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

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Abstract

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.

Keywords

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

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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 (≥95%) of TxA_2 generated by platelet activation is derived from $cPLA_{2\alpha}$.[7]

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 autocoid 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 review boards. Informed consent was obtained from all individuals before blood donation. All experiments were performed on washed platelets, prepared as described previously.[18,19] Unless otherwise noted, all experiments were conducted with a platelet concentration of 2×10^8 platelets/mL and agonist concentrations corresponding to maximal receptor activation with thrombin, PAR1-AP (SFLLRN) or PAR4-AP (AYPGKF) (10 nmol/L, 20 μmol/L, and 200 μmol/L, respectively) based on previous studies.[18,19]

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 $[^2H_8]$12-HETE and 2 ng of $[^2H_4]TxB_2$ 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 10,000g 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, [^{2}H$_8$]AA (20 μmol/L) was added, and platelets were incubated at 37° for 2 minutes. The reaction was stopped by addition of an equal volume of pH 3 water containing 2 ng of [^{2}H$_4$]TxB$_2$ as an internal standard to the platelets. The [^{2}H$_8$]TxB$_2$ 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 [^{2}H$_8$]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 LC/APCI/MS/MS following addition of an internal standard (2 ng of [^{2}H$_8$]12-HETE) as described previously.[20] The concentration of 12-HETE was determined by isotopic dilution.

Measurement of TxB$_2$

Because TxA$_2$ is a relatively unstable metabolite, the stable form, thromboxane B$_2$ (TxB$_2$), was measured as a surrogate for TxA$_2$ formation in human platelets. [^{2}H$_4$]TxB$_2$ (2 ng) was added to samples as an internal standard. Prostaglandins were measured by GC/NICI/MS as described previously.[21] The concentration of TxB$_2$ 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

TxB$_2$ and 12-HETE Biosynthesis Proceed at Different Rates Following PAR Activation

The kinetics of formation of TxB$_2$ 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-AP[19] (Figure 1).

Formation of 12-HETE was considerably and significantly delayed compared with that of TxB$_2$ following activation of platelets with PAR1 ($P<0.0001$), PAR4 ($P<0.0005$), and thrombin ($P<0.005$). Importantly, whereas PAR1-induced TxB$_2$ 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 I, available online at http://atvb.ahajournals.org). This evidence in conjunction with the fact that 12-HETE production continues well after TxB_2 production is maximal indicates that the pool of $cPLA_{2\alpha}$-derived AA that was the substrate for COX-1 had been depleted at a point when AA continued to be provided by $cPLA_{2\alpha}$ coupled to 12-LOX.

PAR1-AP-mediated TxB_2 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 TxB_2 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.

TxB_2 formation mediated by PAR1-AP and that mediated by PAR4-AP each represent ≈50% of TxB_2 levels following platelet activation by thrombin, suggesting that production of TxB_2 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 $cPLA_{2\alpha}$ coupled to COX-1 and 12-LOX,[22,23] 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 $PI(4,5)P_2$ and $PI(3,4,5)P_3$ in platelets.[24] Increasing the concentration of LY resulted in a complete inhibition of TxB_2 formation, with an IC_{50} 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 TxB_2 production when $[^2H_8]$AA was provided to the platelets (Figure 2c). In these conditions, the $cPLA_{2\alpha}$ is bypassed, and the production of eicosanoids is directly proportional to the residual activities of the enzymes catalyzing the production of TxB_2 (COX-1 and TxA_2 synthase). 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 $cPLA_{2\alpha}$ coupled to COX-1 but not for activation of the $cPLA_{2\alpha}$ 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 TxA_2 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 $cPLA_{2\alpha}$ that is coupled to formation of these 2 eicosanoids.

Protein Kinase C Stimulates Platelet Activation But Does Not Contribute to PAR-Mediated Activation of $cPLA_{2\alpha}$

Protein kinase C (PKC) is activated by PARs and has been reported to directly phosphorylate $cPLA_2$.[25] Aggregation and eicosanoid formation were measured by direct addition of 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[26] (Figure 3; Table 1).

