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# Platelet 12-Lipoxygenase Activation via Glycoprotein VI Involvement of Multiple Signaling Pathways in Agonist Control of H(P)ETE Synthesis

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**Abstract**—Lipoxygenases (LOX) contribute to vascular disease and inflammation through generation of bioactive lipids, including 12-hydro(pero)xyeicosatetraenoic acid (12-H(P)ETE). The physiological mechanisms that acutely control LOX product generation in mammalian cells are uncharacterized. Human platelets that contain a 12-LOX isoform (p12-LOX) were used to define pathways that activate H(P)ETE synthesis in the vasculature. Collagen and collagen-related peptide (CRP) (1 to 10  $\mu\text{g}/\text{mL}$ ) acutely induced platelet 12-H(P)ETE synthesis. This implicated the collagen receptor glycoprotein VI (GPVI), which signals via the immunoreceptor-based activatory motif (ITAM)-containing FcR $\gamma$  chain. Conversely, thrombin only activated at high concentrations ( $> 0.2 \text{ U}/\text{mL}$ ), whereas U46619 and ADP alone were ineffective. Collagen or CRP-stimulated 12-H(P)ETE generation was inhibited by staurosporine, PP2, wortmannin, BAPTA/AM, EGTA, and L-655238, implicating *src*-tyrosine kinases, PI3-kinase,  $\text{Ca}^{2+}$  mobilization, and p12-LOX translocation. In contrast, protein kinase C (PKC) inhibition potentiated 12-H(P)ETE generation. Finally, activation of the immunoreceptor tyrosine-based inhibitory motif (ITIM)-containing platelet endothelial cell adhesion molecule (PECAM-1) inhibited p12-LOX product generation. This study characterizes a receptor-dependent pathway for 12-H(P)ETE synthesis via the collagen receptor GPVI, which is negatively regulated by PECAM-1 and PKC, and demonstrates a novel link between immune receptor signaling and lipid mediator generation in the vasculature. (*Circ Res.* 2004;94:1598-1605.)

**Key Words:** platelets ■ lipoxygenase ■ collagen ■ collagen receptor glycoprotein VI

Lipoxygenases (LOX) catalyze lipid hydroperoxide formation from unsaturated fatty acids.<sup>1</sup> Many LOX isoforms are found in the vasculature: some constitutive (eg, p12-LOX in platelets, 5-LOX in neutrophils), others cytokine-inducible (eg, 15-LOX by interleukin-4 (IL-4) in monocytes). Compelling evidence exists for critical involvement of LOXs in vascular and inflammatory diseases including atherosclerosis, cancer, hypertension, and diabetes.<sup>2-13</sup>

Cellular LOXs require direct activation before product generation occurs. Typically, this requires (1) oxidation of the ferrous iron to an intermediate that abstracts hydrogen from unsaturated lipid and (2) translocation of the cytosolic enzyme to nuclear or plasma membrane. This activation is separate from simple regulation of LOX by fatty acid supply, and its requirement for H(P)ETE synthesis is supported by the following evidence: (1)  $\text{Ca}^{2+}$  stimulates membrane binding and arachidonate oxidation by purified 15-LOX in vitro; (2) LOX product generation (in response to  $\text{Ca}^{2+}$  ionophore) is inhibited by membrane translocation inhibitors; and (3) 15-LOX in human monocytes oxidizes membrane-bound

arachidonate after  $\text{Ca}^{2+}$  mobilization, independently of PLA<sub>2</sub>.<sup>14-16</sup> Currently, little is known regarding control of LOX in cells by acute mechanisms. Most studies activate LOX turnover by addition of arachidonate substrate with/without  $\text{Ca}^{2+}$  ionophore.<sup>17,18</sup> These studies implicate  $\text{Ca}^{2+}$ -dependent translocation, serine and tyrosine phosphorylation, and LOX association with accessory proteins such as 5-lipoxygenase activating protein (FLAP), although their involvement in LOX activation using physiological agonists is not established.<sup>18-23</sup> Acute activation of LOXs has been reported for neutrophil 5-LOX by fMLP and p12-LOX in platelets by collagen; however, the receptors involved and intracellular signaling pathways remain uncharacterized.<sup>24-27</sup> Additionally, modulation of LOX activity within cells by agonist-mediated feedback mechanisms has not been examined.

This study utilized washed human platelets as a model to define receptor-dependent and intracellular mechanisms of LOX activation by acute physiological agonists. The data shows (1) collagen or collagen-related peptide (CRP) signal-

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ing via glycoprotein VI (GPVI) acutely activates secretion of 12-H(P)ETE, in contrast to thrombin, ADP, and thromboxane receptor (TP), (2) GPVI-stimulated 12-H(P)ETE synthesis requires *src*-tyrosine kinases, Ca<sup>2+</sup> mobilization, and PI3-kinase, and (3) GPVI-stimulated p12-LOX product generation is suppressed by PKC and PECAM-1. These data reveal a central role for the FcR $\gamma$  chain-associated GPVI collagen receptor in activation of platelet 12-H(P)ETE generation and implicate immune receptors in modulating LOX in the vasculature.

