Discrete Role for Cytosolic Phospholipase $A_2\alpha$ in Platelets: Studies Using Single and Double Mutant Mice of Cytosolic and Group IIA Secretory Phospholipase A_2

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Abstract

Among several different types of phospholipase A_2 (PLA_2), cytosolic PLA_2 ($cPLA_2$)α and group IIA (IIA) secretory PLA_2 ($sPLA_2$) have been studied intensively. To determine the discrete roles of $cPLA_2\alpha$ in platelets, we generated two sets of genetically engineered mice ($cPLA_2\alpha^{-/-}$/$sPLA_2$-$IIA^{-/-}$ and $cPLA_2\alpha^{-/-}$/$sPLA_2$-$IIA^{+/+}$) and compared their platelet function with their respective wild-type C57BL/6J mice ($cPLA_2\alpha^{+/+}$/$sPLA_2$-$IIA^{-/-}$) and C3H/HeN ($cPLA_2\alpha^{+/+}$/$sPLA_2$-$IIA^{+/+}$). We found that $cPLA_2\alpha$ is needed for the production of the vast majority of thromboxane (TX)A_2 with collagen stimulation of platelets. In $cPLA_2\alpha$-deficient mice, however, platelet aggregation in vitro is only fractionally decreased because small amounts of TX produced by redundant phospholipase enzymes sufficiently preserve aggregation. In comparison, adenosine triphosphate activation of platelets appears wholly independent of $cPLA_2\alpha$ and $sPLA_2$-IIA for aggregation or the production of TX, indicating that these phospholipases are specifically linked to collagen receptors. However, the lack of high levels of TX limiting vasoconstriction explains the in vivo effects seen: increased bleeding times and protection from thromboembolism. Thus, $cPLA_2\alpha$ plays a discrete role in the collagen-stimulated production of TX and its inhibition has a therapeutic potential against thromboembolism, with potentially limited bleeding expected.

Key words: knockout mice • platelet aggregation • bleeding • thromboembolism • thromboxane

Introduction

Platelets are required for hemostasis but also contribute in the pathogenesis of thrombotic and inflammatory diseases. A discrete therapeutic change in their properties is needed to maintain safety. Lipid mediators generated from phospholipid membranes, platelet-activating factor, or eicosanoids derived from released arachidonic acid (AA)* are thought to play an important role in their activation (1–4). The inhibition of an AA modifying enzyme, cyclooxygenase, by aspirin continues to be the single most successful therapy at preventing thrombotic disease (5). Thromboxane (TX)A_2 has been most clearly linked to function with the reports of genetic defects causing bleeding tendencies (6–8). There are potentially multiple classes of phospholipase A_2 (PLA_2) that may be involved in AA release: group IIA (IIA) secretory PLA_2 ($sPLA_2$), cytosolic PLA_2s ($cPLA_2$s), group V $sPLA_2$, calcium-independent PLA_2s, and group X $sPLA_2$ (9–13). As well as through the sequential action of phospholipase C (PLC), diglyceride lipase, and monoglyceride lipase, AA can be generated from phosphatidylinositol. However, $cPLA_2\alpha$, acting primarily on phosphatidylcholine, is thought to be the main enzyme involved in AA release on the activation of platelets. The discovery of paralogues, β and γ forms of this enzyme, in addition to the previously cloned $cPLA_2\alpha$ form, has added further complexity (14–16).

Platelet exposure to matrix components such as collagen necessitates a rapid response. In a complex multistage process platelets change shape, degranulate, and then aggregate (17). The binding of platelets to collagen has been reported to involve at least two receptors. Initial binding is to an integrin, $\alpha_2\beta_1$, which can bind at high sheer rates and is reported to activate AA release by PLA_2 enzymes. In vivo,

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**Abbreviations used in this paper:* IIA, group IIA; AA, arachidonic acid; $cPLA_2$, cytosolic phospholipase A_2; HETE, hydroxyeicosatetraenoic acid; PLA_2, phospholipase A_2; PLC, phospholipase C; $sPLA_2$, secretory PLA_2; TX, thromboxane.

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/2002/08/349/9 $5.00
Volume 196, Number 3, August 5, 2002 349–357
http://www.jem.org/cgi/doi/10.1084/jem.20011443

this binding is likely followed by the subsequent binding of collagen to glycoprotein VI (18). Other platelet activators, thrombin, TXA_2, ADP, and platelet-activating factor, act through G protein–coupled receptors on the surface of platelets. The blocking of G protein signaling may effectively inhibit platelet aggregation induced by collagen interfering with the accelerating effects of TXA_2 and ADP released after collagen binding and signaling.

The study of the enzymes involved in AA release is restricted by the unavailability of highly specific inhibitors to the various PLA_2s. Mice lacking both $cPLA_2\alpha$ (engineered) and $sPLA_2$-IIA (spontaneous) were previously generated to study the role of $cPLA_2\alpha$ in vivo (19, 20). Backcrossing these mice into the C3H/HeN mice with a working $sPLA_2$-IIA gene creates a strain with only the $cPLA_2\alpha$ gene inactivated. Both strains of mice were generated in our laboratory and studied to question the specific role of $cPLA_2\alpha$ in platelet activation. In vitro studies were conducted with the simultaneous measurement of degranulation by ATP release and platelet aggregation through impedance allowing the evaluation of the continuous effects of the loss of these enzymes (21, 22). Bleeding times and a thromboembolism model were used to study the effects in vivo.