Intracellular Calcium Contributes to PAR-Induced Eicosanoid Formation

Reduction of cytosolic calcium with 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.

TP Receptor Signaling Contributes to PAR-Induced Eicosanoid Formation

We have previously shown that TxA_2 formed during the first phase of activation stimulates a secondary wave of TxA_2 biosynthesis through autocrine activation of the TP receptor.[7] To investigate the role of the initial biosynthesis of TxA_2 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 TxB_2 formation by thrombin, PAR1-AP and PAR4-AP. SQ-treated platelets, which are able to directly form TxB_2 following PAR activation but are unable to initiate a second wave of activation through the TP receptor, show a significant decrease in total TxB_2 formed; thrombin and PAR4-AP-mediated TxB_2 formation was decreased by 40%, whereas PAR1-AP-mediated TxB_2 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 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 TxA_2 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 nmol/L thrombin, PAR1-AP, or PAR4-AP (Figure 4). Treatment with aspirin or SQ did not alter either maximal or stable platelet aggregation induced by PAR4-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

$cPLA_{2\alpha}$ can be phosphorylated by isozymes of the MAPK family, such as extracellular signal–regulated kinases and p38.[27] 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 TxA_2, 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 TxA_2-dependent collagen-induced aggregation.[28]

Discussion

In the platelet, the same enzyme, phospholipase, $cPLA_{2\alpha}$ (PLA2G4A), delivers AA both to COX-1 for formation of TxA_2 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 $cPLA_{2\alpha}$.[7] Thus, it was of considerable interest to find that PAR-induced signaling to activate the $cPLA_{2\alpha}$ coupled to COX-1 is quite different from PAR-induced signaling to the $cPLA_{2\alpha}$ coupled to 12-LOX. Three lines of evidence delineate the difference between these 2 $cPLA_{2\alpha}$ activation pathways in the platelet: (1) 12-HETE formation continues well after TxB_2 biosynthesis has ceased, (2) PI kinases participate in the lipid signaling pathway for TxA_2 production but not for 12-HETE, and (3) 1-butanol and propranolol inhibit PAR1-induced formation of TxB_2 but do not block 12-HETE production.

The rate of PAR1-induced biosynthesis of TxA_2 is markedly greater than that of 12-HETE. Maximal formation of TxA_2 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 similar to those previously reported for the formation of TxA_2 and 12-HETE following collagen-induced platelet activation.[29] It is clear from the persisting 12-HETE formation long after TxA_2 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 both $PI(4,5)P_2$ and $PI(3,4,5)P_3$,[24] markedly inhibits PAR1 and PAR4-induced biosynthesis of TxA_2. 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 TxA_2 synthase. Thus, the results of the studies with LY also indicate that the PAR signaling that activates $cPLA_{2\alpha}$ coupled to COX-1 is different from the PAR signaling to the $cPLA_{2\alpha}$ coupled to 12-LOX.

Further evidence that the PAR signaling pathway activating the $cPLA_{2\alpha}$ coupled to COX-1 is discrete from that signaling to 12-LOX coupled $cPLA_{2\alpha}$ is derived from the results of the studies with 1-butanol and propranolol. Neither 1-butanol nor propranolol inhibits PI kinases.[30,31] Thus, inhibition of the activation of the $cPLA_2$ 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 $cPLA_2$ coupled to 12-LOX, thus providing additional evidence that this signaling pathway differs from that leading to activation of the $cPLA_2$ coupled to COX-1.

The intracellular localization of COX-1 and 12-LOX is pertinent to a consideration of differential regulation of the $cPLA_{2\alpha}$ 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.[32] 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,[33,34] from which it can be coimmunoprecipitated with

$cPLA_{2\alpha}$.[17] Thus, the differences between PAR-induced signaling to the $cPLA_{2\alpha}$ 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. $cPLA_{2\alpha}$ activity is regulated by several factors in cells, including interaction with membrane lipids and phosphorylation.[35–40] 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 kinases (likely PI[4,5]P2) participates in the activation of the phospholipase coupled to COX-1 (Figure 6). By contrast, activation of the $cPLA_{2\alpha}$ coupled to 12-LOX does not appear to require signaling through a PI kinase. Together, our results support the hypothesis that regulation of $cPLA_{2\alpha}$ in human platelets determines the metabolic pathway of AA by translocating the lipase to separate pools of phospholipids, specifically coupling the lipase to either COX-1 or 12-LOX, or both.

