTA. Results represent mean ± SEM of six separate experiments. $^{**}p < 0.01$ when compared to $iPLA_2$ activity measured in the absence of (*R*)-BEL.

activity in human and mouse lung endothelial cells was determined with radiolabeled phospholipid substrate [100 μM 1-palmitoyl-2-oleoyl plasmenylcholine [oleoyl-9,10-^{3}H] (PlsCho) or phosphatidylcholine (PtdCho)] under Ca^{2+}-replete (1 mM Ca^{2+}) or Ca^{2+}-chelated (4 mM EGTA) conditions by measuring the release of [^{3}H] oleate (Figure 4). Phospholipase A_2 activity in mouse endothelial cells was found to be significantly lower than that in HMVEC-L under all conditions studied (Figure 4). In human and mouse endothelial cells, PLA_2 activity was maximal when Ca^{2+} was chelated (4 mM EGTA) with both PtdCho and PlsCho substrates (Figure 4). In $iPLA_2\beta$-KO lung endothelial cells, PLA_2 activity was significantly lower than that in WT cells under all conditions (Figure 4). No PLA_2 activity was detectable in $iPLA_2\beta$-KO cells under Ca^{2+}-replete conditions with either PtdCho or PlsCho substrate (Figure 4). $iPLA_2$ activity from $iPLA_2\beta$-KO lung endothelial cells measured under Ca^{2+}-chelated conditions was about 60% of that from WT cells (Figure 4). This residual $iPLA_2$ activity from $iPLA_2\beta$-KO cells appears to be attributable to $iPLA_2\gamma$ because it is inhibited by pretreatment with (*R*)-BEL at a concentration of 0.5 μM (Figure 5). More than 10-fold higher BEL concentrations were required to inhibit $iPLA_2$ activity from WT or human endothelial cells. Thus, PLA_2 activity in WT mouse lung endothelial cells is comparable to that in HMVEC-L cells with respect to substrate selectivity, Ca^{2+}-dependence, and sensitivity to inhibition by BEL. In contrast, residual $iPLA_2$ activity in $iPLA_2\beta$-KO mouse lung endothelial cells is attributable to $iPLA_2\gamma$.

Lung endothelial cells isolated from WT or $iPLA_2\beta$-KO mice were prelabeled with [^{3}H] arachidonic acid, washed to remove unincorporated radiolabel, and then stimulated with thrombin

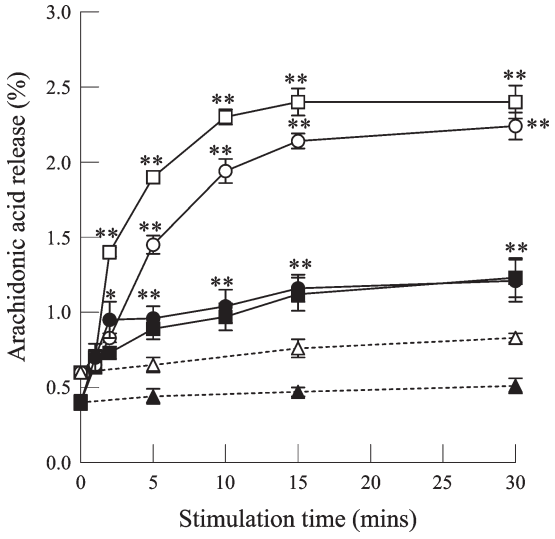

FIGURE 6: Arachidonic acid release from wild type (WT, open symbols) and iPLA$_2\beta$ knockout (KO, filled symbols) mouse lung endothelial cells stimulated with thrombin (open and filled squares, 0.1 IU/mL) or tryptase (open and filled circles, 20 ng/mL). Arachidonic acid release from unstimulated WT (open triangles and dotted lines) and KO (filled triangles, dotted lines) did not increase significantly over the time course studied. Results represent mean ± SEM for 8−10 separate experiments. $^{**}p < 0.01$ when compared to unstimulated release.

