Activation of group VI phospholipase A$_2$ isoforms in cardiac endothelial cells

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Inflammation is a component of several cardiovascular diseases. The endothelium lines the luminal surface of blood vessels and is an integral part of the inflammatory system. In the heart, the endothelium forms a critical barrier between circulating cells and underlying myocardium, and an interaction between endothelial cells and cardiomyocytes is physiologically and pathologically important (22). The endothelium contributes to cardiac homeostasis by regulating vascular permeability and resistance and by releasing mediators such as nitric oxide, endothelin, pro- and anticoagulants, growth factors, and prostaglandins that affect numerous vascular and cardiac functions (26). Owing to its close proximity to both circulating cells and cardiac myocytes, the endothelium can facilitate signal transduction between these cell types (26). The endothelium contributes to this process during inflammation by regulating inflammatory cell transmigration into cardiac tissue and by releasing mediators that are cardioprotective during acute inflammation, but chronic exposure to these mediators could be detrimental (17).

Under physiologic conditions, leukocytes flow across the vascular wall, but a key step in inflammation is leukocyte adherence to endothelium (16). An important regulatory molecule in this process is platelet-activating factor (PAF), which is a potent inflammatory mediator that is active at concentrations as low as 10$^{-12}$ M (13). Activated endothelium produces PAF, which is expressed on the endothelial cell surface and interacts with circulating inflammatory cells via their surface PAF receptors. This interaction tethers the leukocytes to the endothelium and activates them, which results in their degranulation, chemotaxis, and increased expression of other cell surface adherence molecules (16).

PAF production is tightly regulated by the action of phospholipase A$_2$ (PLA$_2$), which comprises a large family of enzymes that hydrolyze the sn-2 ester bond of phospholipids to yield a free fatty acid, e.g., arachidonic acid, and a 2-lysophospholipid, e.g., lyso-PAF. Arachidonic acid can be converted to eicosanoids via the action of oxygenases, and acetylation of lyso-PAF yields PAF (30). Our previous studies have demonstrated an increase in calcium-independent PLA$_2$ (iPLA$_2$) activity in human coronary artery endothelial cells (HCAECs) following stimulation of protease-activated receptor (PAR) by thrombin or tryptase. This has associated with corresponding increases in production of PAF and prostaglandins (19, 30).

Of iPLA$_2$ enzymes identified to date, iPLA$_2$γ and iPLA$_2$β are prominent constituents of mammalian cells. Racemic bro-moeno-lactone (BEL) inhibits iPLA$_2$ activity (6) and has been widely used to examine the potential physiologic and pathologic roles of these enzymes. Of BEL enantiomers, (R)-BEL inhibits iPLA$_2$γ at concentrations tenfold lower than those required to inhibit iPLA$_2$β, and the converse is true for (S)-BEL (7). This permits pharmacologic discrimination between these iPLA$_2$ isoforms, but BEL can also inhibit serine hydrolyases and other enzymes in addition to iPLA$_2$ (24). This has motivated the development of iPLA$_2$ isofrom-specific knock-out (KO) mice to permit genetic discrimination between iPLA$_2$ isoforms to study their potential participation in physiologic and pathologic processes (3, 12).

In the studies described here, we have used cardiac endothelial cells isolated from wild-type (WT) mice and compared them to cells isolated from iPLA$_2$γ-KO and iPLA$_2$β-KO mice to evaluate the roles of these enzymes in production of inflammatory mediators derived from endothelial cell phospholipids, including PAF and prostaglandin I$_2$ (PGL$_2$).
MATERIALS AND METHODS

HCAECs. HCAECs were obtained from Lonza Walkersville (Walkersville, MD). Cells were grown to confluence in EGM-2MV media obtained from Lonza, with 5% fetal bovine serum. Cells were allowed to grow to confluence, achieving a contact-inhibited monolayer of flattened, closely apposed endothelial cells in 4 to 5 days. After achieving confluence, cells were passaged in a 1:3 dilution, and cells from passages 3 to 4 were used for experiments.