In contrast to the difference in the lipid signaling pathways that activate COX-1-coupled $cPLA_{2\alpha}$ but not 12-LOX-coupled $cPLA_{2\alpha}$, calcium is required for the PAR-induced activation of the $cPLA_{2\alpha}$ coupled to both COX-1 and 12-LOX. Also, secondary signaling via the TP receptor contributes to the ultimate PAR1-induced activation of both COX-1-coupled $cPLA_{2\alpha}$ and 12-LOX-coupled $cPLA_{2\alpha}$. TP-induced augmentation of the initial calcium response to PAR1 likely participates in this positive feedback loop regulating the extent of $cPLA_{2\alpha}$ activation. Previous research has shown that aspirin inhibits 12-HETE production by platelets activated by collagen[41] and thrombin,[42] and from that evidence, it was suggested that inhibition of the conversion of 12-hydroperoxyeicosatetraenoic acid to 12-HETE by a peroxidase accounted for this effect of aspirin. The present results indicate that TP receptor signaling contributes substantially to 12-HETE formation in response to PAR1. PAR4-induced 12-HETE formation is not affected by aspirin and is inhibited to a lesser extent by the TP receptor antagonist.

Both the TP receptor and PAR1 are G_q-coupled receptors that signal calcium release via the $PLC\beta$ pathway, which also leads to activation of PKC. Activation of PKC with PMA, however, produced platelet aggregation but did not lead to formation of either TxA_2 or 12-HETE, thus excluding the PKC pathway as a mechanism for activation of $cPLA_{2\alpha}$.

We found that activation of the PAR4 receptor generated TxA_2 more slowly than activation of PAR1 in this direct comparison of the agonists for the 2 receptors. These findings are consistent with previous evaluations in which the effects of PAR1[4] and PAR4[6] agonists were carried out in separate studies. Following stimulation of PAR4, the rate of 12-HETE formation is also less than that resulting from PAR1.

