Protease-Activated Receptor Signaling in Platelets Activates Cytosolic Phospholipase A$_{2\alpha}$ Differently for Cyclooxygenase-1 and 12-Lipoxygenase Catalysis

Michael Holinstat, Olivier Boutaud, Patrick L. Apopa, Joanne Vesci, Manju Bala, John A. Oates, Heidi E. Hamm

Objective—The rate-limiting step in the biosynthesis of thromboxane A$_2$ (TxA$_2$) and 12-hydroxyeicosatetraenoic acid (12-HETE) by platelets is activation of cytosolic phospholipase A$_{2\alpha}$ (cPLA$_{2\alpha}$), which releases arachidonic acid, which is the substrate for cyclooxygenase-1 (COX-1) and 12-lipoxygenase. We evaluated signaling via the human platelet thrombin receptors, protease-activated receptor (PAR) 1 and PAR4, to the activation of cPLA$_{2\alpha}$, which provides a substrate for the biosynthesis of TxA$_2$ and 12-HETE.

Methods and Results—Stimulating washed human platelets resulted in delayed biosynthesis of 12-HETE, which continues after maximal formation of TxA$_2$ is completed, suggesting that 12-HETE is not formed by the same pool of arachidonic acid that provides a substrate to COX-1. PAR1-induced formation of TxA$_2$ was inhibited by the phosphatidylinositol kinase inhibitor LY294002, whereas this inhibitor did not block 12-HETE biosynthesis. Both 1-butanol and propranolol also blocked TxA$_2$ biosynthesis but did not inhibit 12-HETE formation.

Conclusion—The concerted evidence indicates that the platelet thrombin receptors signal activation of cPLA$_{2\alpha}$ coupled to COX-1 by a pathway different from that signaling activation of the cPLA$_{2\alpha}$ coupled to 12-lipoxygenase. (Arterioscler Thromb Vasc Biol. 2011;31:435-442.)

Key Words: aspirin • eicosanoids • thrombosis • 12-lipoxygenase • cyclooxygenase-1 • cPLA2

The importance of thromboxane A$_2$ (TxA$_2$) in arterial thrombosis is indicated by the reduction in myocardial infarction and stroke that results from inhibiting TxA$_2$ biosynthesis with aspirin. In patients with known occlusive vascular disease, aspirin treatment decreases serious vascular events (nonfatal myocardial infarction, nonfatal stroke, and vascular death) by about a quarter.1,2 TxA$_2$ released by activated platelets acts to recruit additional platelets to the thrombus, and it contributes to stabilizing the thrombus. TxA$_2$ biosynthesis is known to be activated by signaling from both of the platelet thrombin receptors, protease-activated receptor (PAR) 1 and PAR4.3-6

The rate-limiting step in TxA$_2$ biosynthesis by platelets is activation of cytosolic phospholipase A$_{2\alpha}$ (cPLA$_{2\alpha}$) with production of free arachidonic acid (AA) as substrate for cyclooxygenase-1 (COX-1). The product of COX-1, prostaglandin H$_2$, is then metabolized to TxA$_2$ by thromboxane synthase. Our investigation of a patient with compound heterozygous null mutations of the cPLA$_{2\alpha}$ gene (PLA2G4A) demonstrated unequivocally that virtually all (\(\geq 95\%\)) of TxA$_2$ generated by platelet activation is derived from cPLA$_{2\alpha}$.

Another major eicosanoid released by platelet activation is 12-hydroxyeicosatetraenoic acid (12-HETE), a product of the oxygenation of AA by 12-lipoxygenase (12-LOX). A number of investigations have suggested autodoid effects of 12-HETE on the platelet, as well as actions on the endothelium and vascular smooth muscle.8-16 As is the case with TxA$_2$ biosynthesis, virtually all of the AA substrate for platelet 12-LOX is derived from activation of cPLA$_{2\alpha}$.7 12-HETE is released by collagen and also by higher concentrations of thrombin.12,17 To better understand the effect of thrombin on 12-HETE release, we have characterized and compared signaling of its 2 receptors, PAR1 and PAR4, with the activation of cPLA$_{2\alpha}$ coupled to 12-LOX.

