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Endothelial Cell Prostaglandin I₂ and Platelet-Activating Factor Production Are Markedly Attenuated in the Calcium-Independent Phospholipase A₂β 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 A₂ (iPLA₂), and this leads to arachidonic acid release and production of prostaglandin I₂ (PGI₂) and platelet-activating factor (PAF). We stimulated lung endothelial cells isolated from iPLA₂β-knockout (KO) and wild type (WT) mice with thrombin and tryptase to determine the role of iPLA₂β in endothelial cell membrane phospholipid hydrolysis. Thrombin or tryptase stimulation of WT lung endothelial cells resulted in increased arachidonic acid release and production of PGI2 and PAF. Arachidonic acid release and PGI₂ production by stimulated iPLA₂β-KO endothelial cells were significantly reduced compared to WT. Measured PLA₂ activity and PGI₂ production by iPLA₂β-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 iPLA₂β 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).

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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 proteaseactivated 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 A₂ (iPLA₂), which results in increased arachidonic acid release and production of prostaglandin I₂ (PGI₂) 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 A2 coexist in mammalian cells, secretory (sPLA2), cytosolic (cPLA₂), and iPLA₂ (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 PLA₂ 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 PLA₂ is expressed constitutively in most cells, demonstrates a preference for arachidonylated phospholipids, and has been demonstrated to play a critical role in agonistinduced eicosanoid production in several cells and tissues. However, in several previous studies, we have demonstrated that the majority of endothelial cell PLA₂ activity is iPLA₂ and that agonist stimulation results in iPLA2 activation, accelerated membrane phospholipid hydrolysis, and the subsequent production of PGI₂ and PAF (13,24–27). Most iPLA₂ activity in mammalian cells resides in the Group VIA and VIB enzymes designated iPLA₂β and iPLA₂γ (28–30). Homology between iPLA₂β and iPLA₂γ 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 iPLA2 activity at concentrations over 1000-fold lower than those required to inhibit cPLA2 and sPLA2 enzymes (32). In addition, (S)-BEL inhibits iPLA₂β preferentially over iPLA₂γ, 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 iPLA2 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 iPLA₂ and have motivated studies of genetic manipulations of iPLA2 enzymes to elucidate their roles in biological processes (36-46).

¹Abbreviations: BEL, bromoenol lactone; EDTA, ethylenediaminete-traacetic acid; EGTA, ethylene glycol tetraacetic acid; HMVEC-L, human microvascular endothelial cells-lung; iPLA2, calcium-independent phospholipase A2; KO, knockout; PAF, platelet-activating factor; PAR-1, protease-activated receptor-1; PAR-2, protease-activated receptor-2; PLA2, phospholipase A2; PGI2, prostaglandin I2; WT, wild type.

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, PGI₂, and PAF by isolated pulmonary endothelial cells upon stimulation with thrombin and tryptase.

EXPERIMENTAL PROCEDURES

iPLA₂β 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% O₂, 5% CO₂. 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 cm² 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 MgCl₂ (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 I₂ 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 MgSO₄, 10 HEPES (pH=7.6), 1.2 CaCl₂, 5.4 KCl, 0.4 KH₂PO₄, 0.3 Na₂HPO₄, and 6.6 glucose. After washing, 0.5mL 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 PGI₂ 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 μ Ci 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 $_2$ PO $_4$, 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 A₂ 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. PLA₂ activity in the lysates was assessed by incubating the cellular protein with 100 µM 1-palmitoyl-2-oleoyl plasmenylcholine [oleoyl-9,10-³H] or 1-palmitoyl-2-oleoyl phosphatidylcholine [oleoyl-9,10-³H] 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 MgSO₄, 10 HEPES (pH 7.4), 1.2 CaCl₂, 5.4 KCl, 0.4 KH₂PO₄, 0.3 Na₂HPO₄, and 6.6 glucose. Cells were incubated with 10 μ Ci [³H] 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 CHCl₃/ CH₃OH, 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 [³H]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 [¹⁴C] 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 $\mu g/mL$, Alexis Biochemicals, Lausen, Switzerland) for 45 min at 37 °C. After washing three times, 2 \times 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 iPLA2 resulting in arachidonic acid release and production of PGI2 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 μ M (R)-BEL resulted in no significant inhibition of thrombin- or tryptase-stimulated PAF production (Figure 1, open bars). In contrast, pretreatment with 5 μ M (R)-BEL resulted in complete inhibition of thrombin- or tryptase-stimulated PAF production (Figure 1, gray bars), suggesting that iPLA2 β activity is required for these responses and that stimulation of HMVEC-L with thrombin or tryptase results in activation of iPLA2 β .