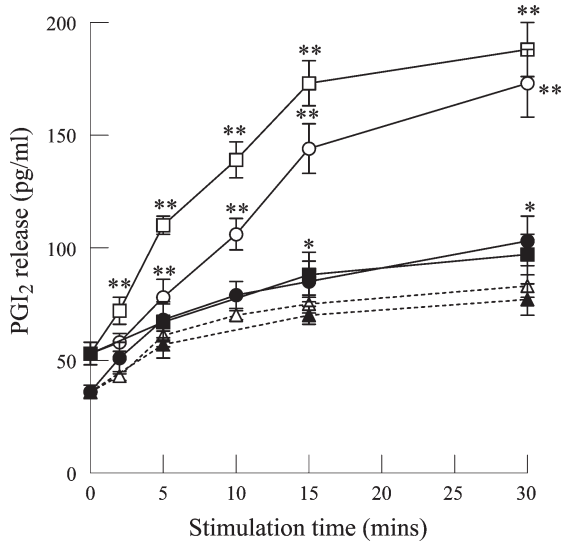

FIGURE 7: Prostaglandin I_2 (PGI_2) release from wild type (WT, open symbols) and iPLA$_2\beta$ knockout (KO, filled symbols) mouse lung endothelial cells stimulated with thrombin (open and filled squares, 0.1 IU/mL) or tryptase (open and filled circles, 20 ng/mL). PGI_2 release from unstimulated WT (open triangles and dotted lines) and KO (filled triangles, dotted lines) did not increase significantly over the time course studied. Results represent mean ± SEM for 8−10 separate experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ when compared to unstimulated release at time 0.

(0.1 IU/mL) or tryptase (20 ng/mL) for up to 30 min. Release of [^{3}H] arachidonate into the incubation medium was measured after various time intervals for 30 min, and both agents were found to induce arachidonic acid release from WT cells after 2 min that continued up to 10 min and then achieved a stable plateau (Figure 6). Under these conditions, iPLA$_2\beta$-KO cells stimulated with thrombin or tryptase released amounts of [^{3}H] arachidonate that were significantly smaller than those from WT cells at all time points between 5 and 30 min (Figure 6).

Incubation of WT lung endothelial cells with thrombin or tryptase also stimulated PGI_2 production and release into the medium that was detectable after 2 min and continued for up to 30 min, and these responses were significantly smaller for iPLA$_2\beta$-KO cells at each tested time point (Figure 7). Pretreatment of WT endothelial cells with (*S*)-BEL significantly inhibited thrombin- and tryptase-induced PGI_2 production, but pretreatment with (*R*)-BEL had no significant effect on these responses (Figure 8). In view of the fact that (*S*)-BEL preferentially inhibits iPLA$_2\beta$ and that (*R*)-BEL preferentially inhibits iPLA$_2\gamma$, the data in Figure 8 suggest that iPLA$_2\beta$ activity is required for thrombin- or tryptase-stimulated PGI_2 production by WT cells but that iPLA$_2\gamma$ activity is not. In contrast, the modest PGI_2 production by thrombin- or tryptase-stimulated iPLA$_2\beta$-KO endothelial cells was prevented by pretreatment with (R)-BEL but was not significantly affected by pretreatment with (*S*)-BEL (Figure 8). This indicates that iPLA$_2\beta$ is not involved in stimulated PGI_2 production in KO cells, as expected, but that the modest increases in PGI_2 production induced by stimulating KO cells with thrombin or tryptase involves the action of iPLA$_2\gamma$.

Incubation of lung endothelial cells isolated from WT mice with thrombin or tryptase induced about a 5-fold rise in PAF production, and these responses were completely prevented by pretreating the cells with racemic BEL (Figure 9), which is consistent with the involvement of an iPLA$_2$ in the responses. In contrast, stimulation of iPLA$_2\beta$-KO endothelial cells with

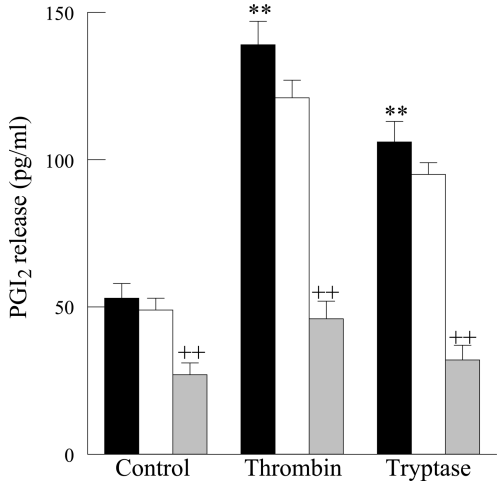

FIGURE 8: Prostaglandin I_2 release from wild type and iPLA$_2\beta$ knockout mouse lung endothelial cells stimulated with thrombin (0.1 IU/mL, 15 min) or tryptase (2 ng/mL, 15 min). Cells were pretreated with the iPLA$_2$ inhibitors (*R*)-bromoenolactone ((*R*)-BEL, 2 μM, 10 min, open bars) or (*S*)-BEL (2 μM, 10 min, gray bars) prior to stimulation with thrombin or tryptase. Data shown are means + SEM for six separate cell cultures. $^{**}p < 0.01$ when compared to unstimulated control values. $^{++}p < 0.01$ when comparing PLA$_2$ inhibitor-treated values with corresponding stimulated data.