Here we present evidence that the platelet thrombin receptors signal activation of the cPLA$_{2\alpha}$ coupled to COX-1 by a pathway different from that signaling activation of cPLA$_{2\alpha}$ coupled to 12-LOX. These findings indicate that the mechanisms providing AA to COX-1 and to 12-LOX are different.

Methods

Human Platelets

Human platelets were obtained from volunteers within the Thomas Jefferson University and Vanderbilt University communities. Studies were approved by the Thomas Jefferson and Vanderbilt institutional

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review boards. Informed consent was obtained from all individuals before blood donation. All experiments were performed on washed platelets, prepared as described previously.16,19 Unless otherwise noted, all experiments were conducted with a platelet concentration of 2 × 10⁸ platelets/mL and agonist concentrations corresponding to maximal receptor activation with thrombin, PAR1-activating peptide (AP) (SFLLRN) or PAR4-AP (AYPGKF) (10 nmol/L, 20 μmol/L, and 200 μmol/L, respectively) based on previous studies.16,19

Platelet Aggregation and Secretion
Platelets were treated with or without 100 μmol/L aspirin for 40 minutes and 100 nmol/L SQ29548 (SQ) for 5 minutes before measurement of aggregation by light transmission (Lumi-Aggregometer model 700, Chronolog Corp) in response to thrombin, PAR1-AP, or PAR4-AP.

Time Course for Eicosanoid Formation
Activated platelets were quenched at various times by the addition of an equal volume of pH 3 water containing 0.5 ng of [²H₄]12-HETE and 2 ng of [²H₄]TxB₂ as internal standards to the platelets. The prostanoids were extracted by adding 1 mL of ethyl acetate and vortexing. The organic phase was separated by centrifugation at 10,000 × g for 5 minutes at 4°C, collected, and stored at −20°C until it was analyzed by mass spectrometry.

Assay of Residual COX-1 Activity
Washed platelets were activated with either PAR1-AP (20 μmol/L) or PAR4-AP (200 μmol/L) for 1 minute. At this time, [²H₄]AA (20 μmol/L) was added, and platelets were incubated at 37°C for 2 minutes. The reaction was stopped by addition of an equal volume of pH 3 water containing 2 ng of [²H₄]TxB₂ as an internal standard to the platelets. The [²H₄]TxB₂ was extracted as described above for the time course experiment and analyzed by mass spectrometry as described below. For each experiment, a control was done in which the same concentration of [²H₄]AA was added without preactivation of the platelets with a PAR agonist peptide. This condition was used to determine the initial activity of the enzyme.

Measurement of 12-HETE
Secretion of 12-HETE was measured from platelet supernatants by liquid chromatography-atmospheric pressure chemical ionization-tandem mass spectrometry (LC/APCI/MS/MS) following addition of an internal standard (2 ng of [²H₄]12-HETE) as described previously.20 The concentration of 12-HETE was determined by isotopic dilution.

Measurement of TxB₂
Because TxA₂ is a relatively unstable metabolite, the stable form, thromboxane B₂ (TxB₂), was measured as a surrogate for TxA₂ formation in human platelets. [²H₄]TxB₂ (2 ng) was added to samples as an internal standard. Prostaglandins were measured by gas chromatography-negative chemical ionization-mass spectrometry (GC/NICI/MS) as described previously.21 The concentration of TxB₂ was determined by isotopic dilution.

Measurement of p38-Mitogen-Activated Protein Kinase
Platelets were stimulated with thrombin or PAR1-AP for the indicated times (from 0 to 10 minutes), lysed with 3× sample buffer (93.72 mmol/L Tris-HCl pH 6.8, 3% SDS, 37.45% glycerol, 0.01% bromophenol blue, 7.5% β-mercaptoethanol), boiled for 10 minutes, and analyzed for total and phosphorylated p38-mitogen-activated protein kinase (MAPK) by Western blot analysis using a LI-COR Odyssey system.