Our results support a key role for $cPLA_2\alpha$ in the production of TXA_2 in platelets but suggest a basic redundancy in its production from other mechanisms. We also found that ADP or TXA_2 activation of platelets appears independent of $cPLA_2\alpha$ and likely leads to the involvement of other PLA_2s. In vivo studies suggest that a specific $cPLA_2\alpha$ inhibitor, in addition to an antiallergic/antiinflammatory effect and a growth suppression effect on intestinal polyp (19, 20, 23–31), will therefore have an antivasoconstrictive effect by dramatically reducing the amount of TXA_2 produced.

Materials and Methods

Reagents. 1 mg/ml equine type I collagen, 1 mM ADP, ATP standard, and Chrono-lume reagent, a bioluminescent luciferase mixture, were purchased from Chrono-log Co. Before use, U46619, a TX receptor agonist in methyl acetate, was evaporated and diluted in PBS (Cayman Chemical). SQ29548, a highly specific TXA_2 receptor antagonist, was dissolved in ethanol for storage and then evaporated and diluted in PBS just before experiments were performed (Cayman Chemical). Indomethacin (Sigma-Aldrich) was dissolved in 0.1 M sodium bicarbonate before use. 12(S)-hydroxyeicosatetraenoic acid (HETE) was purchased from Cayman Chemical.

Mice. Inbred mice were purchased from Central Laboratories for Experimental Animals Japan. As previously described (19), the $cPLA_2\alpha$ gene was disrupted by replacing part of an internal exon with a PGK-neo cassette. The mice generated (F2 of C57BL/6J and 129/Ola) were $cPLA_2\alpha^{-/-}$ and were also discovered to be congenitally defective in $sPLA_2$-IIA due to a frameshift mutation in the $sPLA_2$-IIA gene (32, 33). These mice were backcrossed to the C3H/HeN strain, which were gene sequenced to ensure the presence of a functional $sPLA_2$-IIA gene. Eighth and ninth generation backcrossed C3H/HeN mice were used. Twelfth generation backcrossed C57BL/6J mice were also used in the experiments. All mice were genotyped by a PCR method as previously described (19).

In Vitro Platelet Responses. 20–48-wk-old littermate backcrossed mice matched for sex were studied in pairs ($cPLA_2\alpha^{+/+}$, $cPLA_2\alpha^{-/-}$) to exclude any potential bias. Mice were anesthetized with 25 mg/kg pentobarbital and 25 mg/kg ketamine, and ~1 ml of whole blood was immediately collected in 0.35% (wt/vol) sodium citrate after cardiac puncture. After 30 min, 100 μl whole blood was diluted with 800 μl normal saline and 100 μl Chromo-lume reagent. Aggregation as measured by change in impedance and ATP release from dense granules were monitored continuously by luminometry (Chrono-Log Co.). To allow for comparison between the experiments, aggregation/impedance was adjusted to measure a set change in resistance by changing the baseline and height of a standard impulse. A 2-nM dose of ATP was added at the end of each measurement to standardize ATP. If the baseline measurement of ATP indicated the presence of marked platelet degranulation during cardiac puncture, the experiment was terminated. Continuous measurements were made electronically at every 25 ms, but summarized to the second for analysis. Reagents were added only after 2 min of a flat baseline in both measurements and the ATP level was <0.1 nM.

TXA_2 Assay. After 9 min of reaction, 100 μl of the aliquot was placed on ice. Samples were then spun at 1,000 *g* for 5 min and the supernatant was frozen at −70°C until analysis. For each mouse, 50 μl of the sample was saved to measure the baseline levels before stimulus. TXB_2, a nonenzymic hydrolyzed product of TXA_2, was measured by an enzyme immunoassay kit (Cayman Chemical).

12-HETE Assay. Quantification of 12-HETE was performed by reversed phase HPLC (system GOLD; Beckman Coulter) using a solvent of acetonitrile/methanol/water/acetic acid, 350:150:250:1 (2, 34). Flow rate was 1 ml/min and eluent was monitored at 235 nm. Blood was collected from incisions on femoral vessels of anesthetized mice (25 mg/kg pentobarbital and 25 mg/kg ketamine). After 30 min of incubation at room temperature to allow clotting, serum samples were collected by centrifugation at 500 *g* for 5 min. Samples were extracted with ethylacetate after acidification to a pH of ~5.0 with 0.5 vol of 0.1% formic acid, dried on a centrifuge concentrator, dissolved in the HPLC solvent, and analyzed. To normalize the extraction rate, 0.5 nmol 12(S)-HETE was added to selected samples before extraction and the difference of the area was calculated.

Bleeding Time. Adult mice were restrained in the upright position and the tail was cut 2–3 mm from the tip. The tail was then immersed in saline at 37°C and the bleeding time was defined as the time point at which all visible signs of bleeding from the incision had stopped, or at 10 min (35). Because of a significant difference between males and females in knockout C3H/HeN, only females were used in later studies. 10 mg/kg indomethacin was injected intraperitoneally 1 h before the bleeding times were tested in age-matched female mice.