In conclusion, the PAR-induced signaling pathway that activates the $cPLA_{2\alpha}$ that provides AA to COX-1 is different from that activating the $cPLA_{2\alpha}$ that liberates substrate for 12-LOX. Thus, both COX-1 and 12-LOX are functionally coupled to a discrete subset of platelet $cPLA_{2\alpha}$ that provides AA separately to each of these oxygenases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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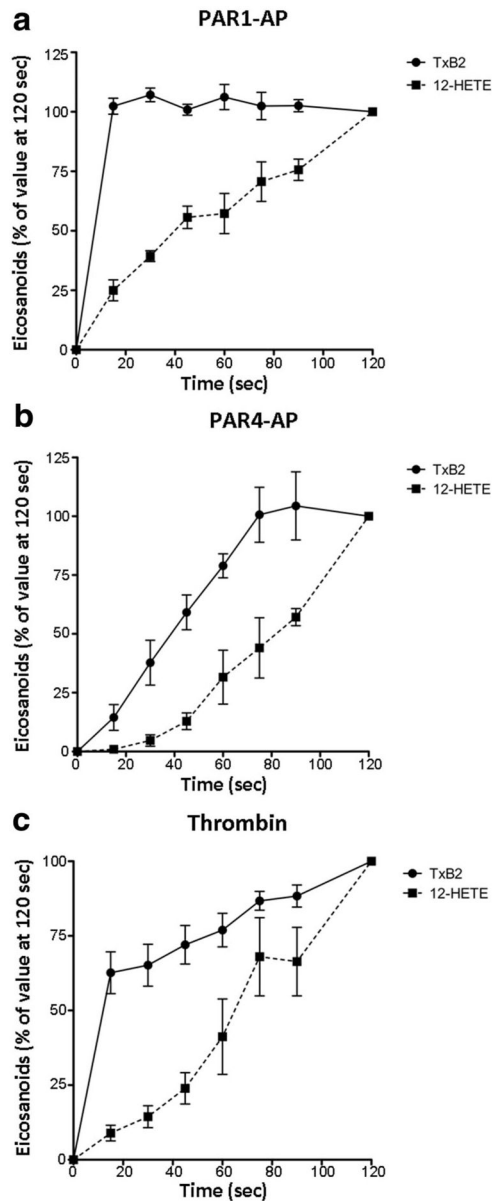
Figure 1.
TxB$_2$ and 12-HETE biosynthesis following PAR activation. Platelets were stimulated for various times with 20 μmol/L PAR1-AP (a), 200 μmol/L PAR4-AP (b), or 10 nmol/L thrombin (c). Following stimulation, platelet activation was quenched at various time points from 0 to 600 seconds. TxB$_2$ (n=6) and 12-HETE (n=5) released into the suspension were measured via mass spectrometry. The 2 curves were found to be statistically significantly different by 2-way ANOVA ($P<0.0001$ for PAR1; $P<0.0005$ for PAR4; $P<0.005$ for thrombin). At 15 seconds, the production of TxB$_2$ was significantly higher than the production of 12-HETE in response to PAR1 and thrombin activation ($P<0.001$, Bonferroni post test) but were not significantly different in response to PAR4 ($P>0.05$, Bonferroni post test).
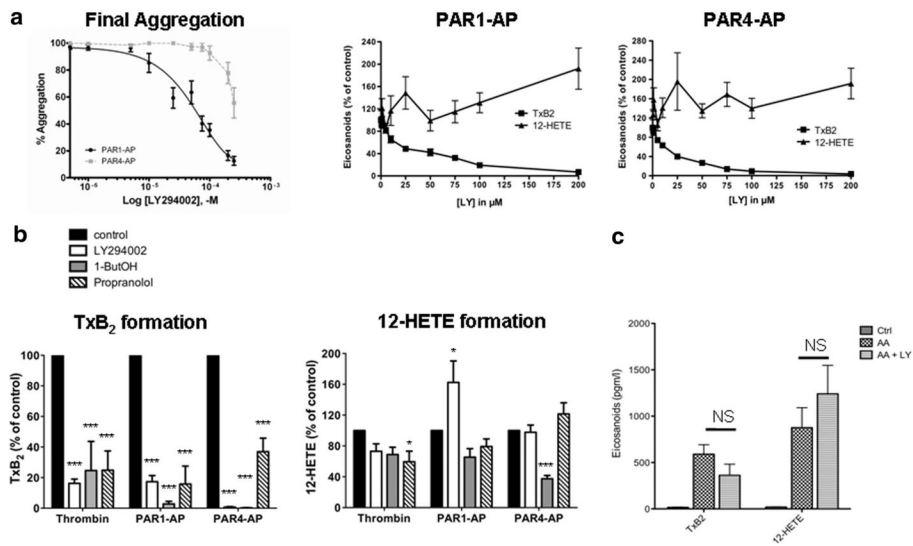
Figure 2.
Regulation of PAR-mediated eicosanoid formation and aggregation by lipid signaling pathways. Platelets were stimulated with 10 nmol/L thrombin, 20 μmol/L PAR1-AP, or 200 μmol/L PAR4-AP, and subsequent eicosanoid formation, platelet aggregation and dense granule secretion were measured. a, Aggregation, TxB_2, 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. Eicosanoid formation was then measured in each condition tested. TxB_2 and 12-HETE: Bars represent mean±SEM ($P<0.001$ and $P<0.05$ for TxB_2 and 12-HETE, respectively, by repeated-measures ANOVA; *$P<0.05$, ***$P<0.001$ compared with control condition by the Dunnett multiple comparison test). NS indicates not significant.