neither thrombin nor tryptase induced a significant increase in PAF production (Figure 9), which is consistent with a requirement for iPLA$_2\beta$ in thrombin- and tryptase-stimulated PAF production by pulmonary endothelial cells. PAF expressed by endothelial cells binds to its cognate receptors on circulating inflammatory cells, leading to cell adherence to an activated endothelial cell monolayer. In this study, we used the murine monocyte/macrophage cell line RAW 264.7 as a cell model to study endothelial cell adherence. As shown in Figure 10, thrombin or tryptase stimulation of lung endothelial cells isolated from

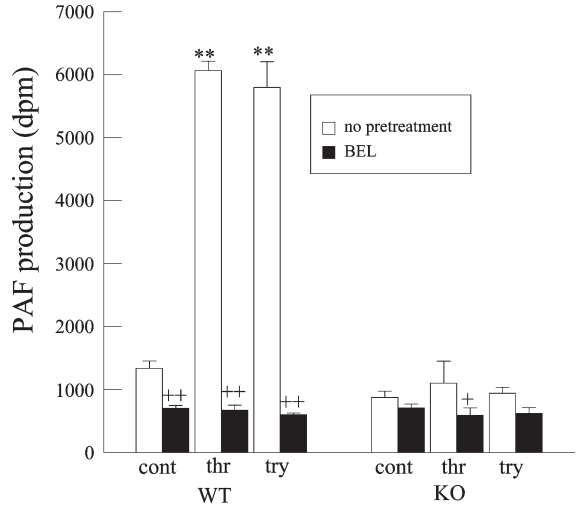

FIGURE 9: Platelet-activating factor (PAF) production in wild type (WT) and iPLA$_2\beta$ knockout (KO) mouse lung endothelial cells stimulated with thrombin (thr, 1.0 IU/mL) or tryptase (try, 20 ng/mL). Cells were pretreated with BEL (filled bars, 5 μM, 10 min) prior to stimulation where indicated. Results represent mean + SEM for six separate experiments. $^{**}p < 0.01$ when compared to unstimulated control (cont). $^{++}p < 0.01$ when comparing BEL-pretreated and corresponding untreated groups.

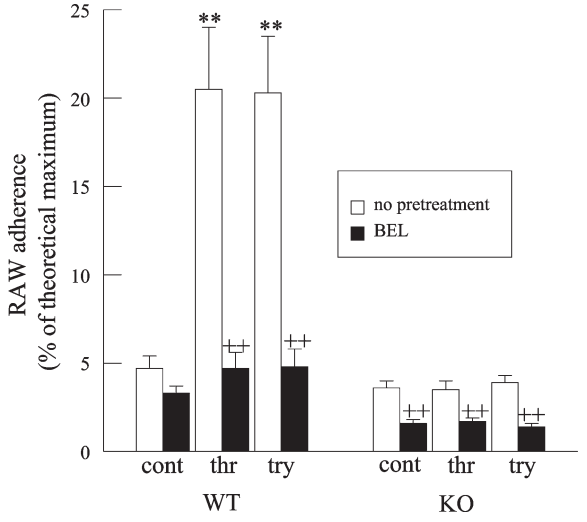

FIGURE 10: Adherence of calcein-labeled RAW 264.7 cells to wild type (WT) and iPLA$_2\beta$ knockout (KO) mouse lung endothelial cell monolayers stimulated with thrombin (thr, 1.0 IU/mL) or tryptase (try, 20 ng/mL). Cells were pretreated with BEL (filled bars, 5 μM, 10 min) prior to stimulation where indicated. Results represent mean + SEM for six separate experiments. $^{**}p < 0.01$ when compared to unstimulated control (cont). $^{++}p < 0.01$ when comparing BEL-pretreated and corresponding untreated groups.

WT mice resulted in a 4-fold increase in RAW cell adherence. Pretreatment with BEL inhibited RAW cell adherence after either tryptase or thrombin stimulation. In contrast, stimulation of lung endothelial cells from iPLA$_2\beta$-KO mice with thrombin or tryptase failed to increase RAW cell adherence to the endothelial cell monolayer (Figure 10). These results are consistent with a requirement for iPLA$_2\beta$ in thrombin and tryptase-stimulated endothelial cell PAF production and inflammatory cell adherence.