Thromboembolism Test. In mice anesthetized with 80 mg/kg sodium pentobarbital, a collagen–ADP mixture (4 ml/kg of a saline-based solution containing 250 mg/ml collagen and 200 μM ADP) was injected into the jugular vein. Survival was evaluated 1 h after injection (36, 37). The dose was pretested to ensure 100% killing of wild-type mice (38). One pair of mice were killed and intubated 2 min after injection. 4% formalin in PBS was injected into the lung, and then the heart and lung were dissected together and placed in 10% formalin in PBS. Serial sections were cut and stained with hematoxylin and eosin for examination. A blindfolded investigator counted 20 areas at 400× for each mouse to determine the percentage of clotted vessels.

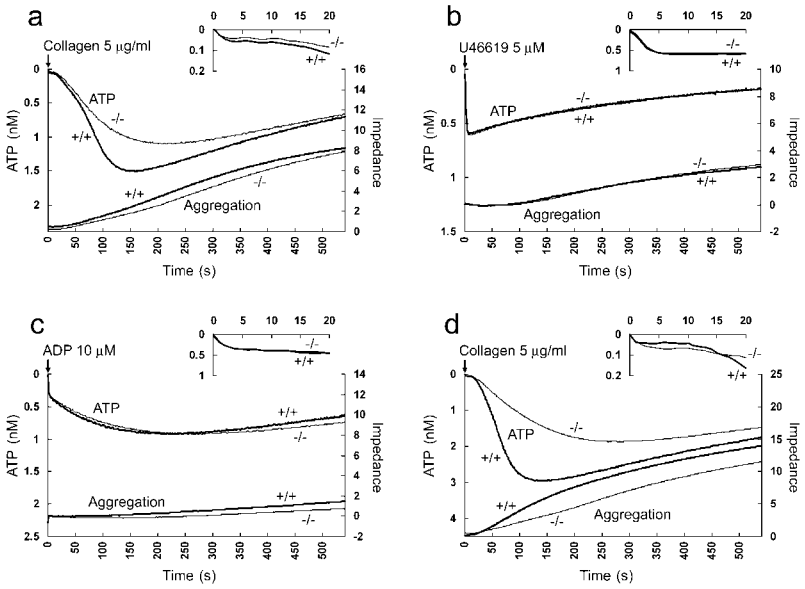

Figure 1. Effect of $cPLA_2\alpha$ on platelet activation. ATP release (left axis) as monitored by luciferase activity is seen on the top of each graph. The first 20 s of the reaction is magnified above. Platelet aggregation (right axis) as measured by impedance is at the bottom. In each case, averaged data from wild-type littermates ($^{+/+}$) are paired with sex-matched $cPLA_2\alpha$-deficient mice ($^{-/-}$). (a) C3H/HeN mice platelet reaction to collagen (n = 9). (b) C3H/HeN reaction to U46619 (n = 7). (c) C3H/HeN reaction to ADP (n = 6). (d) C57BL/6J reaction to collagen (n = 4).

Statistics. All values are expressed as mean ± SD unless stated otherwise. Two-tailed t tests were used to test the significance on continuous data and multiple regression was used to calculate the P-values on nominal data and in multivariable analysis (Quattro Pro).

Results

Degranulation and Aggregation to Collagen. C3H/HeN $cPLA_2\alpha^{-/-}$ $sPLA_2$-$IIA^{+/+}$ mice show a delay in degranulation as measured by ATP release in response to collagen (Fig. 1 a). This accompanies a more concave impedance curve, indicating a slowing in the acceleration phase of platelet aggregation. Aggregation achieved at 9 min was preserved in $cPLA_2\alpha^{-/-}$ platelets. The time to achieve peak ATP release (Fig. 2, left, P = 0.013) and the time to reach 50% of aggregation achieved within 9 min (Fig. 1 a; $cPLA_2\alpha^{+/+}$, 227 ± 43.7 s vs. $cPLA_2\alpha^{-/-}$, 268 ± 42.8 s; P = 0.042) is significantly delayed. Focusing on the first 20 s of the reaction, it appears that the loss of $cPLA_2\alpha$ starts to have an effect on the rate of degranulation at about the 20-s mark (Fig. 1 a). Compared to the changes in response to collagen, the reaction to U46619, a TX agonist, and ADP, both of which work through G protein–mediated receptors, appears to be unchanged (Fig. 1, b and c). C57BL/6J $cPLA_2\alpha^{+/+}$ $sPLA_2$-$IIA^{-/-}$ mice appear to have the same shaped curve as C3H/HeN $cPLA_2\alpha^{+/+}$ $sPLA_2$-$IIA^{+/+}$ mice (Fig. 1 d). C57BL/6J $cPLA_2\alpha^{-/-}$ $sPLA_2$-$IIA^{-/-}$ mice also show a significant delay in the degranulation peak (Fig. 2), and a more concave aggregation curve. The times to reach 50% of aggregation were as follows: $cPLA_2\alpha^{+/+}$ $sPLA_2$-$IIA^{-/-}$, 178 ± 14.6 s versus $cPLA_2\alpha^{-/-}$ $sPLA_2$-$IIA^{-/-}$, 255 ± 67.0 s; P = 0.021. An indirect comparison of the changes between C3H/HeN and C57BL/6J mice using time to peak ATP can be attempted. There is an indication of an additional step delay due to the additional loss of $sPLA_2$ when compared with $cPLA_2\alpha$ loss alone (Fig. 1, a and d, and Fig. 2). The effect of other factors cannot fully be excluded due to the differences in the mouse strains used.