## Materials and Methods

### Reagents

Collagen (Type I) was from Mascia Brunelli, Milan, Italy. Arachidonic acid (AA), U46619, L-655238, 12-hydroxyicosatetraenoic acid (12-HETE), 15-hydroxy-11Z,13E-eicosadienoic acid (15-HEDE), baicalein, and nordihydroguaiaretic acid (NDGA) were from Alexis Chemicals Ltd. Ethylene glycol-O,O'-bis-[2-amino-ethyl]-N,N,N',N'-tetraacetic acid (EGTA), 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid-acetoxymethyl ester (BAPTA/AM), FURA-2/AM, A23187, PP2, p38-MAP kinase inhibitor (MKI), staurosporine, chelerythrine chloride, and bis-indolymaleimide were from Calbiochem Ltd. All other reagents were from Sigma Ltd, unless otherwise stated.

### Preparation of Washed Human Platelets

Platelets were isolated as described<sup>28</sup> from consenting donors who were NSAID-free for 2 weeks before donating. Washed platelets were resuspended in Ca<sup>2+</sup>-free Tyrodes buffer (in mmol/L: 134 NaCl, 12 NaHCO<sub>3</sub>, 2.9 KCl, 0.34 Na<sub>2</sub>HPO<sub>4</sub>, 1 MgCl<sub>2</sub>, 10 HEPES, 5 glucose, pH 7.4).

### Acute Activation of Platelets With Agonists and Sample Preparation for HPLC

3 × 10<sup>8</sup> platelets in 100  $\mu$ L Tyrodes, 1 mmol/L CaCl<sub>2</sub>, were stimulated with either collagen (1 to 10  $\mu$ g/mL), CRP (0.05 to 10  $\mu$ g/mL) (synthesized to a published sequence<sup>29</sup>), convulxin (1  $\mu$ g/mL protein concentration) (a kind gift from Dr S.P. Watson, University of Oxford, UK), thrombin (0.04 to 2.0 U/mL), the calcium ionophore, A23187 (1  $\mu$ mol/L), arachidonic acid (20  $\mu$ mol/L), the thromboxane mimetic, U46619 (10  $\mu$ mol/L), or ADP (5  $\mu$ mol/L) at 37°C with agitation for 10 minutes. Experiments using signaling inhibitors included a 10-minute preincubation step at 37°C before addition of collagen or CRP (both 10  $\mu$ g/mL). Incubations were stopped by the addition of 200  $\mu$ L ice-cold methanol and samples spun at 10 000g for 10 minutes at 4°C. Supernatants containing 12-H(P)ETE were recovered and supplemented with 200 ng 15(S)-HETE per sample as an internal standard.

### Reverse Phase HPLC Analysis of Platelet 12-LOX Products

Samples (equivalent to 2 × 10<sup>7</sup> cells per sample run) were separated on a 150 mm × 4.6 mm, 5  $\mu$ m C<sub>18</sub> ODS2 (Waters Ltd) column using 50% to 90% B over 20 minutes (A=water:acetonitrile:acetic acid, 75:25:0.1, B=methanol:acetonitrile:acetic acid, 60:40:0.1) at 1 mL/min. Absorbance was monitored at 235 nm. Products were identified and quantified using 200 ng 12(S)-HETE standard run in parallel under the same conditions.

### Optical Aggregometry

Aggregation of 4 × 10<sup>8</sup> washed platelets/mL in response to CRP (0.05  $\mu$ g/mL) was measured in siliconized-glass cuvettes at 37°C with constant stirring in a PAP4 optical aggregometer (Bio/Data Corp). Platelets were incubated with anti-PECAM-1 antibody (AB468, 1  $\mu$ g/mL) for 5 minutes, followed by cross-linking for 90 seconds with F(ab')<sub>2</sub> fragments of anti-IgG secondary antibody (30  $\mu$ g/mL). Mouse monoclonal IgG (1  $\mu$ g/mL) plus F(ab')<sub>2</sub> fragments of

anti-IgG secondary antibody (30  $\mu$ g/mL) were utilized as isotype controls. CRP (0.05  $\mu$ g/mL) was then added and aggregation responses measured.

### FURA-2/AM Fluorescence

Washed platelets were incubated at 4 × 10<sup>8</sup>/mL with 2.5  $\mu$ mol/L FURA-2/AM for 45 minutes before washing at 800g for 10 minutes. Fluorescence was measured after agonist addition, using a Perkin Elmer 50B fluorescence spectrophotometer at 37°C with stirring (e<sub>x</sub>, 340 nm, 380 nm; e<sub>m</sub>, 509 nm).

### Statistical Analysis

Data are representative of at least 3 separate donors, with samples run in triplicate (Error bars are for mean ± SEM). Significance was examined using an unpaired *t* test where *P* ≤ 0.05 was considered significant (denoted by \* on figure). Activity in the presence of inhibitors is expressed % relative to the collagen or CRP response, as appropriate.