Mouse endothelial cell isolation and culture. Animal protocols were in strict accordance with the National Institutes of Health guidelines for humane treatment of animals and were reviewed and approved by the Animal Care and Use Committee of St Louis University. iPLA₂β-KO and iPLA₂γ-KO mice were generated by Dr. John Turk’s and Dr. Richard Gross’s research groups, respectively, and have been described in detail previously (3, 12). Mice were bred as heterozygous trios, and WT and KO littersmates were used for endothelial cell isolation. Genotyping for each animal was performed by PCR as described previously (3, 12). Male and female WT, iPLA₂β-KO and iPLA₂γ-KO mice on the C57BL/6J background were used at 10–12 wk of age for endothelial cell isolation. Endothelial cells were isolated from WT and iPLA₂-KO mouse hearts by collagenase digestion. The diced heart muscle was incubated in 2 mg/ml collagenase for 1 h at 37°C, and the digested tissue was passed through a cell strainer. Cells were incubated with murine immunoglobulins to block Fc receptors and then incubated with anti-mouse platelet/endothelial cell adhesion molecule 1 (PECAM-1) coupled to magnetic beads. Cells obtained were cultured until they reached confluence and sorted again using ICAM-2 antibodies coupled with magnetic beads. The eluted cells were washed, resuspended in cell culture medium, and plated in culture. Nonadherent cells were removed the next day, and cells were grown to confluence and passaged at a 1 to 3 dilution. Genotype of each cell culture was verified by real-time RT-PCR analysis (Table 1). Relative quantitation of each iPLA₂ isoform was performed compared with 18s RNA. There was no detectable iPLA₂β and no significant compensation in iPLA₂γ expression in iPLA₂β-KO endothelial cells (Table 1). Likewise, we did not detect iPLA₂γ expression or change in iPLA₂β expression in iPLA₂γ-KO endothelial cells (Table 1).

Stimulation of confluent endothelial cells. All experiments were carried out with confluent monolayers of endothelial cells. Human recombinant skin β-tryptase (Promega, Madison, WI) or thrombin (Sigma Chemical, St. Louis, MO) were diluted with medium (iPLA₂ recombinant skin carboxypeptidase A) to improve recovery and visualize PAF zones on thin-layer chromatography (TLC) plates. The lipids, including PAF, were isolated by chloroform-methanol extraction. The chloroform layer was concentrated by evaporation under N₂, applied to TLC plates containing 7 mmol carrier PAF (β-acetyl-γ-O-hexadecyl-1-α-phosphatidylcholine, Sigma Chemical) to improve recovery and visualize PAF zones on thin-layer chromatography (TLC) plates. The lipids, including PAF, were isolated by chloroform-methanol extraction. The chloroform layer was concentrated by evaporation under N₂, applied to a silica gel 60 TLC plate, and developed in chloroform-methanol-2-propanol-1% acetic acid-water (50/25/8/4 vol/vol). The region corresponding to PAF was scraped, and radioactivity was quantified using liquid scintillation spectrometry. Loss of PAF during extraction and chromatography was corrected for by the addition of a known amount of [³⁰C]PAF as an internal standard. [³⁰C]PAF was synthesized by acetylation of the sn-2 position of lysodipalmitoyl-PAF with [³⁰C]acetic anhydride using 0.33 M dimethylaminopyridine as a catalyst. The synthesized [³⁰C]PAF was purified by HPLC. Analysis of PAF production was validated by the addition of a known amount of hexadecyl-2-acetyl-sn-glycerol-1-phosphoryl choline, 1-O-[acetyl-3H(N)] (Perkin Elmer, Boston, MA) to representative samples.

Prostaglandin release. Endothelial cells were grown to confluence in 16-mm tissueculture dishes. Cells were washed twice with HBSS 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 and were incubated with 50 μCi [³⁰C]acetic acid for 20 min. After the selected time interval for incubation with the appropriate agents, the synthesis and degradation of PAF were determined by comparing ΔCT (where CT is cycle threshold) between iPLA₂ isoforms and 18s RNA for each isolation.