TX Levels. Compared to the small difference in degranulation and aggregation in vitro (Figs. 1 and 2), there is a marked, significant decrease in the amount of TXB_2 production as detected after collagen activation between $cPLA_2\alpha^{+/+}$ and $cPLA_2\alpha^{-/-}$ mice in both C3H/HeN and C57BL/6J strains (Fig. 3). The absolute difference in the C57BL/6J mice is larger (3.5 vs. 1.4 ng/ml), which is consistent with the ATP and aggregation curves. With the increase in TXA_2 upon stimulation, it is also clear that a limited amount of AA is being released in both strains of $cPLA_2\alpha^{-/-}$ mice, indicating the presence of another PLA_2 or an alternative AA-generating pathway. In accord with the aggregation and degranulation data (Fig. 1), there is little difference in TXB_2 production in response to ADP between C3H/HeN wild-type and $cPLA_2\alpha$-deficient

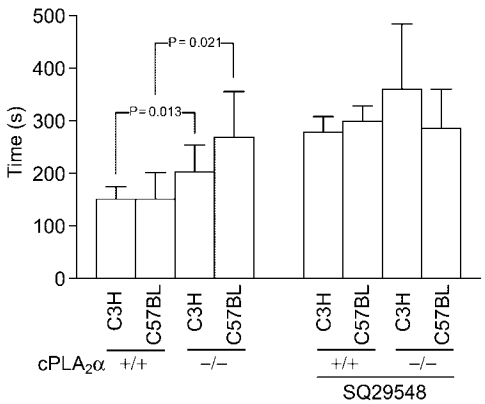

Figure 2. Time to peak ATP after collagen. The mean of individual times to maximum ATP level of each mouse is used in Figs. 1 and 4 plotted by strain, $cPLA_2\alpha$ status, and TXA_2 antagonist, SQ29548. Error bars are SD above the mean and P-values stand for significant differences.

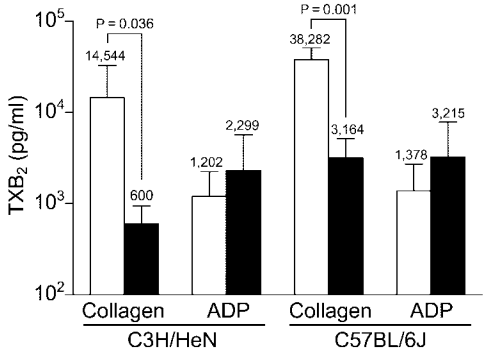

Figure 3. TXB_2 produced after platelet stimulation. Mean change in TXB_2 levels of mice pairs in Fig. 1. Open columns, $cPLA_2\alpha^{+/+}$ mice; shaded columns, $cPLA_2\alpha^{-/-}$ mice. Because of the log scale, actual mean is given above the columns, error bars are SD above the mean, and P-values stand for significant differences.

mice, suggesting a $cPLA_2\alpha$-independent mechanism for TXA_2 production with ADP as a stimulus. Similarly in C57BL/6J platelets stimulated with ADP, there was little difference in ATP release and aggregation (unpublished data), and TXB_2. Therefore, an AA-releasing mechanism, possibly another PLA_2(s) or PLC–diglyceride lipase system independent of both $cPLA_2\alpha$ and $sPLA_2$-IIA, is present in platelets upon the stimulation of G protein–coupled receptor ligands.

Effect of a Specific TX Antagonist. To confirm the presence of additional specific TXA_2 activity generated by $cPLA_2\alpha$/$sPLA_2$-IIA, a specific TXA_2 receptor antagonist, SQ29548, was used (39). Platelet activation appears both TXA_2-dependent and -independent, as SQ29548 was unable to completely block activation in all cases (Fig. 4). Upon examining the TXA_2-dependent component, the results on C3H/HeN $cPLA_2^{-/-}$ $sPLA_2$-$IIA^{+/+}$ mice and C57BL/6J $cPLA_2^{-/-}$ $sPLA_2$-$IIA^{-/-}$ in Fig. 4, c and d, confirm the presence of an alternative redundant enzyme(s) because a persistent inhibiting effect is seen in both mouse strains. In addition, SQ29548 inhibited degranulation and aggregation slightly more in the C57BL/6J mice, where the $sPLA_2$-IIA enzyme activity is missing, than in the C3H/HeN mice (Fig. 4, compare a and c, and b and d). A hint of the additional effect of $sPLA_2$-IIA is also seen when all the times to peak ATP results are graphed (Fig. 2). There appears to be a stepwise delay as successive enzymes are lost or TXA_2 activity is blocked. Statistically, however, multiple regression of the factors that influence time to peak ATP demonstrates an independent significant effect of the loss of $cPLA_2\alpha$ and the inhibitor SQ29548. The effect of the mice strain (the loss of $sPLA_2$-IIA) did not reach statistical significance. Therefore, in these studies we can only conclude that $cPLA_2\alpha^{-/-}$ platelets have an alternative AA-generating mechanism to $cPLA_2\alpha$ and $sPLA_2$-IIA. However, it is interesting to see that as expected, the effect of the inhibitor is smaller in the C57BL/6J strain with only a possible delay in the initiation of the reaction seen (Fig. 4, c and d). There is also a suggestion that the inhibitor effect of SQ29548 occurs earlier than at 20 s when compared with the effect of the loss of $cPLA_2\alpha$ as seen in Fig. 1.