Statistical Analysis
Comparison between experimental groups was made using a t test from the Prism software package. Differences in mean values were considered significant at P<0.05. For comparisons of eicosanoid formation in the different tables, normal distribution was determined by the Shapiro-Wilk test. If data were found to be normally distributed, analyses were done by 1-way ANOVA followed by post-test analyses by the Bonferroni multiple comparison. If the data were found not to be normally distributed, statistical significance was analyzed by 1-way ANOVA with the Kruskal-Wallis test followed by post-test analysis by the Dunn multiple comparison.

Results

TxB₂ and 12-HETE Biosynthesis Proceed at Different Rates Following PAR Activation

The kinetics of formation of TxB₂ and 12-HETE were measured following activation with either the endogenous agonist thrombin or direct activation of the PARs with PAR1-activating peptide (PAR1-AP) or PAR4-AP19 (Figure 1). Formation of 12-HETE was considerably and significantly delayed compared with that of TxB₂ following activation of platelets with PAR1 (P<0.0001), PAR4 (P<0.0005), and thrombin (P<0.005). Importantly, whereas PAR1-induced TxB₂ formation achieved maximal levels within 15 seconds, 12-HETE biosynthesis continued long after this, reaching its maximum only at 120 seconds. Moreover, the residual COX-1 activity at 1 minute after activation with PAR1-AP or PAR4-AP was 55.5±6.6% and 88.7±7.5%, respectively, indicating that the enzyme was still active even at this later time (Supplemental Figure 1, available online at http://atvb.ahajournals.org). This evidence in conjunction with the fact that 12-HETE production continues well after TxB₂ production is maximal indicates that the pool of cPLA₂α-derived AA that was the substrate for COX-1 had been depleted at a point when AA continued to be provided by cPLA₂α coupled to 12-LOX.

PAR1-AP-mediated TxB₂ formation reached 100% within the first 15 seconds following agonist stimulation, whereas PAR4-AP required more than 75 seconds to achieve maximal levels. The kinetics for thrombin-mediated TxB₂ formation was biphasic, with a steep early phase similar to that observed with PAR1-AP and a shallow secondary phase similar to the kinetics observed with PAR4-AP (Figure 1), supporting the hypothesis that in humans the effect of thrombin is mediated by activation of both receptors. The time of maximum formation of 12-HETE did not differ between PAR1 and PAR4 (Figure 1). Interestingly, there was a lag in the formation of 12-HETE induced by PAR4-AP in contrast to the immediate formation of the eicosanoid mediated by PAR1-AP, suggesting an initial fast response of the platelets through PAR1 activation with a delayed response mediated by PAR4.

TxB₂ formation mediated by PAR1-AP and that mediated by PAR4-AP each represent ~50% of TxB₂ levels following platelet activation by thrombin, suggesting that production of TxB₂ by thrombin is mediated by the additive activation of the 2 PARs. In contrast, PAR4-AP induced a significantly higher level of 12-HETE formation compared with PAR1-AP (Table 1). The amount of 12-HETE formed by thrombin exceeded the sum of that formed by PAR1 plus PAR4, suggesting a synergistic effect between the 2 receptors.
Phosphatidylinositol Kinase Products Mediate PAR-Induced Thromboxane Biosynthesis But Not Formation of 12-HETE

To investigate the participation of the phosphatidylinositol (PI) kinases in activating cPLA2 coupled to COX-1 and 12-LOX, platelets were pretreated with increasing concentrations of the PI kinase inhibitor LY294002 (LY), which was shown previously to completely inhibit PAR-mediated formation of both phosphatidylinositol (4,5)-biphosphate (PI[4,5]P2) and phosphatidylinositol (3,4,5)-triphosphate (PI[3,4,5]P3) in platelets. Increasing the concentration of LY resulted in a complete inhibition of TxB2 formation, with an IC50 of approximately 25 μmol/L LY for both PAR1-AP and PAR4-AP (Figure 2a and 2b). In contrast, LY increased 12-HETE formation by 2-fold in a dose-dependent fashion following platelet activation with both PAR1-AP and PAR4-AP. To ascertain that the effects of LY on thromboxane production were not due to direct inhibition of COX-1, we monitored TxB2 production when [3H8]AA was provided to the platelets (Figure 2c). Our results clearly demonstrated that LY at a concentration of 100 μmol/L had no significant effect on either enzyme. Taken together, these results indicate that following PAR signaling, products of the PI kinases are required for activation of the cPLA2 coupled to COX-1 but not for activation of the cPLA2, that provides substrate for 12-LOX.