Measurement of total arachidonic acid release. Endothelial cells were incubated at 37°C with 3 μCi [³⁰C]arachidonic acid for 18 h. This incubation resulted in >70% incorporation of radioactivity into membrane phospholipids. After incubation, endothelial cells were washed three times with Tyrode solution containing 0.36% bovine serum albumin to remove unincorporated [³⁰C]arachidonic acid. Endothelial cells were incubated at 37°C for 15 min before being subjected to experimental conditions. At the end of the stimulation period the supernatant was removed. Endothelial cells were lysed in 10% sodium dodecyl sulfate, and radioactivity in both supernatant and pellet was quantified by liquid scintillation spectrometry.

PAF assay. Endothelial cells grown in 12-well culture dishes were washed twice with HBSS 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 and were incubated with 50 μCi [³⁰C]acetic acid for 20 min. After the selected time interval for incubation with the appropriate agents, the synthesis and degradation of PAF were determined by the addition of 1 μM iPLA₂. The cells and surrounding buffer were removed from the tissue culture plate using a cell scraper and added to Teflon tubes containing 7 mmol carrier PAF (β-acetyl-γ-O-hexadecyl-1-α-phosphatidylcholine, Sigma Chemical) to improve recovery and visualize PAF zones on thin-layer chromatography (TLC) plates. The lipids, including PAF, were isolated by chloroform-methanol extraction. The chloroform layer was concentrated by evaporation under N₂, applied to a silica gel 60 TLC plate, and developed in chloroform-methanol-acetic acid-water (50/25/8/4 vol/vol). The region corresponding to PAF was scraped, and radioactivity was quantified using liquid scintillation spectrometry. Loss of PAF during extraction and chromatography was corrected for by the addition of a known amount of [³⁰C]PAF as an internal standard. [³⁰C]PAF was synthesized by acetylation of the sn-2 position of lysodipalmitoyl-PAF with [³⁰C]acetic anhydride using 0.33 M dimethylaminopyridine as a catalyst. The synthesized [³⁰C]PAF was purified by HPLC. Analysis of PAF production was validated by the addition of a known amount of hexadecyl-2-acetyl-sn-glycerol-1-phosphoryl choline, 1-O-[acetyl-3H(N)] (Perkin Elmer, Boston, MA) to representative samples.
iPLA₂ IN CARDIAC ENDOTHELIAL CELLS

Table 1. Real-time RT-PCR analysis of iPLA₂β, iPLA₂γ, and 18s RNA expression in endothelial cells isolated from the hearts of wild type, iPLA₂β and iPLA₂γ knockout mice

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Thrombin</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>ΔCt iPLA₂β vs. 18s RNA</td>
<td>ΔCt iPLA₂γ vs. 18s RNA</td>
</tr>
<tr>
<td>Wild type</td>
<td>23.8 ± 0.4</td>
<td>24.6 ± 0.2</td>
</tr>
<tr>
<td>iPLA₂β KO</td>
<td>ND</td>
<td>23.7 ± 0.1</td>
</tr>
<tr>
<td>iPLA₂γ KO</td>
<td>22.1 ± 0.1</td>
<td>ND</td>
</tr>
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</table>

Data represent means ± SE for 3 separate cell cultures. Endothelial cells were stimulated with 0.05 IU/ml thrombin for 10 min where indicated. iPLA₂, calcium-independent PLAs; C₅, cycle threshold; ND, not detected.

Prostaglandin release was measured immediately using enzyme-linked immunoassay kits. PGE₂ release was measured as its stable metabolite 6-keto-prostaglandin F₁₅₂₀ (Oxford Biomedical Research, Oxford, MI). PGE₂ release was measured directly (R&D Systems, Minneapolis, MN).

PAF-acetylhydrolase assay. Endothelial cells were grown to confluence and incubated with BEL for 10 min and were then removed from the culture plates in 1.2 mM Ca²⁺-HEPES buffer and sonicated on ice. Cellular protein (25 μg) was incubated with 0.1 mM [acetyl-[^3]H]PAF (10 nCi/mmol) for 30 min at 37°C. The reaction was stopped by the addition of acetic acid and sodium acetate. Released [^3]H acetic acid was isolated by passing the reaction mixture through a C₁₈ silica gel column (J. T. Baker, Phillipsburg, NJ), and eluted radioactivity was measured using a liquid scintillation counter.