DISCUSSION

We have previously reported that stimulation of HMVEC-L with thrombin or tryptase results in release of arachidonic acid and production of PGI_2 and PAF, and we suggested that iPLA$_2$ activation was required to initiate these responses because they were blocked by the iPLA$_2$ inhibitor BEL when administered as a racemic mixture (*13*). In our previous studies, we used racemic BEL pretreatment to demonstrate the activation of iPLA$_2$ (*13*). Here, we have examined which iPLA$_2$ family members might participate in these responses by examining the effects of BEL enantiomers that discriminate between iPLA$_2\beta$ and iPLA$_2\gamma$ and by determining the responses of pulmonary endothelial cells isolated from iPLA$_2\beta$-KO mice. In the case of HMVEC-L cells, arachidonic acid release and production of PGI_2 and PAF induced by stimulation with thrombin or tryptase are all blocked by pretreatment with (*S*)-BEL but not with (*R*)-BEL, which is consistent with a requirement for iPLA$_2\beta$ activity but not for iPLA$_2\gamma$ activity in these responses.

The use of genetically modified mice circumvents the potential nonspecificity of pharmacologic agents such as BEL by selectively eliminating the target gene product, and we have therefore used pulmonary endothelial cells isolated from iPLA$_2\beta$-KO mice and their WT littermates to characterize further the potential involvement of iPLA$_2\beta$ in lung endothelial cell responses to thrombin and tryptase. The iPLA$_2\beta$-KO mouse was described originally in 2004 (*36*) and has been observed to exhibit a number of phenotypic abnormalities at the whole animal and cellular level that impaired male reproductive ability (*36*), impaired insulin secretory responses (*41*, *44*), and acceleration of age-related loss in bone mass and strength (*45*). Vascular smooth muscle cells isolated from iPLA$_2\beta$-KO mice exhibit impaired release of arachidonic acid and production of PGI_2 and reduced proliferative and migratory responses (*46*). We compared iPLA$_2$ activity in HMVEC-L to the activity in WT and iPLA$_2\beta$-KO mouse lung endothelial cells using plasmenylcholine and phosphatidylcholine in the presence and absence of Ca^{2+}. Maximal activity in HMVEC-L and WT mouse lung endothelial cells was observed in Ca^{2+}-chelated conditions. These data agree with our previous studies demonstrating that the majority of PLA$_2$ activity in endothelial cells is iPLA$_2$ (*13*, *24*−*27*). In a previous study, we measured PLA$_2$ activity using several published assay methods and demonstrated that higher activity measurements were made using shorter times of incubation than are more commonly used (*24*). Interestingly, we demonstrated increased PLA$_2$ activity in response to thrombin stimulation when using plasmenylcholine as substrate, but not when using phosphatidylcholine substrate (*24*). We were not able to detect any PLA$_2$ activity in iPLA$_2\beta$-KO endothelial cells under Ca^{2+}-replete conditions, suggesting that iPLA$_2\gamma$ is not active in the presence of 1 mM Ca^{2+}. These data also suggest that cPLA$_2$ activity is minimal in lung endothelial cells.

In the present study, we have demonstrated that lung endothelial cells isolated from iPLA$_2\beta$-KO mice and incubated with thrombin or tryptase exhibit reduced arachidonic acid release and production of PGI_2 and PAF compared to cells isolated from WT littermates. Pretreatment of WT endothelial cells with (*S*)-BEL abolished PGI_2 production in response to thrombin or tryptase but pretreatment with (*R*)-BEL did not affect this response significantly. This is consistent with a requirement for iPLA$_2\beta$ activity but not for iPLA$_2\gamma$ activity in endothelial cell PGI_2 production in response to thrombin and tryptase, and this is supported by the greatly reduced PGI_2 production observed with iPLA$_2\beta$-KO endothelial cells stimulated with thrombin or tryptase. Interestingly, the small amount of PGI_2 produced by the KO cells in response to thrombin

or tryptase was eliminated when the iPLA$_2\beta$-KO cells were pretreated with the iPLA$_2\gamma$ inhibitor (*R*)-BEL, which could reflect compensatory upregulation of iPLA$_2\gamma$ in iPLA$_2\beta$-KO cells, as has been observed in vascular smooth muscle cells isolated from iPLA$_2\beta$-KO mice (*46*).