1-Butanol and Propranolol Inhibit PAR1-Induced Thromboxane Biosynthesis But Not Formation of 12-HETE

Treatment with the lipid signaling inhibitors 1-butanol or propranolol each produced a marked and significant inhibition of TxA2 formation following stimulation with thrombin, PAR1-AP, or PAR4-AP (P < 0.001) (Figure 2b). By contrast, PAR1-induced 12-HETE formation was unaffected by either propranolol or 1-butanol. Only with thrombin signaling did propranolol induce a slight but significant change in 12-HETE levels (P < 0.05), and 1-butanol caused a decrease in 12-HETE only with PAR4-AP (P < 0.001). This evidence, particularly the differences seen with PAR1 signaling, further supports the concept that separate signaling pathways may regulate the activity of the cPLA2, that is coupled to formation of these 2 eicosanoids.

Protein Kinase C Stimulates Platelet Activation But Does Not Contribute to PAR-Mediated Activation of cPLA2

Protein kinase C (PKC) is activated by PARs and has been reported to directly phosphorylate cPLA2. Aggregation and eicosanoid formation were measured by direct addition of 12-myristate-13-acetate (PMA) (a diacylglycerol mimetic known to activate PKC) to the washed platelets (Supplemental Figure II). Although addition of PMA was sufficient to induce platelet aggregation, it was unable to induce either calcium mobilization or downstream eicosanoid formation even at high concentrations (Figure 3; Table 1).

Intracellular Calcium Contributes to PAR-Induced Eicosanoid Formation

Reduction of cytosolic calcium with 1,2-Bis(2-aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid (BAPTA) markedly inhibited the TxA2 biosynthesis that was induced by thrombin, and by the PAR1 and PAR4 agonists (Table 2).
BAPTA also inhibited the production of 12-HETE by these agonists, but to a lesser extent.

**Thromboxane Prostanoid (TP) Receptor Signaling Contributes to PAR-Induced Eicosanoid Formation**

We have previously shown that TxA₂ formed during the first phase of activation stimulates a secondary wave of TxA₂ biosynthesis through autocrine activation of the TP receptor.7 To investigate the role of the initial biosynthesis of TxA₂ on eicosanoid formation and platelet function, we pretreated washed platelets with or without aspirin (a cyclooxygenase inhibitor) or SQ (a TP antagonist) before stimulation with thrombin, PAR1-AP or PAR4-AP (Figure 3). As expected, treatment with aspirin completely inhibited TxB₂ formation by thrombin, PAR1-AP and PAR4-AP. SQ-treated platelets, which are able to directly form TxB₂ following PAR activation but are unable to initiate a second wave of activation through the TP receptor, show a significant decrease in total TxB₂ formed; thrombin and PAR4-AP-mediated TxB₂ formation was decreased by 40%, whereas PAR1-AP-mediated TxB₂ formation was inhibited by approximately 80% (Figure 3a). 12-HETE formation in the presence of aspirin and SQ also showed a differential effect based on type of activation (Figure 3b). In both thrombin and PAR4-AP conditions, treatment with SQ had only a partial inhibitory effect on thrombin, PAR1-AP or PAR4-AP (Figure 3).

<table>
<thead>
<tr>
<th>Table 1. Eicosanoid Formation in Human Platelets</th>
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<tr>
<td>Eicosanoid</td>
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<tr>
<td>TxB₂, ng/mL (n)</td>
</tr>
<tr>
<td>12-HETE, ng/mL (n)</td>
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</table>

Levels of eicosanoid formation were measured in human platelets following stimulation with 10 nmol/L thrombin, 20 μmol/L PAR1-AP, 200 μmol/L PAR4-AP, or 1 μmol/L PMA for 10 minutes. Eicosanoid levels at baseline (without stimulation) were subtracted from each eicosanoid value. Statistical significance was analyzed by 1-way ANOVA with Kruskal-Wallis test followed by post-test analysis by Dunn’s multiple comparison.