Statistical analysis. All studies were repeated with at least four separate cell cultures. Data were analyzed using Student’s t-test or one-way analysis of variance followed by post hoc analysis using Dunnett’s test. Differences were regarded as significant at P < 0.05 and highly significant at P < 0.01. Error bars in the figures represent the standard error of the mean.

RESULTS

PLA₂ activity in cardiac endothelial cells. PLA₂ activity was measured in cardiac endothelial cells isolated from WT and iPLA₂-KO mice, using (16:0,[^3]H)18:1) plasmenylcholine or phosphatidylincholine substrates in the presence (1 mM Ca²⁺) or absence (4 mM EGTA) of Ca²⁺ (Table 1), and most cardiac endothelial cell PLA₂ activity did not require the presence of Ca²⁺. Mouse iPLA₂β- and iPLA₂γ-KO endothelial cells exhibited less PLA₂ activity than WT cardiac endothelial cells under all conditions studied (Table 2). When enzyme (50 μg of cellular protein) was incubated with increasing concentrations of (R)-BEL (0.05 to 1.0 μM for 10 min), the iPLA₂ activity in WT cardiac endothelial cells and HCAECs was not inhibited at concentrations of 1 μM or less (Fig. 1), which indicates that iPLA₂γ does not contribute significantly to their total PLA₂ under these conditions. Endothelial cells isolated from iPLA₂γ-KO mice demonstrated a lower iPLA₂ activity than cells from WT mice or HCAECs, but they also demonstrated no inhibition of iPLA₂ activity at (R)-BEL concentrations below 1 μM. iPLA₂β-KO endothelial cells had significantly less iPLA₂ activity than WT endothelial cells or HCAECs, but the residual iPLA₂β-KO endothelial cell iPLA₂ activity was sensitive to inhibition by (R)-BEL at concentrations below 1 μM, suggesting that the residual activity is attributable to iPLA₂γ that may be upregulated to compensate for the absence of iPLA₂β. These data indicate that cardiac endothelial cell PLA₂ activity is largely Ca²⁺-independent and that iPLA₂β is the predominant iPLA₂ isoform in WT cells.

Thrombin (0.05 IU/ml, 10 min) or tryptase (20 ng/ml, 10 min) stimulation of mouse cardiac endothelial cells resulted in a significant increase in iPLA₂ activity (Table 2). Increased thrombin- or tryptase-stimulated iPLA₂ activity was similar between WT and iPLA₂γ-KO endothelial cells, whereas that in iPLA₂β-KO endothelial cells was smaller. Activation of iPLA₂ activity by thrombin (0.05 IU/ml, 10 min) did not result in a significant change in iPLA₂ mRNA expression (Table 1),

Table 2. PLA₂ activity in cell lysate from WT and iPLA₂-KO cardiac endothelial cells under control, thrombin- (0.05 IU/ml, 10 min), or tryptase-stimulated (20 ng/ml, 10 min) conditions

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Control</th>
<th>Thrombin</th>
<th>Tryptase</th>
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<tbody>
<tr>
<td></td>
<td>EGTA</td>
<td>Ca²⁺</td>
<td>EGTA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasmenylcholine 16:0,[^3]H]18:1</td>
<td>4.62 ± 0.58</td>
<td>0.69 ± 0.04</td>
<td>6.73 ± 0.53*</td>
</tr>
<tr>
<td>Phosphatidylincholine 16:0,[^3]H]18:1</td>
<td>7.25 ± 1.03</td>
<td>0.44 ± 0.23</td>
<td>10.49 ± 0.83*</td>
</tr>
<tr>
<td>iPLA₂β-KO</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasmenylcholine 16:0,[^3]H]18:1</td>
<td>2.81 ± 0.04§</td>
<td>0.51 ± 0.05</td>
<td>3.51 ± 0.16*</td>
</tr>
<tr>
<td>Phosphatidylincholine 16:0,[^3]H]18:1</td>
<td>4.67 ± 0.34§</td>
<td>0.61 ± 0.17</td>
<td>5.93 ± 0.43</td>
</tr>
<tr>
<td>iPLA₂γ-KO</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasmenylcholine 16:0,[^3]H]18:1</td>
<td>2.03 ± 0.17§</td>
<td>0.32 ± 0.09†</td>
<td>5.64 ± 0.38*</td>
</tr>
<tr>
<td>Phosphatidylincholine 16:0,[^3]H]18:1</td>
<td>3.93 ± 0.21§</td>
<td>0.42 ± 0.08</td>
<td>7.22 ± 0.51†</td>
</tr>
</tbody>
</table>