Serum 12-HETE Levels. Next, we determined the contribution of $cPLA_2\alpha$ on the production of 12-HETE, another AA metabolite. As shown in Fig. 5 a, elution of 12-HETE was observed at 11.9 min on HPLC analysis. The absorbance spectrum was indistinguishable from authentic 12(S)-HETE, and coelution was also observed when they were mixed (not depicted). As shown in Fig. 5 b, the calculated concentration of 12-HETE in serum from C3H/HeN $cPLA_2\alpha^{-/-}$ adult female mice was 0.72 ± 0.28 μM ($n = 14$), significantly lower than $cPLA_2\alpha^{+/+}$ (2.4 ± 1.5 μM; $n = 13$; $P < 0.001$) and $cPLA_2\alpha^{+/-}$ (1.8 ± 0.83 μM; $n = 15$; $P < 0.05$). A similar significant difference was observed for C57BL/6J adult male mice: $cPLA_2\alpha^{+/+}$ (3.2 ± 1.6 μM; $n = 6$) versus $cPLA_2\alpha^{-/-}$ (0.44 ± 0.25 μM; $n = 6$; $P = 0.01$; Fig. 5 b).

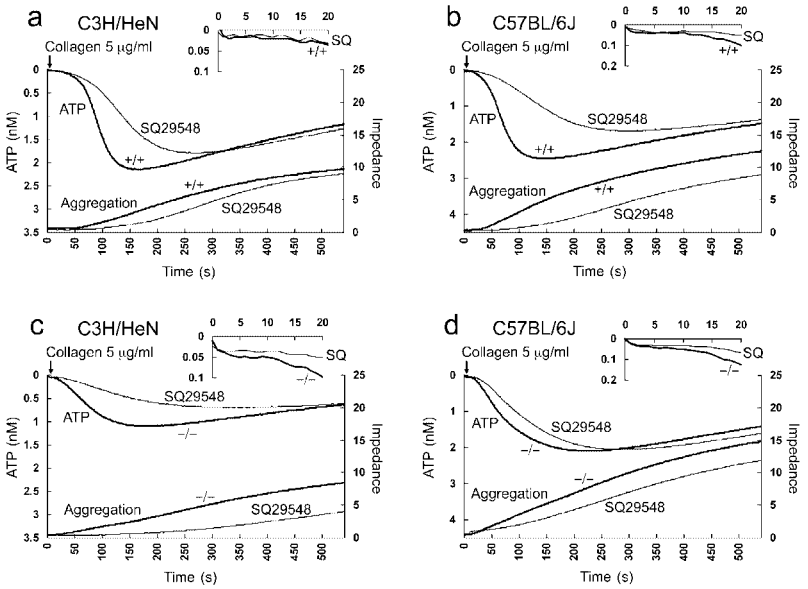

Figure 4. Effect of a TXA_2 receptor antagonist, SQ29548, on platelet activation. ATP release (left axis) as monitored by luciferase activity is seen on the top of each graph. The first 20 s of the reaction is magnified above. Platelet aggregation (right axis) as measured by impedance is at the bottom. In each case, responses to collagen are compared with and without SQ29548 from pooled data from wild-type ($^{+/+}$) or $cPLA_2\alpha$-deficient mice ($^{-/-}$). (a) C3H/HeN $cPLA_2\alpha^{+/+}$ mice ($n = 3$), (b) C57BL/6J $cPLA_2\alpha^{+/+}$ mice ($n = 3$), (c) C3H/HeN $cPLA_2\alpha^{-/-}$ mice ($n = 2$), and (d) C57BL/6J $cPLA_2\alpha^{-/-}$ mice ($n = 2$).

Marked Changes in Bleeding Time. Compared to the in vitro aggregation results, the differences in bleeding time correlate with TXB_2 levels. Bleeding times in both C3H/HeN and C57BL/6J mice were significantly increased (Fig. 6 a). However, because of a marked difference between male and female mice (unpublished data), only female mice were used for comparison between strains and interventions. Bleeding times, like the in vitro aggregation results, show that the $cPLA_2\alpha^{-/-}$ C57BL/6J mice have longer bleeding times than C3H/HeN females and cyclooxygenase inhibition with indomethacin leads to a similar increase in bleeding time.