*P<0.05 compared with thrombin; †PAR1-AP vs PAR4-AP not significantly different; ‡P<0.05 for PAR1-AP vs PAR4-AP.

Figure 2. Regulation of PAR-mediated eicosanoid formation and aggregation by lipid signaling pathways. Platelets were stimulated with 10 nmol/L thrombin, 20 μmol/L PAR1-AP, or 200 μmol/L PAR4-AP, and subsequent eicosanoid formation, platelet aggregation and dense granule secretion were measured. a, Aggregation, TxB₂ and 12-HETE levels were measured following stimulation with PAR1-AP or PAR4-AP in platelets treated with increasing concentrations of LY from 0 to 250 μmol/L. b, Washed platelets were treated with 100 μmol/L LY, 0.4% 1-butanol (1-ButOH), 100 μmol/L propranolol, or vehicle followed by stimulation for 10 minutes with thrombin, PAR1-AP, or PAR4-AP. c, Washed platelets were treated with 100 μmol/L LY or vehicle followed by stimulation for 10 min with 10 μmol/L arachidonic acid (AA). Eicosanoid formation was then measured in each condition tested. Eicosanoid formation was then measured in each condition tested. TxB₂ and 12-HETE: Bars represent mean±SEM (P<0.001 and P<0.05 for TxB₂ and 12-HETE, respectively, by repeated-measures ANOVA; *P<0.05, ***P<0.001 compared with control condition by the Dunnnett multiple comparison test). NS indicates not significant.
12-HETE formation, and aspirin treatment resulted in an increase in 12-HETE formation. In contrast, PAR1-AP activated platelets showed a significant decrease in 12-HETE formation in the presence of either SQ or aspirin, indicating that TxA2 feedback on TP mediates the production of 12-HETE following PAR1 (Table 3).

To determine whether the observed differences in regulation of eicosanoid formation might be related to the phases of platelet activation, platelet aggregation was measured in the presence of aspirin or SQ following stimulation with increasing concentrations of thrombin, PAR1-AP, PAR4-AP and PAR4-AP (Figure 4). Treatment with aspirin or SQ did not alter either maximal or stable platelet aggregation induced by PAR1-AP, whereas thrombin and PAR1-AP-stimulated platelet aggregation was significantly attenuated by aspirin or SQ at lower concentrations of each agonist.

The Time Course of p38 MAPK Phosphorylation
cPLA2 can be phosphorylated by isozymes of the MAPK family, such as extracellular signal–regulated kinases and p38. We therefore sought to determine whether the time course for PAR-mediated activation of MAPK would correlate with the time course of eicosanoid production. Our results show that detectable p38-MAPK phosphorylation is initiated after 30 seconds following thrombin or PAR stimulation and reaches maximum levels within 5 minutes post-stimulation (Figure 5). This is not concordant with the rapid release of TxA2, particularly that in response to PAR1-AP, and it is consistent with the findings of Kuliopulos et al demonstrating no effect of a p38 MAPK inhibitor on TxA2-dependent collagen-induced aggregation.

Discussion
In the platelet, the same enzyme, phospholipase, cPLA2 (PLA2G4A), delivers AA both to COX-1 for formation of TxA2 and to 12-LOX for formation of 12-HETE, as was demonstrated by the virtual absence of agonist-induced biosynthesis of both of these eicosanoids in a patient with null mutations of cPLA2. Thus, it was of considerable interest to find that PAR-induced signaling to activate the cPLA2 coupled to COX-1 is quite different from PAR-induced signaling to the cPLA2 coupled to 12-LOX. Three lines of evidence delineate the difference between these 2 cPLA2 activation pathways in the platelet: (1) 12-HETE formation continues well after TxB2 biosynthesis has ceased, (2) PI kinases participate in the lipid signaling pathway for TxA2 production but not for 12-HETE, and (3) 1-butanol and propranolol inhibit PAR1-induced formation of TxB2 but do not block 12-HETE production.