Data represent means ± SE for separate measurements from 4 different cell isolations. PLA₂ activity (in nmol/mg protein⁻¹·min⁻¹) was measured using plasmenylcholine or phosphatidylincholine substrates in the absence (4 mM EGTA) or presence (1 mM Ca²⁺) of calcium. Substrate composition is represented as a:b, c:d, where a:b and c:d represent the chain length: number of double bonds for the aliphatic groups at the sn-1 and sn-2 position, respectively, of the corresponding phospholipid substrate molecule. *P < 0.05, †P < 0.01 when comparing stimulated to unstimulated activity. §P < 0.05, ‡P < 0.01 when comparing knockout (KO) activity to wild type (WT).
suggesting that the increase in iPLA2 activity is a result of activation of a latent enzyme in endothelial cells. These findings caused us to hypothesize that iPLA2 might play a central role in lipid signaling in cardiac endothelial cells, and we next measured arachidonic acid release and production of PGI2 and PAF in WT and iPLA2-KO mouse cardiac endothelial cells.

**Arachidonic acid release.** Arachidonic acid released from WT and iPLA2-KO mouse cardiac endothelial cells was measured following treatment with tryptase (20 ng/ml) or thrombin (0.05 IU/ml) for up to 30 min (Fig. 2). WT endothelial cells released significantly greater amounts of arachidonic acid after stimulation, whereas both iPLA2β- and iPLA2γ-KO cardiac endothelial cells released smaller amounts of arachidonic acid, which indicates that PAR stimulation-induced hydrolysis of arachidonic acid from endothelial cell membrane phospholipids is catalyzed predominantly by both iPLA2β and iPLA2γ.

**PGI2 production.** Prostacyclin (PGI2) is the predominant prostaglandin generated by endothelial cells as a result of sequential arachidonic acid release from membrane phospholipids and subsequent hydrolysis by cyclooxygenases and prostacyclin synthase. Since iPLA2 isoforms catalyze arachidonic acid release in cardiac endothelial cells, they may also be involved in PGI2 production. To examine the contributions of iPLA2 isoforms in cardiac endothelial cell PGI2 production, we compared the time course of PGI2 release from WT, iPLA2β-KO, and iPLA2γ-KO cardiac endothelial cells stimulated with thrombin (0.05 IU/ml) or tryptase (20 ng/ml) for up to 30 min (Fig. 3). WT endothelial cells exhibited increased PGI2 production after stimulation, and iPLA2γ-KO endothelial cells exhibited a similar response. In contrast, iPLA2β-KO cardiac endothelial cells exhibited significantly attenuated thrombin-stimulated PGI2 production compared with WT and iPLA2γ-KO endothelial cells (Fig. 3), and the majority of thrombin-stimulated PGI2 release appears to require iPLA2β activity. Tryptase-stimu-

lated PGI2 release was similar for iPLA2β-KO and iPLA2γ-KO cardiac endothelial cells but was significantly less than that from WT hearts (Fig. 3). These data suggest that the signaling pathways for coupling PAR-1 receptor activation and iPLA2 production differ from those for PAR-2 receptors.

We next determined the effects of BEL enantiomers on thrombin- or tryptase-stimulated WT cardiac endothelial cell PGI2 production and found that both (R)-BEL (5 μM, 10 min) and (S)-BEL (5 μM, 10 min) inhibited PGI2 production and that the latter was more effective, which indicates that both iPLA2β and iPLA2γ are involved in PGI2 production (Fig. 4). When endothelial cells were incubated with racemic BEL (10 μM, 10 min), PGI2 production was inhibited completely following stimulation with thrombin or tryptase (Fig. 4). Prostaglandin E2 release from WT cardiac endothelial cells was significantly increased by thrombin (0.05 IU/ml, 10 min) or tryptase (20 ng/ml, 10 min) stimulation (Fig. 5). Cardiac
endothelial cells isolated from iPLA2γ/H9253 or iPLA2β/H9252-KO mice demonstrated smaller increases in PGE2 production in response to protease stimulation (Fig. 5). Thus PGI2 and PGE2 production are reduced similarly in iPLA2-KO endothelial cells, suggesting that this is a direct result of decreased arachidonic acid release.