Similarly, stimulation of HMVEC-L with thrombin or tryptase resulted in a significant increase in prostaglandin I_2 (PGI₂) release (Figure 2, black bars). Pretreating HMVEC-L with 5 μ M (R)-BEL resulted in no significant inhibition of thrombin- or tryptase-stimulated PGI₂ production and release (Figure 2, open bars). In contrast, pretreatment with 5 μ M (S)-BEL resulted in complete inhibition of thrombin- or tryptase-stimulated PGI₂ production (Figure 2, gray bars), suggesting that iPLA₂ β activity is required for PGI₂ production and release and that stimulation of HMVEC-L with thrombin or tryptase results in activation of iPLA₂ β .

We next isolated endothelial cells from the lungs of WT and iPLA₂β-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 A₂ activity in human and mouse lung endothelial cells was determined with radiolabeled phospholipid substrate [100 µM 1-palmitoyl-2-oleoyl plasmenylcholine [oleoyl-9,10-3H] (PlsCho) or phosphatidylcholine (PtdCho)] under Ca²⁺replete (1 mM Ca²⁺) or Ca²⁺-chelated (4 mM EGTA) conditions by measuring the release of [³H] oleate (Figure 4). Phospholipase A₂ 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, PLA₂ activity was maximal when Ca²⁺ was chelated (4 mM EGTA) with both PtdCho and PlsCho substrates (Figure 4). In iPLA₂β-KO lung endothelial cells, PLA2 activity was significantly lower than that in WT cells under all conditions (Figure 4). No PLA₂ activity was detectable in iPLA₂β-KO cells under Ca²⁺-replete conditions with either PtdCho or PlsCho substrate (Figure 4). iPLA₂ activity from iPLA₂β-KO lung endothelial cells measured under Ca²⁺-chelated conditions was about 60% of that from WT cells (Figure 4). This residual iPLA₂ activity from iPLA₂β-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 iPLA₂ activity from WT or human endothelial cells. Thus, PLA₂ 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 iPLA₂ activity in iPLA₂ β -KO mouse lung endothelial cells is attributable to iPLA₂ γ .

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 (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 PGI₂ 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 iPLA₂ β -KO cells at each tested time point (Figure 7). Pretreatment of WT endothelial cells with (S)-BEL significantly inhibited thrombin- and tryptase-induced PGI₂ 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 iPLA₂ β and that (R)-BEL preferentially inhibits iPLA₂ γ , the data in Figure 8 suggest that iPLA₂ β activity is required for thrombin- or tryptase-stimulated PGI₂ production by WT cells but that iPLA₂ γ activity is not. In contrast, the modest PGI₂ production by thrombin- or tryptase-stimulated iPLA₂ β -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 iPLA₂ β is not involved in stimulated PGI₂ production in KO cells, as expected, but that the modest increases in PGI₂ production induced by stimulating KO cells with thrombin or tryptase involves the action of iPLA₂ γ .