## Results

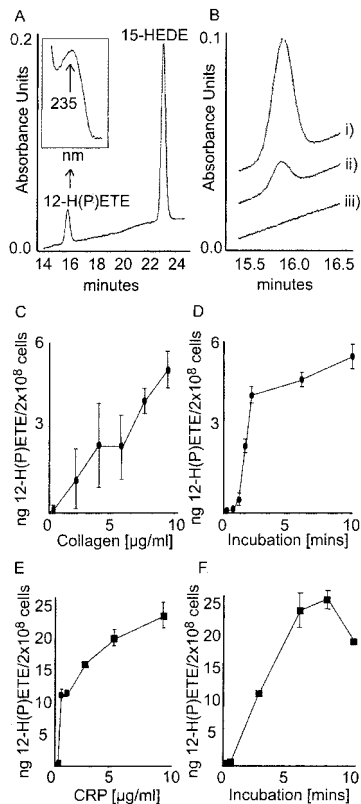
### Collagen and CRP Stimulate p12-LOX Product Generation in Washed Human Platelets

Incubation of platelets with collagen or CRP led to 12-H(P)ETE synthesis in a dose- and time-dependent manner (Figure 1). 12-H(P)ETE generation in response to agonists was confirmed by coelution with (1) authentic 12-H(P)ETE standard, and (2) 12-H(P)ETE generated after arachidonate activation of platelets. Furthermore, its synthesis was inhibited by LOX inhibitors baicalein or NDGA (Figure 2A). Peroxide analysis of platelet lysates determined that 12-H(P)ETE comprised predominantly 12-HETE, with 12-H(P)ETE content being 2.7 ± 1.5% or 0.7 ± 0.02% for collagen or CRP, respectively (not shown). Collagen treatment showed a lag-phase of approximately 25 seconds before a rapid linear increase over 2 minutes, followed by a slower phase of product generation from 2 to 10 minutes (Figure 1D). CRP showed a similar lag phase with product generation increasing linearly over 1 to 5 minutes (Figure 1F). Synthesis of 12-H(P)ETE did not require addition of exogenous fatty acid or Ca<sup>2+</sup> ionophore, indicating that arachidonate substrate originated from internal phospholipid hydrolysis. A crude fraction of venom from *Crotalus durissus terrificus* containing convulxin, which selectively activates GPVI, also efficiently stimulated 12-H(P)ETE generation (Table).

Incubation of platelets with thrombin (< 0.2 U/mL) failed to stimulate 12-H(P)ETE synthesis (Table). Nonphysiological concentrations of thrombin, up to 2.0 U/mL did stimulate, although far less than collagen or CRP (not shown). Finally, neither ADP nor the thromboxane A<sub>2</sub> mimetic, U46619, activated p12-LOX when added alone (Table). This was in comparison to arachidonate or A23187 activated 12-H(P)ETE synthesis, as previously shown.<sup>14</sup>

### Ca<sup>2+</sup> Mobilization Is Necessary but Not Sufficient for GPVI Stimulation of 12-H(P)ETE Synthesis

To examine whether Ca<sup>2+</sup> mobilization is required or sufficient for GPVI-stimulated p12-LOX product generation, platelets were first activated in Ca<sup>2+</sup>-free buffer with EGTA (1 mmol/L) to chelate extracellular Ca<sup>2+</sup>, with/without BAPTA/AM (10  $\mu$ mol/L). With EGTA alone, there was a significant 75% decrease in collagen activation of p12-LOX. Additional prein-