PAF production. In WT endothelial cells a significant increase in PAF production in response to thrombin or tryptase was observed (Fig. 6). Pretreatment with (R)-BEL did not affect PAF production, but pretreatment with (S)-BEL significantly reduced PAF production to a level similar to that for the untreated control, suggesting that iPLA2β activity is required for protease-stimulated cardiac endothelial cell PAF production. To exclude a significant contribution of iPLA2γ, PAF production by tryptase- or thrombin-stimulated iPLA2γ-KO cells was compared with that of WT and iPLA2β-KO endothelial cells (Fig. 6). As with WT endothelial cells, iPLA2γ-KO cells exhibited increased PAF production upon protease stimulation, and this was unaffected by pretreatment with (R)-BEL. In contrast, pretreatment with (S)-BEL inhibited PAF production completely. Similarly, tryptase or thrombin stimulation of iPLA2β-KO endothelial cells resulted in no increase in PAF production (Fig. 6).

PAF-acetylhydrolase activity. To determine whether variations in PAF-acetylhydrolase (PAF-AH), the enzyme responsible for PAF catabolism, might constrain net PAF production by cardiac endothelial cells, we measured PAF-AH activity in WT and iPLA2β-KO cells without or with BEL pretreatment (Fig. 7) and observed no significant difference in basal PAF-AH activity between WT and iPLA2β-KO cells and no effect of BEL on their PAF-AH activity (Fig. 7).
lipid-derived mediators are involved in endothelial cell signaling events (21). In its basal state, the endothelium is required to control inflammation and coagulation, but in response to destructive stimuli, endothelial cells produce a variety of vasoactive and proinflammatory mediators, including the bioactive lipids PAF and PGI2 (27). These compounds are formed from the precursors arachidonic acid and lyso-PAF that are generated by the action of iPLA2 on membrane phospholipid substrates. The generation of these precursors is the rate-limiting step for formation of eicosanoids and PAF.

Human coronary artery endothelial cells (HCAECs) and cardiac endothelial cells isolated from WT mice have comparable levels of iPLA2 activity, and it is sensitive to inhibition by relatively high concentrations of (R)-BEL. In contrast, iPLA2β-KO mouse cardiac endothelial cells have much lower levels of iPLA2 activity that is sensitive to inhibition by low concentrations of (R)-BEL, suggesting that this residual activity is attributable to iPLA2γ. Our previous studies have demonstrated that tryptase or thrombin activate HCAEC iPLA2, resulting in arachidonic acid release, and production of PGE2 and PGI2 (19). Activation of endothelial cell iPLA2 and the resultant release of free arachidonic acid can lead to the formation of multiple eicosanoids that are involved in inflammation. Endothelial cell arachidonic acid is metabolized by cytochrome P450 to produce epoxyeicosatrienoic acids in response to shear stress, stretch, and bradykinin (4). Leukotrienes have been implicated in the inflammatory component of atherosclerosis (1) and can induce proinflammatory signaling via activation of specific leukotriene receptors (20). PGI2 is the predominant prostaglandin produced by endothelial cells and has been implicated in acute and chronic pain (18). PGI2 contributes to normal inflammatory responses by promoting vasodilation and increasing vascular permeability. PGI2 is also cardioprotective since it inhibits platelet and lymphocyte adhesion to endothelium, restricts vascular smooth muscle cell proliferation and migration, and prevents production of profibrotic growth factors (28).