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 iPLA₂ in the responses. In contrast, stimulation of iPLA₂β-KO endothelial cells with neither thrombin nor tryptase induced a significant increase in PAF production (Figure 9), which is consistent with a requirement for iPLA₂β 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 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 iPLA₂β-KOmice 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 iPLA₂ β in thrombin and tryptasestimulated 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 iPLA₂ activation was required to initiate these responses because they were blocked by the iPLA₂ inhibitor BEL when administered as a racemic mixture (13). In our previous studies, we used racemic BEL pretreatment to demonstrate the activation of iPLA₂ (13). Here, we have examined which iPLA₂ family members might participate in these responses by examining the effects of BEL enantiomers that discriminate between iPLA₂ β and iPLA₂ γ and by determining the responses of pulmonary endothelial cells isolated from iPLA₂ β -KO mice. In the case of HMVEC-L cells, arachidonic acid release and production of PGI₂ 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 iPLA₂ β activity but not for iPLA₂ γ 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 iPLA₂β-KO mice and their WT littermates to characterize further the potential involvement of iPLA₂ β in lung endothelial cell responses to thrombin and tryptase. The iPLA₂ β -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 iPLA₂β-KO mice exhibit impaired release of arachidonic acid and production of PGI₂ and reduced proliferative and migratory responses (46). We compared iPLA₂ activity in HMVEC-L to the activity in WT and iPLA₂β-KO mouse lung endothelial cells using plasmenylcholine and phosphatidylcholine in the presence and absence of Ca²⁺. Maximal activity in HMVEC-L and WT mouse lung endothelial cells was observed in Ca²⁺-chelated conditions. These data agree with our previous studies demonstrating that the majority of PLA2 activity in endothelial cells is iPLA₂ (13,24–27). In a previous study, we measured PLA₂ 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 PLA₂ 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 PLA₂ activity in iPLA₂ β -KO endothelial cells under Ca²⁺replete conditions, suggesting that iPLA₂ γ is not active in the presence of 1 mM Ca²⁺. These data also suggest that cPLA₂ 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 PLA₂ (iPLA₂) activities in HMVEC-L cells and WT and KO mouse lung endothelial cells are of interest. The much greater sensitivity of HMVEC-L iPLA₂ activity to inhibition by (*S*)-BEL compared to (*R*)-BEL is consistent with iPLA₂ β being the

predominant contributor to total iPLA2 activity in those cells, and this is also true for WT mouse lung endothelial cells. In contrast, iPLA₂β-KO endothelial cell iPLA₂ activity is much more susceptible to inhibition by (R)-BEL, suggesting that iPLA₂ γ is responsible for the residual iPLA₂ activity in iPLA₂ β -KO cells and may be upregulated in those cells. Although iPLA₂ activity in iPLA₂β-KO is approximately 60% of that in WT endothelial cells, PAF production is inhibited completely, and PGI₂ production is inhibited by at least 70%. Since our data suggest that the residual activity in iPLA₂β-KO endothelial cells is due to iPLA₂ γ , we propose that endothelial cell iPLA₂ β is primarily responsible for membrane phospholipid hydrolysis in response to stimulation, whereas iPLA₂ γ may play an alternative role. In previous studies, iPLA₂ γ 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 iPLA₂γ 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 iPLA₂ 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 iPLA2 might be involved in these responses. In contrast, we observe no increase in PAF production by iPLA₂β-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 iPLA₂β 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 iPLA₂β 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 PGI₂ production.

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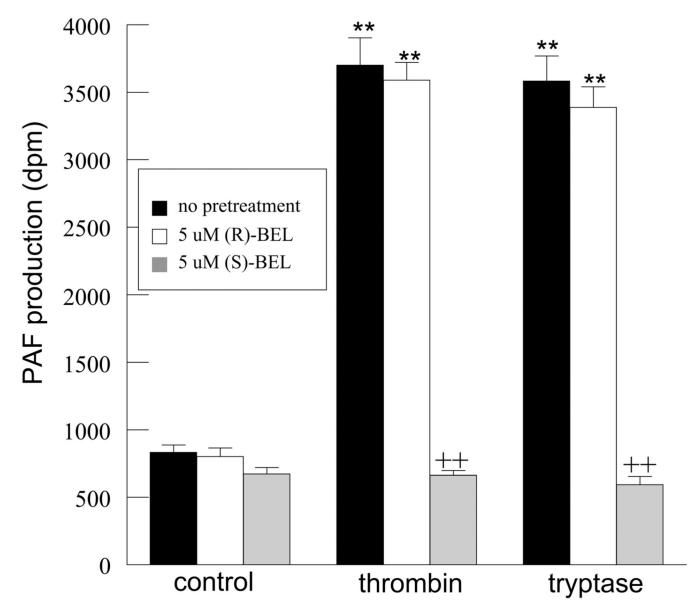


FIGURE 1. Effect of pretreatment with (*R*)-bromoenol lactone (5 μM, 10 min, (*R*)-BEL) or (*S*)-BEL (5 μM, 10 min) on platelet-activating factor (PAF) production in human pulmonary microvascular endothelial cells stimulated with thrombin (1 IU/mL, 10 min) or tryptase (20 ng/mL, 10 min). Data are expressed as mean + SEM for six separate cell cultures. **p < 0.01 when compared to control values. ++p < 0.01 when comparing PLA₂ inhibitor-treated values with corresponding stimulated data.