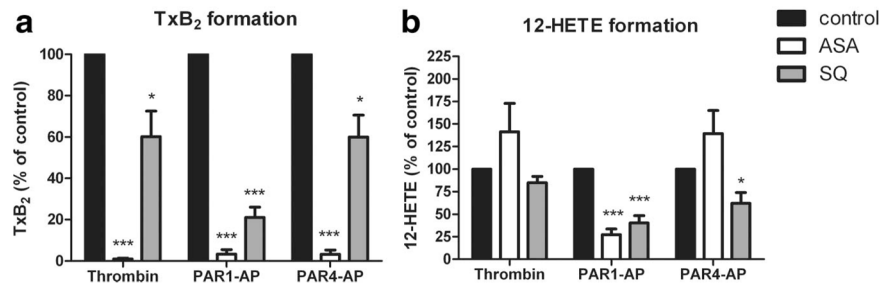
Figure 3.
TP receptor signaling contributes to PAR-mediated eicosanoid formation. Platelets were treated with or without 100 μmol/L aspirin (ASA) for 40 minutes or 100 nmol/L SQ for 5 minutes followed by stimulation under stirring conditions with 10 nmol/L thrombin, 20 μmol/L PAR1-AP, or 200 μmol/L PAR4-AP. Following stimulation, eicosanoids released into the suspension were measured via mass spectrometry. a, TxB_2 was measured following stimulation with the different agonists (n=6). Treatment with ASA resulted in complete inhibition of TxB_2 formation relative to control conditions (***P<0.001). Treatment with SQ resulted in partial inhibition of TxB_2 formation with a higher degree of inhibition following PAR1-AP (***P<0.001) relative to thrombin or PAR4-AP-treated conditions (*P<0.05) as compared with control. b, 12-HETE was measured following stimulation with the different agonists (n=6). Treatment with ASA resulted in a complete increase of 12-HETE formation following stimulation with thrombin or PAR4-AP and a significant decrease in 12-HETE formation following stimulation with PAR1-AP (***P<0.001). Treatment with SQ resulted in partial inhibition of TxB_2 formation following stimulation with thrombin (not significant) or PAR4-AP (*P<0.05) but a high level of inhibition following treatment with PAR1-AP (***P<0.001) as compared with control.
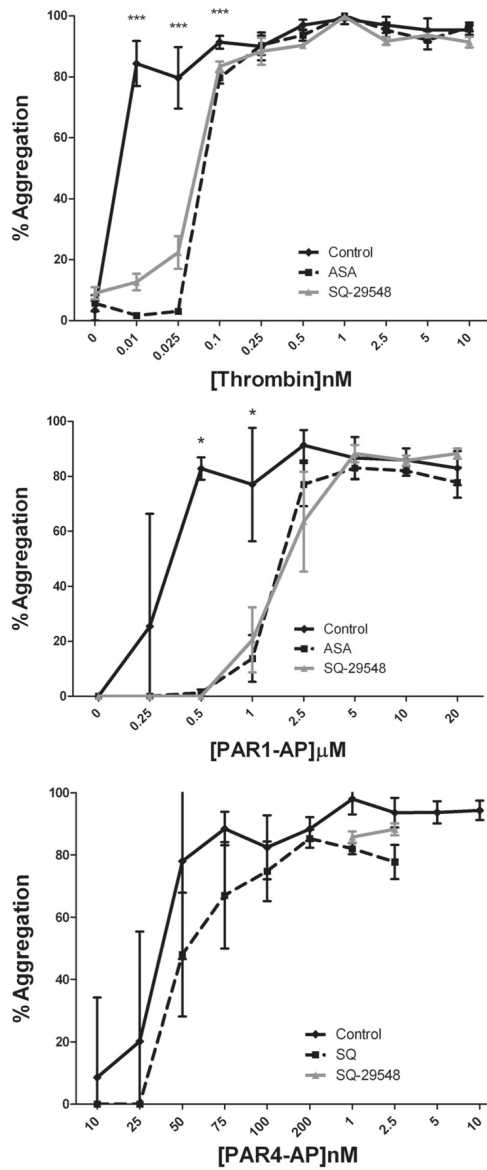
Figure 4.
Inhibition of TP signaling significantly attenuates PAR1-mediated platelet aggregation. Platelets were treated with or without 100 μmol/L aspirin (ASA) for 40 minutes or 100 nmol/L SQ for 5 minutes. Following treatment, platelet aggregation was measured following stimulation with increasing concentrations of thrombin (0 to 10 nmol/L), PAR1-AP (0 to 20 μmol/L), or PAR4-AP (0 to 200 μmol/L). Treatment with ASA or SQ significantly shifted PAR1-AP- and thrombin-mediated platelet aggregation at lower concentrations but had minimal effects on thrombin or PAR4-AP-induced platelet aggregation. n=8 (***$P<0.001$, *$P<0.05$).