Our data here demonstrate that iPLA2β activity contributes to tryptase- or thrombin-stimulated cardiac endothelial cell

DISCUSSION

In this study we have demonstrated that the majority of cardiac endothelial cell iPLA2 activity is attributable to iPLA2β, an enzyme that has been cloned from hamster (25), rat (10), mouse (2), and human cells (9), inter alia. This isoform is classified as the group VIA PLA2 (11, 23) that is expressed in various species, and as proteolysis products, including a 70-kDa variant, inter alia (9). Each of these variants contains a GXSXG lipase consensus motif and a stretch of seven to eight ankyrin-like repetitive sequence motifs that mediate protein interactions in other proteins (25). Studies involving pharmacological inhibition of iPLA2β with BEL have suggested its involvement in modulating arachidonic acid release from vascular cells and vasomotor tone, among other processes (5, 14). BEL, like other pharmacologic inhibitors, has a number of recognized off-target effects and probably also has such effects that are not yet recognized. This has motivated the preparation of genetically modified cell lines and animals with altered iPLA2 expression to characterize the participation of the enzyme in various biological processes (3, 12, 14). In this study we have demonstrated that iPLA2β is the predominant Ca2+-independent PLA2 in WT and iPLA2 KO mouse cardiac endothelial cells and that iPLA2β activity is responsible for most of the protease-stimulated PAF, PGE2, and PGI2 production by these cells. However, cardiac endothelial cells from iPLA2γ-KO mice indicate that iPLA2γ can also contribute to prostaglandin production. These findings with cells from genetically modified mice were corroborated by experiments with BEL enantiomers to strengthen the hypothesis that iPLA2β is involved in protease-stimulated cardiac endothelial cell production of PAF, PGE2, and PGI2.

Vascular endothelium plays important roles of vascular homeostatic processes and responses to injury, and phospho-

Fig. 6. Platelet-activating factor (PAF) production in WT, iPLA2β-KO, and iPLA2γ-KO cardiac endothelial cells unstimulated or stimulated with tryptase (try, 20 ng/ml, 10 min) or thrombin (thr, 0.05 IU/ml, 10 min), with or without (R)- or (S)-BEL (5 μM, 10 min) pretreatment. Cont, control; dpm, disintegrations per minute. Results represent means ± SE of 4 independent experiments.

Fig. 7. PAF-acetylhydrolase (AH) activity in WT and iPLA2β-KO cardiac endothelial cells without or with BEL (5 μM, 10 min) pretreatment.
PGI<sub>2</sub> production and thereby would exert a cardioprotective effect. iPLA<sub>2</sub>γ may also contribute to PGI<sub>2</sub> production because its pharmacological inhibition resulted in a reduction in PGI<sub>2</sub> production, and iPLA<sub>2</sub>γ-KO mouse cardiac endothelial cells exhibited a reduction in protease-stimulated PGI<sub>2</sub> production that could reflect coupling with cyclooxygenases, PARs, or other PL<sub>A</sub>2 family members. That only a small amount of arachidonic acid is released from iPLA<sub>2</sub>β-KO endothelial cells suggests that the reduction in PGI<sub>2</sub> production by these cells results from the low availability of free arachidonic acid substrate for cyclooxygenase, and it is unlikely that members of the PL<sub>A</sub>2 family other than iPLA<sub>2</sub> make significant contributions to this substrate pool under these conditions. We have observed different patterns of PGI<sub>2</sub> production here in response to thrombin compared with tryptase, and this suggests that distinct populations of PAR may be differentially coupled to signaling events involved in PL<sub>A</sub>2 activation, arachidonate release, and prostanoid generation. This sequence requires further scrutiny to clarify the mechanisms whereby distinct proteases couple to their cognate receptors to initiate the cascade of intracellular events that culminate in the production of lipid mediators that influence vascular biologic processes.