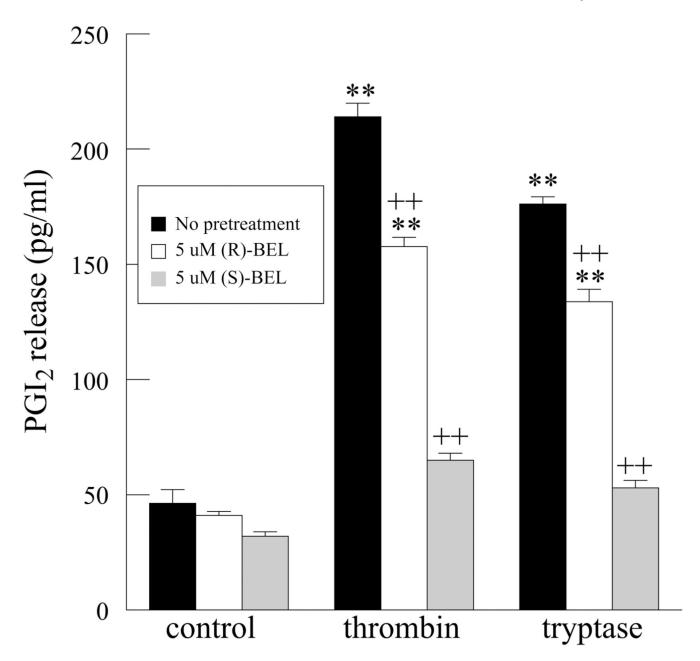


FIGURE 2. Effect of pretreatment with (*R*)-bromoenol lactone (5 μM, 10 min, (*R*)-BEL) or (*S*)-BEL (5 μM, 10 min) on prostaglandin I2 (PGI₂) release from human pulmonary microvascular endothelial cells stimulated with thrombin (1 IU/mL, 10 min) or tryptase (20 ng/mL, 10 min). Data are expressed as mean + SEM for four separate cell cultures. **p < 0.01 when compared to control values. ++p < 0.01 when comparing PLA₂ inhibitor-treated values with

corresponding stimulated data.

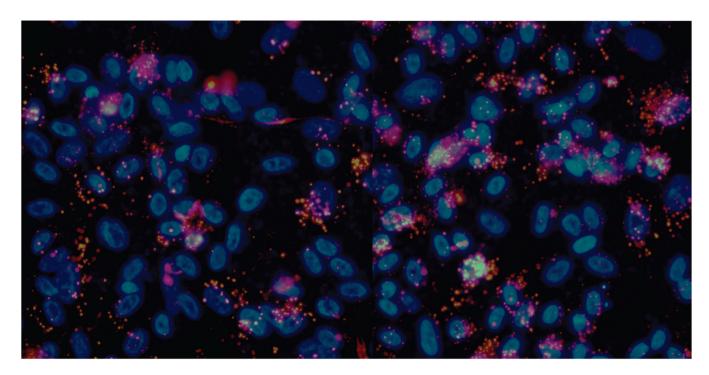
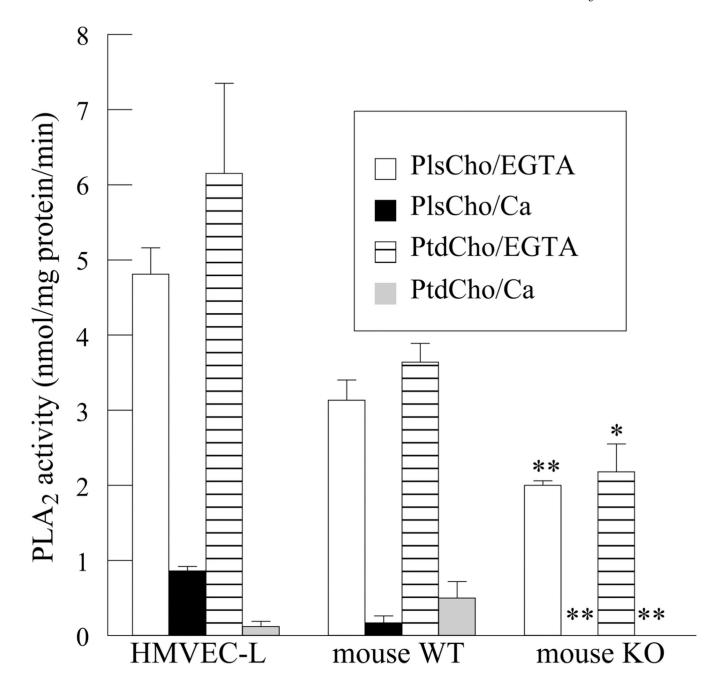