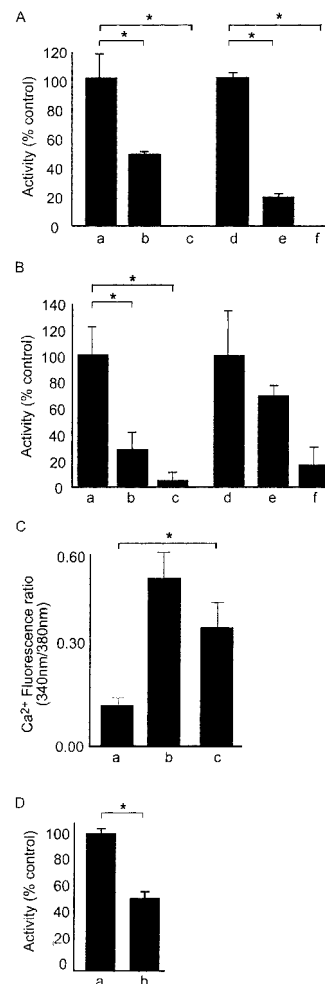


**Figure 1.** Collagen and CRP stimulate 12-H(P)ETE synthesis in a dose- and time-dependent manner in washed human platelets. **A**, Generation of 12-H(P)ETE by collagen-activated platelets.  $3 \times 10^8$  platelets in Tyrodes buffer (pH 7.4) and 1 mmol/L  $\text{CaCl}_2$  were activated with 10  $\mu\text{g}/\text{mL}$  collagen. LOX products were separated on reverse-phase HPLC as described in Materials and Methods. An inset, UV spectrum of platelet-derived 12-H(P)ETE peak showing absorption maximum at 235 nm. **B**, Generation of 12-H(P)ETE by collagen or CRP-activated platelets. Overlaid chromatograms showing 12-H(P)ETE generation for (i) CRP (10  $\mu\text{g}/\text{mL}$ ), (ii) collagen (10  $\mu\text{g}/\text{mL}$ ), and (iii) untreated platelets. **C**, Dose dependence of collagen activation of p12-LOX. Platelets were incubated with 1 to 10  $\mu\text{g}/\text{mL}$  collagen for 10 minutes. **D**, Time dependence of collagen activation of p12-LOX. Platelets were incubated with 10  $\mu\text{g}/\text{mL}$  collagen for 1 to 10 minutes. **E**, Dose dependence of CRP activation of p12-LOX. Platelets were incubated with 1 to 10  $\mu\text{g}/\text{mL}$  CRP for 10 minutes. **F**, Time dependence of CRP activation of p12-LOX. Platelets were incubated with 10  $\mu\text{g}/\text{mL}$  CRP for 1 to 10 minutes.

cubation with BAPTA/AM virtually abolished 12-H(P)ETE synthesis (Figure 2B). Similarly, a substantial decrease in p12-LOX activity in response to CRP was observed after  $\text{Ca}^{2+}$  chelation, although differences were not statistically significant. Separately,  $\text{Ca}^{2+}$  mobilization in response to collagen (10  $\mu\text{g}/\text{mL}$ ), CRP (10  $\mu\text{g}/\text{mL}$ ), or thrombin (0.1 U/mL) was determined and compared. It was found that thrombin and CRP caused similar elevations in cytosolic  $\text{Ca}^{2+}$ , which were approximately two-fold higher than collagen (Figure 2C). Because thrombin is ineffective at stimulating 12-H(P)ETE generation at this concentration,  $\text{Ca}^{2+}$  mobilization is required, but not sufficient to stimulate p12-LOX turnover.

#### L-655238 Inhibits CRP-Mediated p12-LOX Product Generation

When incubated with washed platelets, the translocation inhibitor L-655238 (10  $\mu\text{mol}/\text{L}$ ) significantly inhibited CRP-stimulated 12-H(P)ETE generation (Figure 2D).



**Figure 2.** Effects of LOX, calcium signaling inhibitors, and a LOX translocation inhibitor on collagen/CRP-mediated p12-LOX activity. All samples were analyzed for 12-H(P)ETE content as described. **A**, Inhibition of p12-LOX by LOX inhibitors. Platelets were preincubated with the LOX inhibitors baicalein (0.3  $\mu\text{mol}/\text{L}$ ) or NDGA (60  $\mu\text{mol}/\text{L}$ ) before adding collagen or CRP. Platelets: a, +collagen; b, +collagen+baicalein; c, +collagen+NDGA; d, +CRP; e, +CRP+baicalein; f, +CRP+NDGA. **B**, Requirement for calcium signaling in collagen or CRP activation of p12-LOX. Platelets were preincubated with calcium chelators (1 mmol/L EGTA and/or 10  $\mu\text{mol}/\text{L}$  BAPTA/AM) before addition of collagen or CRP. Platelets: a, +collagen; b, +collagen+EGTA; c, +collagen+EGTA+BAPTA/AM; d, +CRP; e, +CRP+EGTA; f, +CRP+EGTA+BAPTA/AM. **C**, Calcium mobilization in response to agonists. Platelets were labeled with FURA-2 as described in Materials and Methods. Background fluorescence ratios were subtracted from all samples. Platelets: a, +collagen (10  $\mu\text{g}/\text{mL}$ ); b, +CRP (10  $\mu\text{g}/\text{mL}$ ); c, +thrombin (0.1 U/mL). **D**, LOX translocation inhibitor, L-655238, inhibits CRP-mediated p12-LOX product generation. Platelets were preincubated with 10  $\mu\text{mol}/\text{L}$  L-655238 before adding CRP. Platelets: a, +CRP; b, +CRP+L-655238. \* $P \leq 0.05$  using unpaired *t* test.

#### CRP and Collagen Stimulation of 12-H(P)ETE Generation Requires Signaling by Tyrosine Kinases

To examine the potential role of tyrosine kinases in p12-LOX activation, staurosporine and the *src*-family tyrosine kinase inhibitor, PP2, were utilized.<sup>30,31</sup> Preincubating platelets with 20  $\mu\text{mol}/\text{L}$  staurosporine before collagen or CRP addition caused an approximate 85% inhibition of H(P)ETE genera-

### Quantification of 12-H(P)ETE Generation by Platelets

Treatment	[ng 12-H(P)ETE per $2 \times 10^8$ platelets]
Platelets only	0.00
Platelets+collagen (10 $\mu$ g/mL)	6.50 $\pm$ 1.50
Platelets+CRP (10 $\mu$ g/mL)	30.61 $\pm$ 0.29
Platelets+thrombin (0.2 U/mL)	0.50 $\pm$ 0.70
Platelets+convulxin (1 $\mu$ g/mL)	19.50 $\pm$ 4.80
Platelets+A23187 (1 $\mu$ mol/L)	17.03 $\pm$ 0.10
Platelets+arachidonate (20 $\mu$ mol/L)	94.80 $\pm$ 6.10
Platelets+U46619 (10 $\mu$ mol/L)	0.90 $\pm$ 0.90
Platelets+ADP (5 $\mu$ mol/L)	0.04 $\pm$ 0.05