The endothelium regulates trafficking of cells from blood to tissues via cell surface adherence molecules (16). In this setting, PAF is considered an important inflammatory mediator, and it is expressed on the surfaces of endothelial cells where it regulates inflammatory cell endothelial transmigration and causes increased vascular permeability. PAF is involved in cardiac anaphylaxis, ischemia-reperfusion cardiac injury, and atherogenesis, among other (patho)physiologic processes. PAF interacts with endothelial cell PAF receptors (PAFR) and with PAFR on inflammatory cells that include neutrophils, macrophages, and eosinophils. The interaction of PAF with circulating leukocyte PAFRs results in leukocyte activation, adhesion, and migration, resulting in leukocyte recruitment to sites of injury. PAF also facilitates NF-κB translocation to the nucleus and may thereby contribute to early inflammatory cytokine generation (13). Leukocyte activation also results in generation of reactive oxygen species, lipid mediators, cytokines, and degradative enzymes. These mediators are released to restrict and eradicate the inflammatory stimulus but can subsequently cause tissue injury in inflammatory diseases (15). Oxidants and free radicals modify proteins and nucleic acids and activate the immune and inflammatory systems to produce deleterious effects.

PAF synthesis and degradation are highly regulated processes. PAF-acetylhydrolase (PAF-AH) is a PL<sub>A</sub>2 family member that catalyzes hydrolysis of PAF to biologically inactive lyso-PAF (8). We have previously demonstrated that thrombin or tryptase stimulation of HCAECs results in PAF production by an iPLA<sub>2</sub>-independent process, and inhibition of HCAEC PAF-AH activity increases the net amount of PAF produced (6). In the studies described here, stimulation of WT mouse cardiac endothelial cells with tryptase or thrombin resulted in increased PAF production. Experiments with BEL enantiomers to selectively inhibit iPLA<sub>2</sub> isoforms indicated that iPLA<sub>2</sub>β is important for protease-stimulated cardiac endothelial cell PAF production but that iPLA<sub>2</sub>γ made little contribution. These findings are consistent with the observations that iPLA<sub>2</sub>β-KO mouse cardiac endothelial cells failed to respond to tryptase or thrombin stimulation by increasing PAF production. As expected, iPLA<sub>2</sub>γ-KO mouse cardiac endothelial cells did respond to protease stimulation by increasing PAF production but were unable to so when pretreated with the iPLA<sub>2</sub>β inhibitor (S)-BEL. Together these findings suggest that iPLA<sub>2</sub>β is almost exclusively responsible for PAF production in cardiac endothelial cells.

To determine whether variations in PAF-AH activity might explain the low PAF production by iPLA<sub>2</sub>β-KO mouse cardiac endothelial cells, PAF-AH activity was directly measured, and WT and iPLA<sub>2</sub>β-KO mouse cardiac endothelial cells exhibited similar levels PAF-AH activity. Pretreatment of the cells with BEL failed to affect PAF-AH activity, and together these findings indicate that net PAF production under these conditions is not governed by variations in the level of PAF-AH activity. Because PAF is responsible for inflammatory cell transmigration and activation and plays important roles in cardiac (patho)physiologic processes, interventions targeted at affecting local PAF levels based on an understanding of factors modulating its production may prove to be therapeutically beneficial.

In summary, our studies demonstrate that iPLA<sub>2</sub>β and iPLA<sub>2</sub>γ have distinct roles in cardiac endothelial cell prostacyclin and PAF production in response to inflammatory stimuli. To the best of our knowledge, this is the first study to examine the role of iPLA<sub>2</sub>β in cardiac endothelial cell lipid mediator production that uses genetically modified mice and that does not rest primarily on pharmacologic inhibitors with known and potential off-target effects. Our data demonstrate that iPLA<sub>2</sub>β is the predominant iPLA<sub>2</sub> isoform in cardiac endothelial cells, that iPLA<sub>2</sub>β is solely responsible for protease-stimulated cardiac endothelial PAF production, and that iPLA<sub>2</sub>β also accounts for majority of the PGI<sub>2</sub> production under these conditions. These findings indicate that iPLA<sub>2</sub>β is an important component of the inflammatory signaling via participation in the production of two major lipid modulators of inflammation. Although inflammation can represent a protective response to remove injurious insults and to initiate healing, prolongation of the inflammatory response can cause tissue destruction and impair wound healing. Better understanding of the processes governing production and persistence of inflammatory mediators such as PAF and eicosanoids may permit development of means to manipulate these processes in therapeutically beneficial ways. Our studies suggest that interventions targeted at modulating the activity of iPLA<sub>2</sub> isoforms could be useful in that regard.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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