FIGURE 3.Lung endothelial cell cultures isolated from wild type (left panel) and knockout (right panel) mice. Cultures were stained with rabbit antifactor VIII antibody followed by goat antirabbit Alexa Fluor 568 (red) and with DAPI (blue) to localize cell nuclei.



Phospholipase A_2 (PLA₂) activity in human pulmonary vascular endothelial cells (HMVEC-L) and endothelial cells isolated from the lungs of wild type (WT) and iPLA₂ β knockout (KO) mice. Activity was measured using 100 μ M 1-palmitoyl-2-oleoyl plasmenylcholine [oleoyl-9,10-³H] (PlsCho) or phosphatidylcholine (PtdCho) substrate in the presence (1 mM

FIGURE 4.

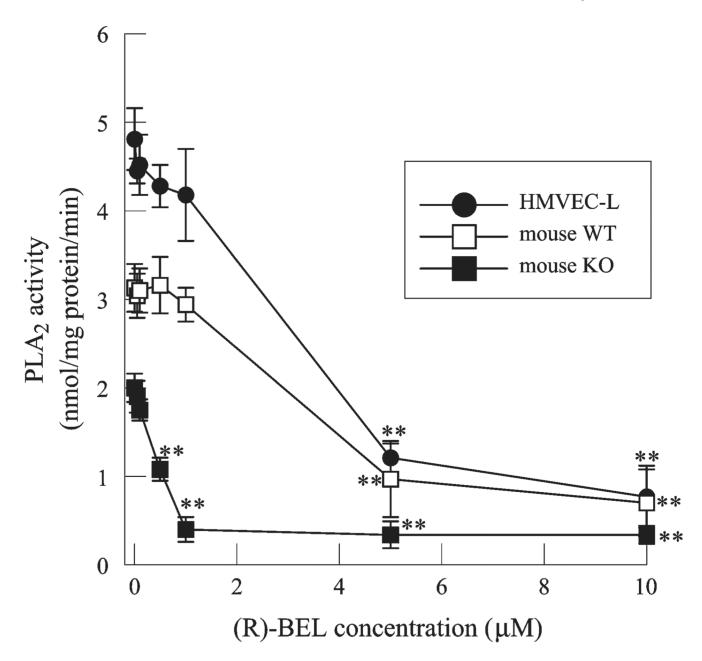


FIGURE 5. Calcium-independent phospholipase A_2 (iPLA₂) activity in human pulmonary vascular endothelial cells (HMVEC-L) and endothelial cells isolated from the lungs of wild type (WT) and iPLA₂ β knockout (KO) mice. Protein was incubated with indicated concentrations of (*R*)-bromoenol lactone (BEL) for 10 min prior to activity assay measurements. Activity was measured using 100 μ M 1-palmitoyl-2-oleoyl plasmenylcholine [oleoyl-9,10-³H] in the presence of 4 mM EGTA. Results represent mean \pm SEM of six separate experiments. **p <

0.01 when compared to iPLA₂ activity measured in the absence of (R)-BEL.