The rate of PAR1-induced biosynthesis of TxA2 is markedly greater than that of 12-HETE. Maximal formation of TxA2 is reached in ~15 seconds after the PAR1 peptide, whereas formation of 12-HETE increases gradually over 120 seconds. These differences between the rates of formation and the time at which maximal formation is achieved are very large. However, when PAR1 stimulation is stopped (Figure 5), the 12-HETE decrease is rapid (~10 seconds), whereas the TxB2 measurement shows a long plateau of 120 seconds. The reason for this is not known, although it is possible that the 12-HETE measurement may reflect some time delay in the detection system.

Table 2. Calcium-Dependent Eicosanoid Biosynthesis by Human Platelets

<table>
<thead>
<tr>
<th>Eicosanoids</th>
<th>Thrombin</th>
<th>PAR1-AP</th>
<th>PAR4-AP</th>
</tr>
</thead>
<tbody>
<tr>
<td>TxB2, % inhibition by BAPTA (n)</td>
<td>89 (9)*</td>
<td>93 (9)*</td>
<td>95 (9)*</td>
</tr>
<tr>
<td>12-HETE, % inhibition by PA (n)</td>
<td>81 (9)</td>
<td>57 (9)*</td>
<td>79 (9)*</td>
</tr>
</tbody>
</table>

Eicosanoids were measured in human platelets following stimulation with 10 nmol/L thrombin, 20 μmol/L PAR1-AP, or 200 μmol/L PAR4-AP for 10 minutes. Eicosanoids produced in presence of 20 μmol/L BAPTA are expressed as percentage inhibition compared with control conditions (no BAPTA present) and represent the calcium-dependent eicosanoid biosynthesis. Statistical significance was analyzed by repeated ANOVA (P<0.0001) followed by post-test analysis by Bonferroni’s multiple comparison.

Table 3. TP-Dependent Eicosanoid Biosynthesis by Human Platelets

<table>
<thead>
<tr>
<th>Eicosanoids</th>
<th>Thrombin</th>
<th>PAR1-AP</th>
<th>PAR4-AP</th>
</tr>
</thead>
<tbody>
<tr>
<td>TxB2, % inhibition by SQ (n)</td>
<td>40 (8)*</td>
<td>79 (8)*</td>
<td>40 (8)*</td>
</tr>
<tr>
<td>12-HETE, % inhibition by SQ (n)</td>
<td>NS (8)</td>
<td>60 (8)*</td>
<td>38 (8)*</td>
</tr>
</tbody>
</table>

Eicosanoids were measured in human platelets following stimulation with 10 nmol/L thrombin, 20 μmol/L PAR1-AP, or 200 μmol/L PAR4-AP for 10 minutes. Eicosanoids produced in presence of the TP receptor antagonist were expressed as percent inhibition compared to control condition (no antagonist present) and represent the TP-dependent eicosanoid biosynthesis. Data were analyzed by repeated ANOVA (P<0.005) followed by post-test analysis by Dunn’s multiple comparison.

Difference with eicosanoid in control was significant: *P<0.05, †P<0.0005, ‡P<0.0001. NS indicates not significant.
similar to those previously reported for the formation of TxA2 and 12-HETE following collagen-induced platelet activation. It is clear from the persisting 12-HETE formation long after TxA2 production has ceased that the AA substrate is not provided by the same mechanisms to COX-1 and to 12-LOX after PAR1 activation.

LY249002, a PI kinase inhibitor that blocks formation of PI(4,5)P2 and PI(3,4,5)P3, markedly inhibits PAR1 and PAR4-induced biosynthesis of TxA2. In contrast, LY does not inhibit PAR1 or PAR4-induced formation of 12-HETE. Indeed, LY actually increases PAR1-induced 12-HETE formation. Also, our results indicate that LY does not have direct effects on the enzymatic activities of either COX-1 or TxA2 synthase. Thus, the results of the studies with LY also indicate that the PAR signaling that activates cPLA2/H9251 coupled to COX-1 is different from the PAR signaling to the cPLA2/H9251 coupled to 12-LOX.