Protective Effect of $cPLA_2\alpha$ Loss on Thromboembolism. In the thromboembolism test there was an increase in survival that is significant in $cPLA_2\alpha^{-/-}$ mice (Fig. 6 b). After the dissection of the heart and lungs together, the $cPLA_2\alpha^{-/-}$ lungs were of a much darker red than the $cPLA_2\alpha^{+/+}$, suggesting increased vasoconstriction in the presence of $cPLA_2\alpha$. However, both were engorged with blood compared with normal lungs due to clots. Blinded counts show an equal percent of clotted vessels in the wild type and knockout ($cPLA_2\alpha^{+/+}$, 76% vs. $cPLA_2\alpha^{-/-}$, 82%). The percentage is similar to previous studies (37). In $cPLA_2\alpha^{+/+}$ mice, small vessels were mostly clotted with evidence of arterial constriction and platelet clots in large arteries (Fig. 6 c, top and bottom right). In $cPLA_2\alpha^{-/-}$ mice, perhaps because of the lack of TXA_2-induced arterial constriction, platelet clots were allowed to reach larger collecting veins (Fig. 6 c, top and bottom left). The large number of platelet clots seen in knockout mice indicates that platelets are able to aggregate well, as suggested by the in vitro studies. The collection of clots in the venous side of the pulmonary system in mice lacking $cPLA_2\alpha$ appears to be more forgiving than clotting of the arterial side of the pulmonary artery system, which explains the reduction in mortality.

Discussion

By using platelets of two sets of genetically engineered mice, we found that $cPLA_2\alpha$ plays a critical role in TXA_2 production but a relatively redundant autocrine role in platelet aggregation due to an alternative AA-releasing mechanism. The lack of $cPLA_2\alpha$ leads to a dramatic loss of

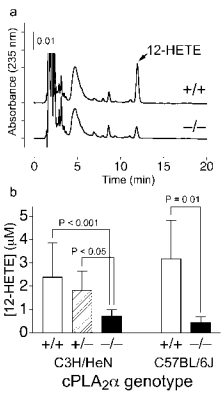

Figure 5. Serum 12-HETE content. (a) Representative HPLC chart. $^{+/+}$, a $cPLA_2\alpha^{+/+}$ mouse (3.6 μM); $^{-/-}$; a $cPLA_2\alpha^{-/-}$ mouse (1.0 μM). (b) Mean levels of 12-HETE in serum. Open columns, $cPLA_2\alpha^{+/+}$ mice; hatched column, $cPLA_2\alpha^{+/-}$ mice; shaded columns, $cPLA_2\alpha^{-/-}$ mice. Error bars are SD above the mean and P-values stand for significant differences.