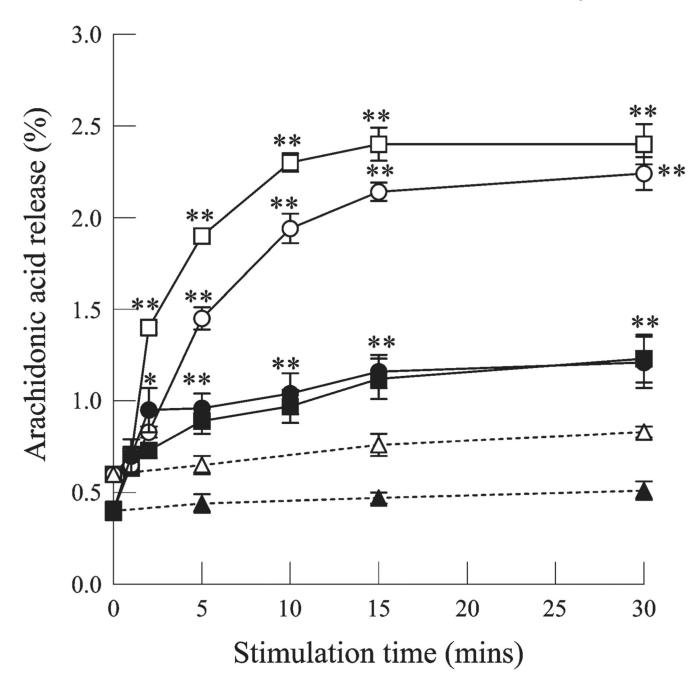


FIGURE 6. Arachidonic acid release from wild type (WT, open symbols) and iPLA $_2\beta$ knockout (KO, filled symbols) mouse lung endothelial cells stimulated with thrombin (open and filled squares, 0.1 IU/mL) or tryptase (open and filled circles, 20 ng/mL). Arachidonic acid release from unstimulated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

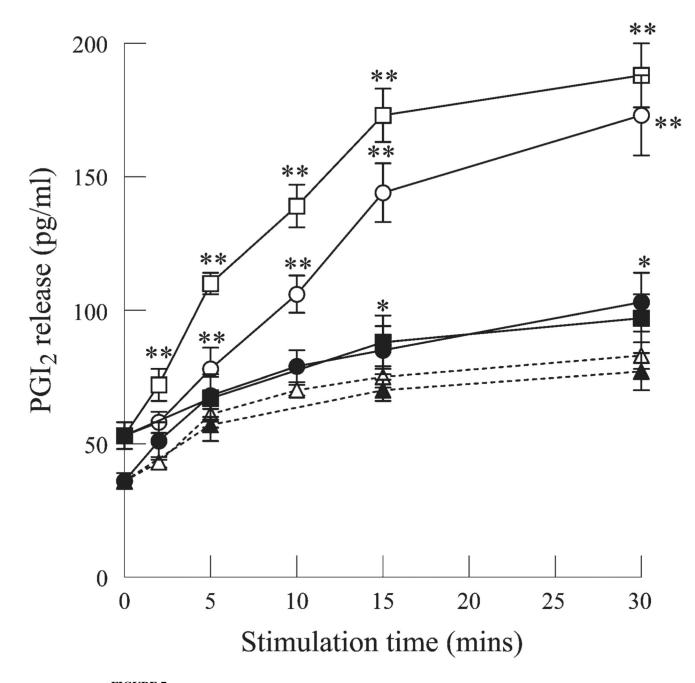


FIGURE 7. Prostaglandin I_2 (PGI₂) release from wild type (WT, open symbols) and iPLA₂β knockout (KO, filled symbols) mouse lung endothelial cells stimulated with thrombin (open and filled squares, 0.1 IU/mL) or tryptase (open and filled circles, 20 ng/mL). PGI₂ release from unstimulated WT (open triangles and dotted lines) and KO (filled triangles, dotted lines) did not increase significantly over the time course studied. Results represent mean \pm SEM for 8–10 separate experiments. *p < 0.05, **p < 0.01when compared to unstimulated release at time 0.