Platelets were stimulated with various agonists before being analyzed by reverse-phase HPLC for 12-H(P)ETE. Data shown are for mean $\pm$ SEM, n=3.

tion. (Figure 3A). Preincubating platelets with PP2 (0 to 50  $\mu$ mol/L) before collagen or CRP addition caused significant dose-dependent inhibition of p12-LOX product generation. (Figure 3B).

### CRP Mediated 12-H(P)ETE Synthesis Is Only Partially Affected by p38-MAP Kinase Inhibition

Preincubating platelets with MKI (100 nmol/L) partially inhibited CRP, but was without effect on collagen-activated H(P)ETE synthesis (Figure 3C).

### CRP and Collagen Stimulation of 12-H(P)ETE Synthesis Requires Signaling by PI3-Kinase

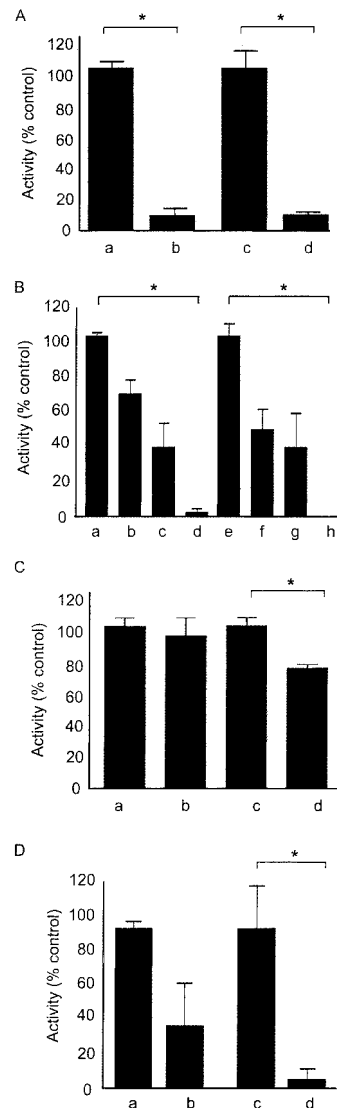
After GPVI activation, PI3-kinase binds to the phosphorylated Fc $\gamma$  chain and yields PIP<sub>3</sub>.<sup>32</sup> Preincubation of platelets with the PI3-kinase inhibitor wortmannin (100 nmol/L) substantially inhibited collagen and CRP-mediated p12-LOX activity (Figure 3D), implicating this pathway in GPVI-dependent p12-LOX activation.

### PKC Inhibitors Potentiate Agonist-Mediated 12-H(P)ETE Generation

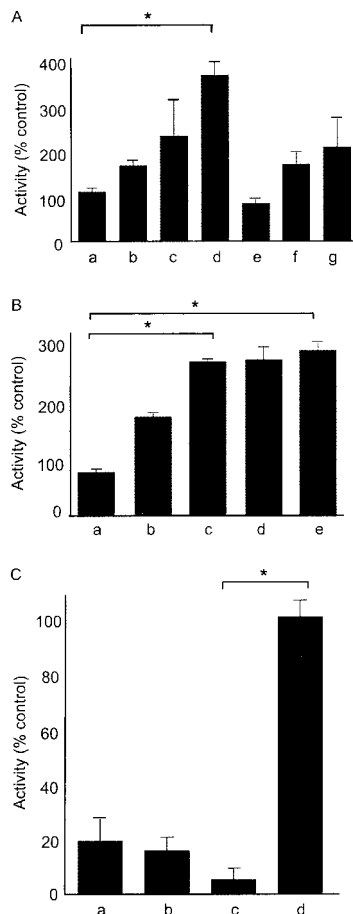
PKC is associated with phosphorylation of cytosolic PLA<sub>2</sub> and Ca<sup>2+</sup> mobilization in platelets.<sup>33,34</sup> Two specific PKC inhibitors, bis-indolymaleimide and chelerythrine chloride (1 to 10  $\mu$ mol/L), potentiated collagen and CRP-mediated p12-LOX. Also, bis-indolymaleimide inclusion with U46619, but not ADP, stimulated 12-H(P)ETE synthesis to levels similar to collagen alone (Figure 4C).