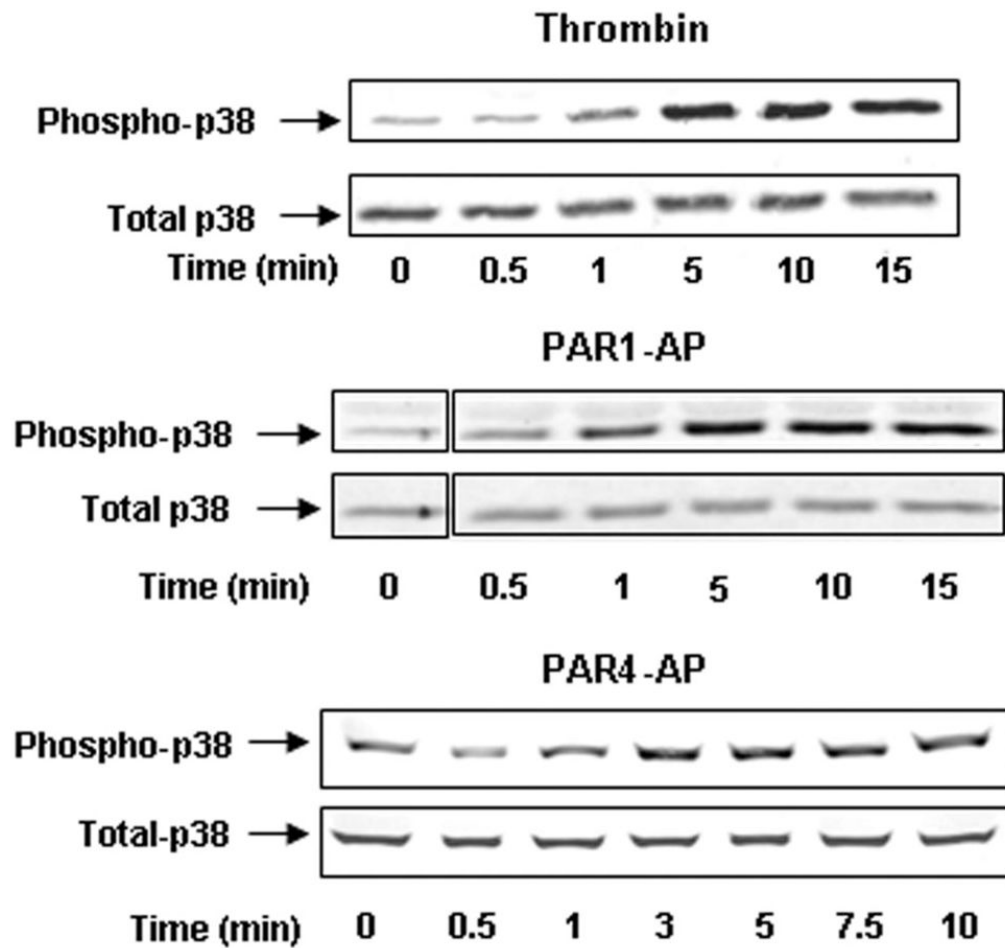**Figure 5.**
PKC does not play a role in PAR-mediated eicosanoid formation. Platelets were stimulated with 10 nmol/L thrombin, 20 μmol/L PAR1-AP, or 200 μmol/L PAR4-AP and subsequently measured for p38 phosphorylation. Platelets were stimulated from 0 to 15 minutes, and phosphorylation of p38 MAPK was measured. p38 was phosphorylated within 1 minute, with maximum phosphorylation occurring at 5 minutes following stimulation. Total cPLA$_2$ was also measured to ensure that equal protein was loaded for each condition (n=2).
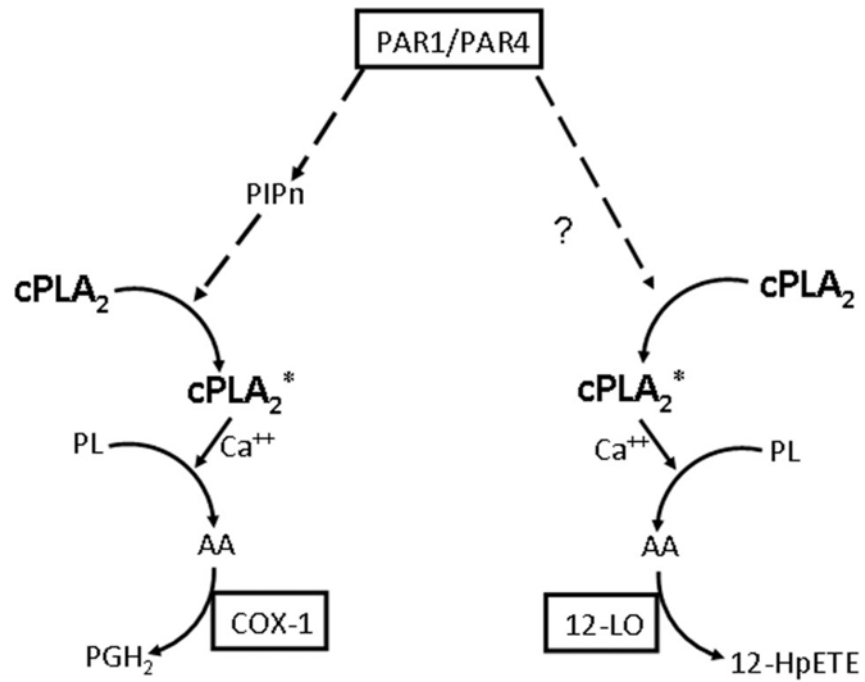
Figure 6.
Hypothetical model for PAR1-mediated formation of eicosanoids. cPLA2* indicates activated cPLA2; cPLA2, nonactivated cPLA2; PIP, phosphatidyl inositols; PL, phospholipids; PGH2, prostaglandin H2; 12-HpETE, 12-hydroperoxyeicosatetraenoic acid.