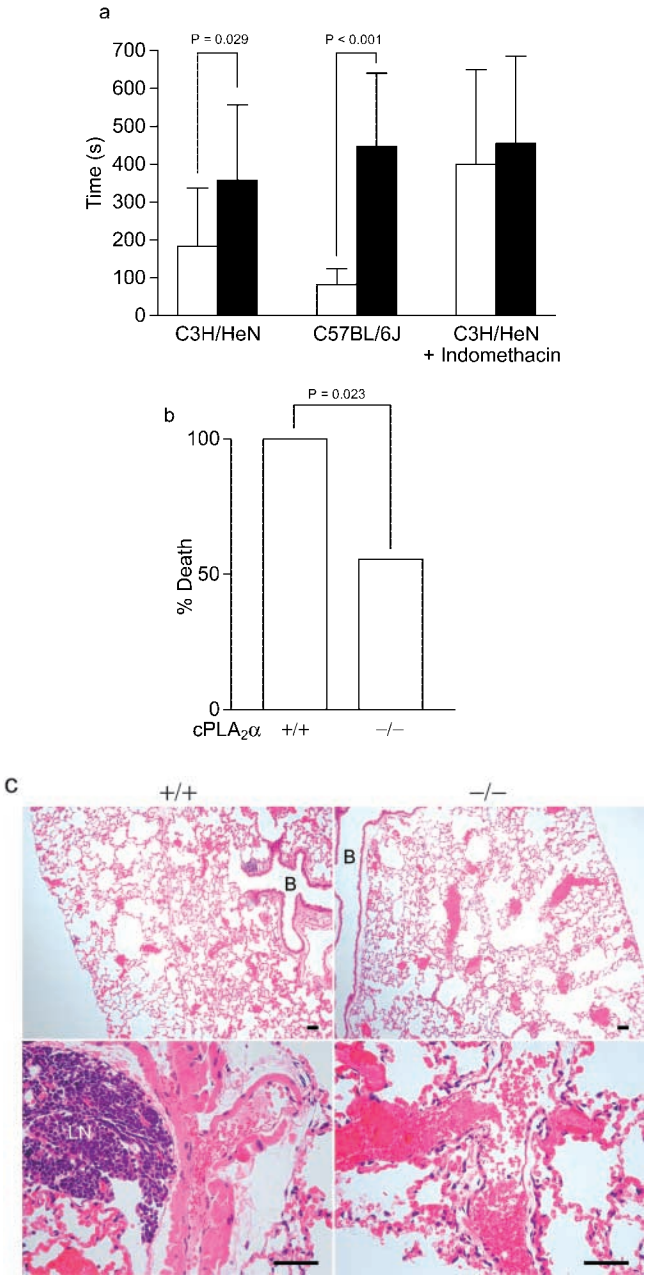

Figure 6. In vivo effects of $cPLA_2\alpha$. (a) Bleeding time. Open columns, $cPLA_2\alpha^{+/+}$ female mice; shaded columns, $cPLA_2\alpha^{-/-}$ female mice. C3H/HeN mice ($cPLA_2\alpha^{+/+}$, $n = 15$; $cPLA_2\alpha^{-/-}$, $n = 10$), C57BL/6J ($cPLA_2\alpha^{+/+}$, $n = 7$, wild-type nonlittermates; $cPLA_2\alpha^{-/-}$, $n = 7$), and C3H/HeN mice treated with indomethacin (six mice in each group stratified by bleeding time). Error bars are SD above the mean and P-values for significant differences between $cPLA_2\alpha^{+/+}$ and $cPLA_2\alpha^{-/-}$ are shown in each case. (b) Death in thromboembolism test of C3H/HeN littermate mice matched for sex and eight or ninth generation backcrossed. (c) Lung histology of $cPLA_2\alpha^{-/-}$ and $cPLA_2\alpha^{+/+}$ hematoxylin and eosin stain. In $cPLA_2\alpha^{-/-}$ mice at a low magnification (top) there are multiple platelet clots seen, causing red blood cell congestion in small and medium vessels. At a higher magnification (bottom), draining veins can be seen with platelet clots. In $cPLA_2\alpha^{+/+}$ mice at a low magnification (top), smaller vessels appear to be clotted. At a higher magnification a muscular artery is seen with platelet clots. B, bronchi; LN, lymph node. Bars, 100 μM.

TXA_2 production from collagen-activated mouse platelets in vitro. This loss contrasts to a small but significant delay in ATP release and a small but significant loss in the acceleration of platelet aggregation. The TXA_2 results, however, correlate well with a marked increase in bleeding time in mice lacking $cPLA_2\alpha$. This discrepancy is best explained if the vasoconstrictive effect of TXA_2 is considered. The test of bleeding time transects large veins and arteries in the mice tail. The veins, in particular, could be seen bleeding profusely in some $cPLA_2\alpha^{-/-}$ males where the diameter of the tail is considerably larger than that of the female, whereas in $cPLA_2\alpha^{-/-}$ mice bleeding could stop abruptly at 60–90 s due to vasoconstriction. The need for more vasoconstriction normally induced by TXA_2 would help explain the difference in bleeding time between the $cPLA_2\alpha^{-/-}$ C3H/HeN males (large diameter vessels) and females (smaller diameter vessels; unpublished data). This explanation fits well with the biological effects of TXA_2 inhibition with aspirin. Acute vascular syndromes are being prevented but with well-preserved aggregation at low intermittent dosing, i.e., inhibiting much, but not all, of the TXA_2. Small amounts of TXA_2 might be required early by platelets to activate themselves effectively, whereas the larger amounts produced mostly by $cPLA_2\alpha$ are intended for vasoconstriction. All of the current results support a dual function for TXA_2 and a discrete role for $cPLA_2\alpha$ in mouse platelets. (a) There are low levels of TXA_2 in both strains of knockout mice and therefore relatively well-preserved aggregation. (b) ADP, TXA_2 (Fig. 1, b and c, and Fig. 3), and probably other G protein–coupled receptor ligands, independent of $cPLA_2\alpha$, stimulate TXA_2 production, suggesting a redundant source of low level AA release. (c) In both strains of mice, a specific TXA_2 antagonist appeared to extend the delay in aggregation in proportion to the level of TXA_2 expected to be generated and also appeared to have an earlier effect. (d) There is a stepwise increase in bleeding times due to the blocking of successive enzyme loss between $cPLA_2\alpha^{-/-}$ and $cPLA_2\alpha^{-/-}$/$sPLA_2$-$IIA^{-/-}$ mice, similar to the in vitro aggregation pattern. (e) During thromboembolism, the preservation of the ability to form platelet clots in $cPLA_2\alpha^{-/-}$ mice with decreased lethality is probably due to the modification of vascular effects.

These results suggest that although $cPLA_2\alpha$ is responsible for the vast majority of TXA_2 production in activated platelets after collagen stimulation, there are redundant enzymes in platelets that can maintain basic platelet aggregation with relatively small amounts of TXA_2. The loss of this baseline autocrine TXA_2 effect leads to a more profound loss of platelet function. At least two independent mechanisms of TXA_2 production are apparent in mouse platelets with different PLA_2 enzymes, production levels and physiological effects. Additional support for this is the log fold difference in the dose of TXA_2 agonist required for smooth muscle contraction and platelet activation, even though the TXA_2 receptors appear to be the same (40, 41). There is mounting evidence for the dual role, an intracellular role and an intercellular/extracellular role, for any given lipid mediator (42). The $cPLA_2\alpha$ enzyme appears to play a critical part in generating lipid mediators for extracellular roles in platelets. It is also possible that platelets can utilize AA provided by serum lipids and neighboring cells.

The loss of $cPLA_2\alpha$ also appears to be important in the release of AA needed to produce other lipid mediators that may augment platelet aggregation/TXA_2 production (2). The loss of 12-HETE production evident in mice without $cPLA_2\alpha$ provides another mechanism by which the absence of $cPLA_2\alpha$ could act. The amount of 12-HETE dependence on $cPLA_2\alpha$ during aggregation may also apply to 12-lipoxygenase activity in other cell types and could contribute to the antiatherosclerosis/antiangiogenesis effect of $cPLA_2\alpha$ inhibition (43–46).