Further evidence that the PAR signaling pathway activating the cPLA2α coupled to COX-1 is discrete from that signaling to 12-LOX coupled cPLA2α is derived from the results of the studies with 1-butanol and propranolol. Neither 1-butanol nor propranolol inhibits PI kinases. Thus, inhibition of the activation of the cPLA2 coupled to COX-1 by these agents occurs at a site in the signaling pathway different from the PI kinases. Importantly, neither 1-butanol nor propranolol inhibits PAR1-induced activation of the cPLA2α coupled to 12-LOX, thus providing additional evidence that this signaling pathway differs from that leading to activation of the cPLA2 coupled to COX-1.

The intracellular localization of COX-1 and 12-LOX is pertinent to a consideration of differential regulation of the
cPLA<sub>2α</sub> coupled to each of the enzymes. Platelet COX-1 is localized to the intracellular membrane complex with characteristics of the endoplasmic reticulum that is referred to as the dense tubular system. On the other hand, the 12-LOX is predominantly localized in the cytosol of rat platelets that have been isolated so as to minimize ex vivo activation, and it undergoes calcium- and thrombin-dependent translocation to a membrane fraction, from which it can be coimmunoprecipitated with cPLA<sub>2α</sub>. Thus, the differences between PAR-induced signaling to the cPLA<sub>2α</sub> providing substrate to COX-1 and that coupled to 12-LOX may occur in the context of localization of the 2 oxygenases at separate sites in the platelet. cPLA<sub>2α</sub> activity is regulated by several factors in cells, including interaction with membrane lipids and phosphorylation. Although these mechanisms have been well described in vitro, their relevance in vivo is still debated. Our results suggest that, in human platelets, a product of the PI <sub>3,4,5</sub>P<sub>3</sub> pathway that activate the cPLA<sub>2α</sub> activity is regulated by several factors in cells, including interaction with membrane lipids and phosphorylation. Although these mechanisms have been well described in vitro, their relevance in vivo is still debated. Our results suggest that, in human platelets, a product of the PI <sub>3,4,5</sub>P<sub>3</sub> pathway activates the cPLA<sub>2α</sub> activity is regulated by several factors in cells, including interaction with membrane lipids and phosphorylation. Although these mechanisms have been well described in vitro, their relevance in vivo is still debated. Our results suggest that, in human platelets, a product of the PI <sub>3,4,5</sub>P<sub>3</sub> pathway.

In conclusion, the PAR-induced signaling pathway that activates the cPLA<sub>2α</sub> that provides AA to COX-1 is different from that activating the cPLA<sub>2α</sub> that liberates substrate for 12-LOX. Thus, both COX-1 and 12-LOX are functionally coupled to a discrete subset of platelet cPLA<sub>2α</sub> that provides AA separately to each of these oxygenases.

**Acknowledgments**

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**Disclosures**

None.

**References**


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Supplemental Material

Michael Holinstat et al. PAR signaling in platelets activates cPLA₂ differently for COX-1 and 12-LOX catalysis

Legends for Supplementary Figures

Figure I: residual COX-1 activity following platelet activation with PAR-APs.
Platelets were activated with PAR1-AP (20 µM), PAR4-AP (200 µM) or vehicle for 1 min. At this time [²H₈] arachidonic acid (20 µM) was added. After 2 min, the reaction was stopped as described in material and methods and TxB₂ was analyzed by mass spectrometry. *: p < 0.05 with control (ANOVA with Dunn’s multiple comparison test).

Figure II: PMA induces platelet aggregation but not release of eicosanoids.
a) Platelets were treated with 100 nM PMA, 1000 nM PMA or vehicle and aggregation was measured for 10 minutes. PMA induced full platelet aggregation at both concentrations tested (N=7). b) Platelet calcium mobilization was measured following stimulation with 20 nM thrombin, 40 µM PAR1-AP, 400 µM PAR4-AP or 1000 nM PMA at the EC₁₀₀ for calcium mobilization. Thrombin, PAR1-AP and PAR4-AP showed their characteristic curves for calcium mobilization, while PMA was unable to induce calcium mobilization even at a high concentration.
Figure I

Residual COX-1 activity

Ctrl  PAR1-AP  PAR4-AP