Table 1

Eicosanoid Formation in Human Platelets

	Thrombin	PAR1-AP	PAR4-AP	PMA
TxB$_2$, ng/mL (n)	289.8±41.4 (19)	124.0±25.4[*†] (19)	134.6±25.2[*†] (19)	9.3±6.1 (4)
12-HETE, ng/mL (n)	200.6±32.1 (16)	33.3±6.2[*‡] (16)	82.9±10.7[*‡] (16)	2.4±0.3 (4)

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.

Table 2

Calcium-Dependent Eicosanoid Biosynthesis by Human Platelets

	Thrombin	PAR1-AP	PAR4-AP
TxB$_2$, % inhibition by BAPTA (n)	89 (9)[*]	93 (9)[*]	95 (9)[*]
12-HETE, % inhibition by PABTA (n)	81 (9)[*]	57 (9)[*‡]	79 (9)[*]

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.

[*] $P<0.001$ vs control;

[‡] PAR1-AP vs thrombin or PAR4-AP.

Table 3

TP-Dependent Eicosanoid Biosynthesis by Human Platelets

	Thrombin	PAR1-AP	PAR4-AP
TxB$_2$, % inhibition by SQ (n)	40 (8)[*]	79 (8)[†]	40 (8)[*]
12-HETE, % inhibition by SQ (n)	NS (8)	60 (8)[‡]	38 (8)[*]

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.