The delay in α granule release, as reflected in ATP, associates with and precedes the delay in aggregation and therefore may play a mechanistic role. There appears to be a more generalized delay in degranulation in cells missing $cPLA_2\alpha$ (25, 26). The delay in degranulation is not present with a TX agonist or ADP and is reproduced with a specific antagonist, which suggests that a reduction in the pace of the reaction is the only explanation needed for the delay in ATP release in these studies.

Rather than representative data, electronic summarized graphs are chosen in our experiments to block out the multiple factors that can influence platelet studies in mice. Variations in activation and platelet numbers during cardiac puncture cannot be easily standardized. As an alternative to the current approach, dilution of whole blood to a standard platelet count would add the variation caused by the dilution of plasma factors. During preliminary studies on third generation mice, small differences in platelet response were seen. Therefore, steps such as blocking for age and sex were used to decrease all other possible sources of variation. The presence of similar results in both strains of mice and the complimentary findings with the use of a specific antagonist to TXA_2 validates the approach. In vitro platelet data must, however, be viewed with some skepticism (47). In mice where careful controlled collection of blood is difficult, special care to correlate in vitro and in vivo experiments is needed. Although the use of different strains of mice help strengthen the generalization of the results, the comparisons between the two strains have to be carefully interpreted. The base levels of TXA_2 and ATP levels between C3H/HeN and C57BL/6J in response to collagen are different. Without a C57BL/6J mouse with a functional $sPLA_2$-IIA as a control, conclusions on the role of this enzyme in platelets cannot be certain. The addition, however, of studies using the C57BL/6J does show that $sPLA_2$-IIA is not the only alternative AA-generating enzyme to $cPLA_2\alpha$ in platelets.

Previous platelet studies with engineered mice put these new findings in context. Mice with $G\alpha q$ deficiency have severe loss of platelet functions with little evidence for redundant processes for this key protein (48). In knocking out P2Y1, an ADP receptor, evidence was found for a second, partially redundant receptor that was recently cloned (36, 49, 50). The surprise finding, the disruption of CD39,

which is an ATP diphosphohydrolase, was an inhibition of platelet aggregation (51). AA released by PLA_2s may also be acted upon by 12-lipoxygenase to form 12-HETE. Knockout studies suggest that this might be a factor that plays a unique role in ADP-stimulated platelets (2). In TXA_2 receptor knockout mice, findings very consistent with the current results were found (1). The bleeding times were also prolonged with the loss of TXA_2 receptor, which contrasted with human receptor deficiency. However, the test for bleeding time in humans and that used in mice is very different. The vasoconstriction caused by TXA_2 may have a significant effect on the bleeding times measured on mice tails compared to the human tests where aggregation in small skin vessels is tested. A delay in collagen activation compared to ADP activation was noted similarly to our paper. The current, more sensitive studies confirm a delay in the initiation of aggregation but also suggest that a stepwise loss of TXA_2 has effects on aggregation, with the complete loss of all TXA_2 having a more pronounced effect on aggregation. The current results cannot be directly compared with those of the TXA_2 receptor knockout because of different methodologies. It cannot be excluded, however, that an additional mechanism, which is TXA_2 receptor independent, is present. Studies involving multiple knockouts and specific inhibitors are needed to take the entire process apart.

The use of specific inhibitors to intracellular signaling proteins and knockout studies has provided some insights into how $cPLA_2\alpha$ might be regulated in platelets. First, G protein–coupled receptors to ADP, thrombin and TXA_2, appear to work through Gq proteins and activate PLC βs (48, 52, 53). Second, the recently cloned glycoprotein VI appears to require coactivation of the integrin $\alpha_2\beta_1$ to initiate a reaction. That reaction involves *lyn* and *fyn* Src family kinases that affect p72 Syk, leading to a pathway through SLP-76 to PLCγ2 (17, 54–57). Third, the signaling though $\alpha_2\beta_1$ involves actin polymerization and probably involves the activation of an Src kinase leading to a mitogen-activated protein kinase–dependent $cPLA_2$ activation (58–61). Therefore, platelet activation appears to involve at least three different pathways that interact with one another. $cPLA_2\alpha$ activation would appear to be related to the pathway through integrin $\alpha_2\beta_1$ and an alternative mechanism, most likely another PLA_2(s), through G protein–coupled receptors (ADP or TXA_2, etc.). The picture that emerges is that platelet activation is a complex multichanneled process involving multiple surface receptors and intracellular signaling pathways that mostly behave in vivo with increasing intensity. The loss of any particular protein may thus have a broad or distinct effect depending on its unique placement in a complex scheme.

Although these results in mouse platelets provide insights into human platelet function, in vivo use of specific inhibitors of $cPLA_2\alpha$ (62) and $sPLA_2$-IIA are needed to confirm these results in humans.

We are grateful to Drs. T. Yokomizo and S. Ishii, and members of the laboratory for discussion.

This work is supported in part by a grant-in-aid from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, and grants from Uehara Memorial Foundation and Yamanouchi Foundation of Metabolic Disorders.

Submitted: 21 August 2001
Revised: 22 May 2002
Accepted: 19 June 2002

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