### Requirement of PGHS-1 or ADP for Collagen and CRP Activation of p12-LOX Product Generation

Although unable to directly activate 12-H(P)ETE synthesis alone (Table), secondary mediators including ADP and U46619, may modulate GPVI-dependent activation.<sup>35,36</sup> Indomethacin (10  $\mu$ mol/L) or aspirin (10 mmol/L) were without effect on collagen stimulation, but caused a significant decrease in the CRP response, suggesting that GPVI activation of 12-H(P)ETE generation may involve signaling by TXA<sub>2</sub> (Figure 5A). Preincubation of platelets with 1 U/mL apyrase caused significant inhibition of collagen or CRP-



**Figure 3.** Tyrosine kinase and PI3-kinase inhibitors block collagen or CRP activation of p12-LOX. A p38-MAP kinase inhibitor partially inhibits CRP-mediated p12-LOX activity. All samples were analyzed for 12-H(P)ETE content as described. A, Staurosporine inhibits collagen or CRP activation of p12-LOX. Platelets were preincubated in the presence of staurosporine (20  $\mu$ mol/L) before adding collagen or CRP. Platelets: a, +collagen; b, +collagen+staurosporine; c, +CRP; d, +CRP+staurosporine. B, Src-family tyrosine kinase inhibition blocks collagen or CRP activation of p12-LOX. Platelets were preincubated with the src-family tyrosine kinase inhibitor, PP2 (0 to 50  $\mu$ mol/L), before adding collagen or CRP. Platelets: a, +collagen; b, +collagen+PP2 (5  $\mu$ mol/L); c, +collagen+PP2 (10  $\mu$ mol/L); d, +collagen+PP2 (50  $\mu$ mol/L); e, +CRP; f, +CRP+PP2 (5  $\mu$ mol/L); g, +CRP+PP2 (10  $\mu$ mol/L); h, +CRP+PP2 (50  $\mu$ mol/L). C, p38-MAP kinase inhibition partially blocks CRP-mediated p12-LOX activation. Platelets were preincubated with the p-38 MAP kinase inhibitor, MKI (100 nmol/L), before addition of collagen or CRP. Platelets: a, +collagen; b, +collagen+MKI; c, +CRP; d, +CRP+MKI. D, PI3-kinase inhibition blocks collagen or CRP activation of p12-LOX. Platelets were preincubated with the PI3-kinase inhibitor, wortmannin (100 nmol/L), before the addition of collagen or CRP. Platelets: a, +collagen; b, +collagen + wortmannin; c, +CRP; d, +CRP+wortmannin. \* $P \leq 0.05$  using unpaired *t* test.

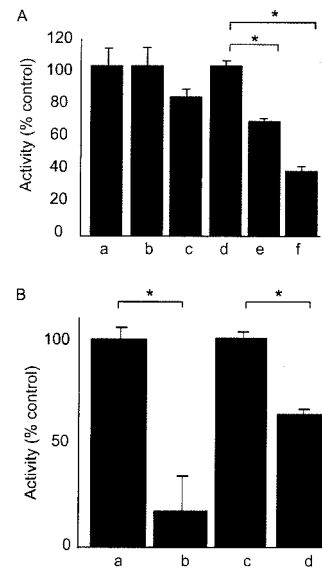


**Figure 4.** PKC inhibition potentiates collagen or CRP-mediated p12-LOX product generation, and enhances U46619 (but not ADP)-mediated p12-LOX activity. All samples were analyzed for 12-H(P)ETE content as described. Platelets were preincubated in the presence of PKC inhibitors chelerythrine chloride or bis-indolymaleimide (both 1 to 10  $\mu\text{mol/L}$ ) before adding collagen or CRP. A, Collagen activation of p12-LOX is potentiated by PKC inhibition. Platelets: a, +collagen; b, +collagen+chelerythrine (1  $\mu\text{mol/L}$ ); c, +collagen+chelerythrine (3  $\mu\text{mol/L}$ ); d, +collagen+chelerythrine (10  $\mu\text{mol/L}$ ); e, +collagen+bis-indolymaleimide (1  $\mu\text{mol/L}$ ); f, +collagen+bis-indolymaleimide (3  $\mu\text{mol/L}$ ); g, +collagen+bis-indolymaleimide (10  $\mu\text{mol/L}$ ). B, CRP activation of p12-LOX is potentiated by PKC inhibition. Platelets: a, +CRP; b, +CRP+chelerythrine (5  $\mu\text{mol/L}$ ); c, +CRP+chelerythrine (10  $\mu\text{mol/L}$ ); d, +CRP+bis-indolymaleimide (5  $\mu\text{mol/L}$ ); e, +CRP+bis-indolymaleimide (10  $\mu\text{mol/L}$ ). C, Bis-indolymaleimide potentiates U46619-mediated (but not ADP-mediated) p12-LOX activity. Platelets: a, +ADP (5  $\mu\text{mol/L}$ ); b, +ADP+bis-indolymaleimide (20  $\mu\text{mol/L}$ ); c, +U46619 (10  $\mu\text{mol/L}$ ); d, +U46619+bis-indolymaleimide (20  $\mu\text{mol/L}$ ). \* $P \leq 0.05$  using unpaired  $t$  test; for C, % control refers to collagen response.

stimulated activity (Figure 5B). However inhibition of the collagen response was greater, suggesting ADP signaling may be more important in collagen activation of 12-H(P)ETE synthesis via receptors other than GPVI.