In that regard, our observations on the differential sensitivity to BEL enantiomers of Ca^{2+}-independent PLA$_2$ (iPLA$_2$) activities in HMVEC-L cells and WT and KO mouse lung endothelial cells are of interest. The much greater sensitivity of HMVEC-L iPLA$_2$ activity to inhibition by (*S*)-BEL compared to (*R*)-BEL is consistent with iPLA$_2\beta$ being the predominant contributor to total iPLA$_2$ activity in those cells, and this is also true for WT mouse lung endothelial cells. In contrast, iPLA$_2\beta$-KO endothelial cell iPLA$_2$ activity is much more susceptible to inhibition by (*R*)-BEL, suggesting that iPLA$_2\gamma$ is responsible for the residual iPLA$_2$ activity in iPLA$_2\beta$-KO cells and may be upregulated in those cells. Although iPLA$_2$ activity in iPLA$_2\beta$-KO is approximately 60% of that in WT endothelial cells, PAF production is inhibited completely, and PGI_2 production is inhibited by at least 70%. Since our data suggest that the residual activity in iPLA$_2\beta$-KO endothelial cells is due to iPLA$_2\gamma$, we propose that endothelial cell iPLA$_2\beta$ is primarily responsible for membrane phospholipid hydrolysis in response to stimulation, whereas iPLA$_2\gamma$ may play an alternative role. In previous studies, iPLA$_2\gamma$ has been demonstrated to preferentially hydrolyze the *sn*-1 fatty acid when the *sn*-2 fatty acid of phospholipids is polyunsaturated, resulting in the production of polyunsaturated lysophospholipids (*48*). The generation of an iPLA$_2\gamma$ KO mouse resulted in an animal with growth retardation, cold intolerance, and reduced exercise endurance, suggesting that this isoform maintains efficient bioenergetic mitochondrial function (*49*). Taken together, these data suggest distinct and separate roles for iPLA$_2$ isoforms.

Prostaglandin I_2 is generated by the pulmonary endothelium and macrophages and binds to the IP receptor, which is coupled to the Gs subunit of a G-protein. In a murine model of pulmonary inflammation, the PGI_2−IP complex has been reported to suppress Th2-mediated allergic inflammatory reactions (*50*). PGI_2 also has antithrombotic effects in vivo, and bronchodilator effects on human airways in vitro, although it is less potent than PGE_2 in the latter regard (*51*). In the lungs of IP-deficient mice, increased inflammatory leukocyte infiltrations, and Th2 cytokine levels, have been observed in response to prolonged allergen exposure that are accompanied by goblet cell hyperplasia and subepithelial cells fibrosis, and these responses are greatly augmented compared to WT mice (*52*). These observations suggest that PGI_2 plays regulatory roles in allergen-induced airway inflammation and remodeling and point to the potential utility of pharmacologic PGI_2 agonists in the therapy of allergic asthma.

We have demonstrated here that pulmonary endothelial cells isolated from WT mice greatly increase PAF production when stimulated with thrombin or tryptase and that these responses are blocked by pretreatment with BEL. These observations are similar to our previously reported findings with HMVEC-L cells (*13*) and suggest that an iPLA$_2$ might be involved in these responses. In contrast, we observe no increase in PAF production by iPLA$_2\beta$-KO endothelial cells stimulated with thrombin or tryptase, and together these observations strongly suggest that pulmonary endothelial cell PAF production in response to thrombin or tryptase is dependent on iPLA$_2\beta$ activity. We have demonstrated previously that HMVEC-L PAF production is associated with increased neutrophil adherence to endothelial cells and that such adherence is prevented by pretreating the endothelial cells with BEL or by blocking the neutrophil PAF receptor with the compound CV3988 (*13*). Neutrophils are the most abundant cell type in the airways of normal and asthmatic subjects (*53*), and increased numbers of neutrophils are associated with more severe airway obstruction (*4*). Neutrophils are also prominent during acute asthma exacerbations (*54*) and may regulate both initiation and resolution of attacks (*53*). Platelet-activating factor can play a central role in the propagation of chronic inflammatory conditions by increasing systemic, pulmonary, and microvascular permeability and disrupting vascular integrity (*55*). Additionally, PAF stimulates migration of eosinophils into the airways (*56*) and induces airway smooth muscle contraction and hyperreactivity in otherwise healthy subjects (*14*, *15*). Increased numbers of eosinophils in airway secretions are a characteristic feature of asthma and are associated with disease severity (*57*, *58*). Taken together, the data presented here and previously suggest that a selective and systemically bioavailable iPLA$_2\beta$ inhibitor could represent a potentially useful therapeutic tool in inflammatory airway disease. Such an agent might reduce inflammatory cell recruitment to the airways while sparing a component of pulmonary endothelial cell PGI_2 production.

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