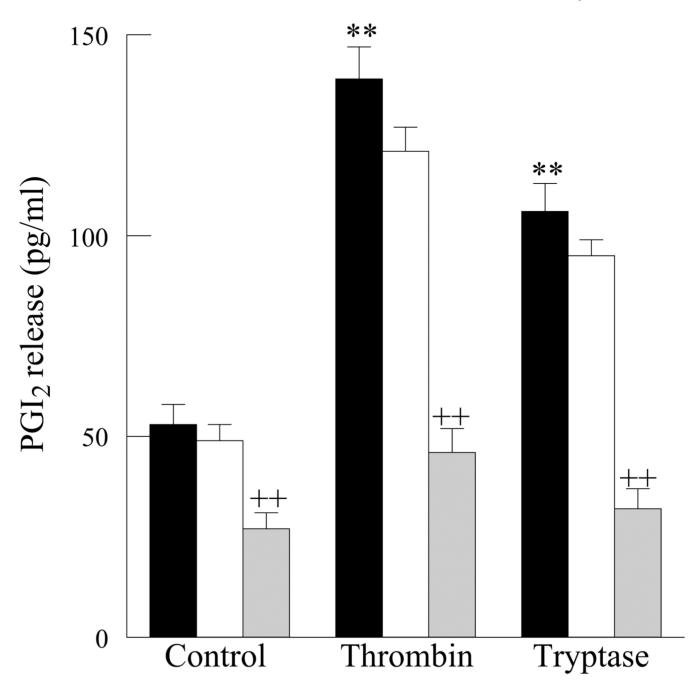


FIGURE 8.

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 μ M, 10 min, open bars) or (S)-BEL (2 μ M, 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.

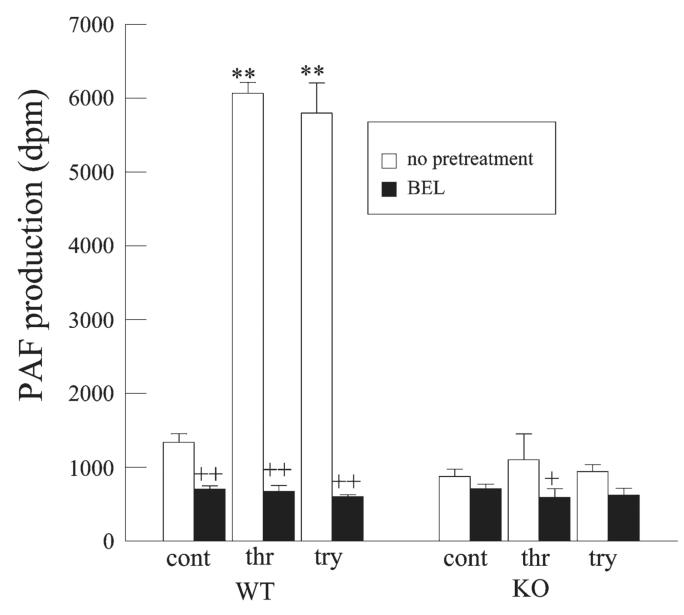


FIGURE 9.

Platelet-activating factor (PAF) production in wild type (WT) and iPLA₂ β 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 μ M, 10 min) prior to stimulation where indicated. Results represent mean + SEM for six separate experiments. **p < 0.01when compared to unstimulated control (cont). ++p < 0.01 when comparing BEL-pretreated and corresponding untreated groups.

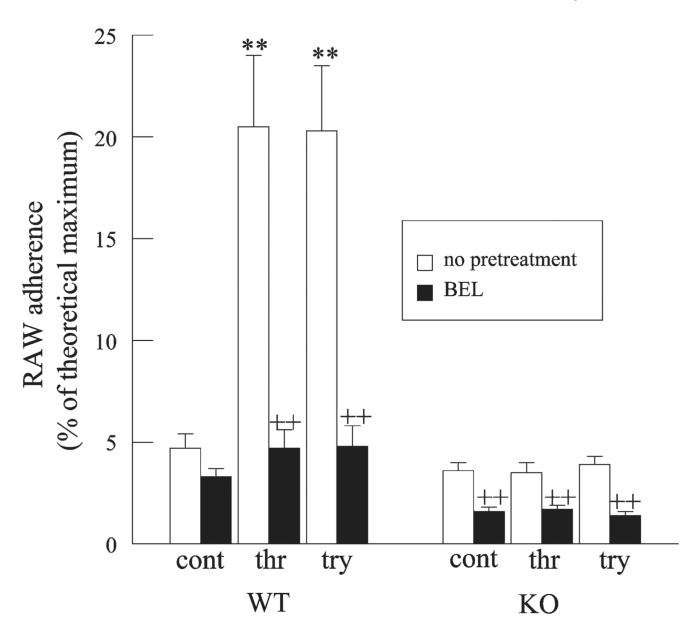


FIGURE 10.

Adherence of calcein-labeled RAW 264.7 cells to wild type (WT) and iPLA₂ β 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 μ M, 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.