#### Alterations in 12-H(P)ETE Esterification Do Not Explain Changes in p12-LOX Product Detection

After their generation, H(P)ETEs can be esterified into membrane phospholipids. To examine whether changes in lipid esterification or hydrolysis in the presence of inhibitors



**Figure 5.** Role of secondary mediators in collagen or CRP stimulation of p12-LOX. All samples were analyzed for 12-H(P)ETE content as described. A, PGHS-1 signaling is involved in CRP but not collagen activation of p12-LOX. Platelets were preincubated with either indomethacin (10  $\mu\text{mol/L}$ ) or aspirin (10  $\text{mmol/L}$ ) before adding collagen or CRP. Platelets: a, +collagen; b, +collagen+indomethacin; c, +collagen+aspirin; d, +CRP; e, +CRP+indomethacin; f, +CRP+aspirin. B, Apyrase inhibits collagen or CRP-mediated p12-LOX activation. Platelets were incubated with the ADP scavenger, apyrase (1 U/mL), before adding collagen or CRP. Platelets: a, +collagen; b, +apyrase+collagen; c, +CRP; d, +apyrase+CRP. \* $P \leq 0.05$  using unpaired  $t$  test.

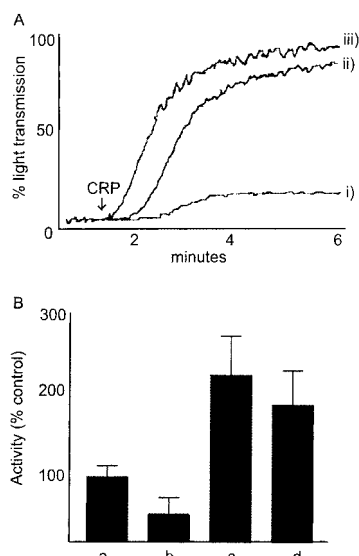
could account for the data herein, samples were analyzed for 12-H(P)ETE before and after alkaline hydrolysis. No significant differences were found for controls or samples treated with PP2, wortmannin, bis-indolymaleimide, or aspirin, indicating that 12-H(P)ETE esterification is not taking place during the time course of our assays (data not shown).

#### Activation of PECAM-1 in Platelets Inhibits CRP-Mediated p12-LOX Activity

PECAM-1 signaling is stimulated by receptor oligomerization, which is achieved in platelets by antibody cross-linking.<sup>37</sup> Stimulation of PECAM-1 before CRP addition caused marked inhibition of platelet aggregation, as previously found (Figure 6A). Similarly PECAM-1 activation inhibited CRP-mediated 12-H(P)ETE synthesis by approximately 50% (Figure 6B). Conversely, isotype control antibody added with cross-linking F(ab')<sub>2</sub> or cross-linking F(ab')<sub>2</sub> added alone caused potentiation of p12-LOX activity. This reveals PECAM-1 as a negative regulator of GPVI-dependent p12-LOX activation.

#### Discussion

Using human platelets, a novel receptor-dependent signaling pathway for 12-H(P)ETE synthesis was characterized that utilizes the collagen receptor GPVI. Requirements for GPVI-activation of p12-LOX included *src*-tyrosine kinases, PI3-kinase, Ca<sup>2+</sup> mobilization, and p12-LOX translocation, whereas PKC and PECAM-1 were inhibitory.



**Figure 6.** PECAM-1 activation inhibits CRP-mediated p12-LOX activation in washed platelets. Washed platelets were incubated with anti-PECAM antibody (AB468), anti-mouse IgG isotype control, and cross-linking F(ab')<sub>2</sub> anti-IgG secondary antibody, activated by CRP, and 12-H(P)ETE levels determined. A, Optical aggregometry of washed platelets ( $4 \times 10^8$ /mL) treated with (i) CRP+anti PECAM-1+F(ab')<sub>2</sub> anti-IgG, (ii) CRP+isotype control+F(ab')<sub>2</sub> anti-IgG, and (iii) CRP alone. B, Washed platelets were incubated with (a) CRP alone, (b) CRP+anti PECAM-1+F(ab')<sub>2</sub> anti-IgG; (c) CRP+isotype control+F(ab')<sub>2</sub> anti-IgG; (d) CRP+F(ab')<sub>2</sub> anti-IgG.

Collagen or CRP activated 12-H(P)ETE synthesis in a dose- and time-dependent manner (Figure 1), implicating FcR $\gamma$  chain-coupled GPVI as a specific p12-LOX-activating pathway in human platelets. Collagen utilizes several cell surface receptors including: GPVI,  $\alpha_2\beta_1$  integrin, GP1b, and bound von Willebrand factor.<sup>38,39</sup> However, activation by CRP indicates that GPVI signaling alone is sufficient to activate p12-LOX. Conversely, thrombin did not activate unless added in amounts far higher than required for platelet aggregation and degranulation. Finally, neither TP receptor signaling nor ADP could activate 12-H(P)ETE generation alone (Table, Figure 5), although U46619 did stimulate in the presence of the PKC inhibitor, bis-indolymaleimide, suggesting PKC may negatively regulate PGHS-1-dependent activation of p12-LOX activity in platelets (Figure 4C).

GPVI is a platelet-specific collagen receptor whose importance in vascular collagen signaling is highlighted by observations that sequence dimorphisms are associated with increased risk of myocardial infarction.<sup>40,41</sup> GPVI closely associates with an ITAM present on the FcR $\gamma$  chain that activates tyrosine kinases via adapter molecules such as SLP-76.<sup>32,42–44</sup> Activating GPVI triggers PI3-kinase turnover, whose products activate phospholipase C $\gamma$ 2 (PLC $\gamma$ 2).<sup>45,46</sup> PLC forms DAG and IP<sub>3</sub>, which activate PKC and Ca<sup>2+</sup> mobilization, respectively.<sup>47,48</sup> GPVI also causes phosphorylation of p38-MAP kinase, which can directly activate platelet cytosolic PLA<sub>2</sub>, a potential source of arachidonate substrate for p12-LOX.<sup>34,49</sup>

To explore requirements for GPVI activation of p12-LOX, signaling inhibitors were also utilized. Potent inhibition of

CRP-mediated 12-H(P)ETE synthesis by BAPTA/AM and EGTA implicated Ca<sup>2+</sup> mobilization, which could activate p12-LOX by stimulation of p12-LOX translocation (Figure 2B).<sup>50,51</sup> In agreement, a translocation inhibitor, L-655238, substantially inhibited GPVI-dependent 12-H(P)ETE synthesis similar to previous reports using collagen (Figure 2D).<sup>15</sup> However, Ca<sup>2+</sup> mobilization is not in itself sufficient for p12-LOX activation, because a concentration of thrombin that did not cause 12-H(P)ETE synthesis caused significantly greater Ca<sup>2+</sup> mobilization than collagen (Figure 2C).

GPVI stimulation of 12-H(P)ETE generation was inhibited by PP2, staurosporine, and wortmannin, implicating *src*-tyrosine kinases and PI3-kinase in p12-LOX activation (Figure 3). Although these pathways are already known to be activated via GPVI, they have not been previously reported to activate H(P)ETE synthesis in mammalian cells, and provide novel insights into intracellular signaling pathways that modulate LOX product formation. In contrast, an inhibitor of p38 MAP kinase (MKI) did not inhibit collagen, and only slightly inhibited CRP-dependent H(P)ETE generation (Figure 3C).

Secondary mediators such as TXA<sub>2</sub> and ADP are involved in collagen signaling in platelets and were found to variably regulate collagen or CRP activation of p12-LOX (Figure 5).<sup>52</sup> Apyrase significantly inhibited CRP and collagen-mediated 12-H(P)ETE synthesis, but with a greater effect on collagen activation (Figure 5B). Indomethacin or aspirin partially blocked CRP but not collagen-mediated 12-H(P)ETE generation (Figure 5A). Collectively, this indicates that stimulation of H(P)ETE synthesis via GPVI involves TP signaling, but collagen activation may involve additional receptors signaling independently of the TP receptor and a greater involvement of ADP.<sup>38,39</sup>

Two PKC inhibitors dose-dependently enhanced collagen/CRP-mediated 12-H(P)ETE synthesis, indicating that PKC negatively regulates GPVI-dependent p12-LOX product generation (Figure 4A). Also, PKC inhibition potentiated U46619 but not ADP stimulation of 12-H(P)ETE generation (Figure 4C). It has been previously shown that PKC is required for U46619 but not ADP-induced platelet activation.<sup>53</sup> Inhibition by PKC may involve cross-talk between PKC $\delta$  or  $\theta$  and Bruton's or Fyn tyrosine kinases in platelets, which was recently described.<sup>54–56</sup>

In the vasculature, the balance between two distinct signaling motifs, termed ITAM and ITIM, has impact on regulating vascular cell function.<sup>57</sup> In platelets, ITAM present on the FcR $\gamma$  chain coupled to GPVI is activated through phosphorylation by *src*-family tyrosine kinases. This is inhibited by proteins possessing ITIM, for example, PECAM-1.<sup>58,59</sup> Herein, cross-linking studies utilizing antibodies specific for PECAM-1 demonstrated substantial suppression of GPVI-stimulation of 12-H(P)ETE synthesis. Unexpectedly, isotype controls (omitting anti-PECAM-1 antibodies), showed that cross-linking antibodies alone could potentiate CRP stimulation of 12-H(P)ETE synthesis. Although not understood, this may result from interactions of F(ab')<sub>2</sub> anti-IgG secondary antibody with sites on the platelet surface unrelated to PECAM-1. The observation that cross-linking using specific anti-PECAM-1 antibody markedly inhibits GPVI-mediated 12-H(P)ETE synthesis demonstrates a novel

function for PECAM-1 and a role for ITIM/ITAM-containing immune receptors in modulating 12-H(P)ETE synthesis (Figure 6).

This study presents the first detailed intracellular characterization of a specific receptor-dependent signaling pathway that acutely activates LOX turnover in mammalian cells, and provides a novel link between immune receptor signaling and lipid oxidation in the vasculature.

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