Pharmacological Inhibition of Phospholipase D Protects Mice From Occlusive Thrombus Formation and Ischemic Stroke—Brief Report

David Stegner,* Ina Thielmann,* Peter Kraft, Michael A. Frohman, Guido Stoll, Bernhard Nieswandt

Objective—We recently showed that mice lacking the lipid signaling enzyme phospholipase (PL) D1 or both PLD isoforms (PLD1 and PLD2) were protected from pathological thrombus formation and ischemic stroke, whereas hemostasis was not impaired in these animals. We sought to assess whether pharmacological inhibition of PLD activity affects hemostasis, thrombosis, and thrombo-inflammatory brain infarction in mice.

Approach and Results—Treatment of platelets with the reversible, small molecule PLD inhibitor, 5-fluoro-2-indolyl des-chlorohalopemide (FIPI), led to a specific blockade of PLD activity that was associated with reduced α-granule release and integrin activation. Mice that received FIPI at a dose of 3 mg/kg displayed reduced occlusive thrombus formation upon chemical injury of carotid arteries or mesenterial arterioles. Similarly, FIPI-treated mice had smaller infarct sizes and significantly better motor and neurological function 24 hours after transient middle cerebral artery occlusion. This protective effect was not associated with major intracerebral hemorrhage or prolonged tail bleeding times.

Conclusions—These results provide the first evidence that pharmacological PLD inhibition might provide a safe therapeutic strategy to prevent arterial thrombosis and ischemic stroke. (Arterioscler Thromb Vasc Biol. 2013;33:2212-2217.)

Key Words: 5-fluoro-2-indolyl des-chlorohalopemide ■ cerebral stroke ■ phospholipase D ■ thrombosis

Platelet plug formation at sites of vascular injury is essential for normal hemostasis, but under pathological conditions it can lead to full vessel occlusion resulting in myocardial infarction or ischemic stroke, which are both leading causes of death worldwide.1-3 Platelet inhibition is commonly used for the prevention of secondary stroke, but its use is often limited because of increased risk of intracranial hemorrhages.4 Therefore, novel therapeutic approaches with a better safety profile are required. On the basis of animal models, targeting glycoprotein Ib (GPIb) or its principal ligand von Willebrand factor has been suggested as such an alternative approach.5-7

Phospholipase D (PLD) is a lipid signaling enzyme that catalyzes the hydrolysis of phosphatidylcholine to phosphatidic acid and choline.8 Phosphatidic acid can be dephosphorylated to form diacylglycerol, thereby generating a second important intracellular signaling molecule. The 2 mammalian PLD isoforms, PLD1 and PLD2, are widely expressed and are 50% identical in amino acid sequence.8,9 PLD1 and PLD2 locate to distinct intracellular membrane compartments and have been implicated in many important signaling pathways and cell biological processes.8,10 In platelets, both PLD isoforms are present and become activated upon stimulation with various agonists.8,11 Recently, we demonstrated that PLD1 contributes to GPIb-dependent platelet integrin activation, whereas absence of both PLD isoforms leads to a defect in α-granule release in addition to the defects observed in PLD1-deficient platelets.12 Moreover, Pld1−/−/Pld2−/− mice are protected from arterial thrombosis and ischemic stroke but do not exhibit hemostatic defects.11 These findings have led to the proposal that PLD could serve as a target for safe antithrombotic therapy,13 but in vivo evidence in support of this concept has not been reported yet.

For many years, inhibition of PLD-dependent generation of phosphatidic acid was achieved using 1-butanol; however, it has now been compellingly demonstrated that this approach creates off-target effects, making it difficult to interpret reports that drew conclusions using this tool.14,15 In recent years, however, PLD inhibition has become achievable using small molecule inhibitors.16 An inhibitor screen identified 5-fluoro-2-indolyl des-chlorohalopemide (FIPI) as a potent and reversible inhibitor of both PLD isoforms, with a half-life of 5.5 hours in vivo and moderate bioavailability.14,17 Further studies confirmed its specificity and effectiveness for inhibiting PLD through observation of PLD-dependent phenotypes in FIPI-treated cells.14,18-20 Moreover, FIPI has become an essential tool for studying the physiological relevance of PLD in therapeutic...
settings. FIPI is an analog of the psychiatric drug halopemide, a dopamine receptor antagonist. FIPI is 2 orders of magnitude more potent than halopemide for PLD2 inhibition; nonetheless, halopemide is used clinically at levels that should accomplish full PLD inhibition, suggesting that PLD inhibition in humans can be achieved without overt toxicity. We recently demonstrated that FIPI treatment prevents tumor growth and metastasis in mice to the same extent as genetic ablation of Pld1. In that study, mice received FIPI for ≤10 days with no apparent toxicity. This indicates, together with the overall unaltered appearance of Pld1−/−/Pld2−/− mice, that the absence or blockade of PLD activity is compatible with normal development and physiology. Here, we show that pharmacological inhibition of PLD activity with FIPI specifically reduced PLD-dependent α-granule release and integrin activation resulting in decreased thrombosis and infarct progression during acute stroke in mice without affecting hemostasis.

Materials And Methods
Materials and Methods are available in the online-only Supplement.

Results
FIPI Treatment Abolishes PLD Activity and Leads to Defective Integrin Activation and α-Granule Release
It has been shown that platelets of Pld1−/−/Pld2−/− mice display defective integrin activation and α-granule release on platelet stimulation with intermediate concentration of thrombin. To assess whether the small, reversible PLD inhibitor FIPI specifically inhibits PLD and whether this leads to the same effects as observed in the PLD1/2 double-deficient mice, we analyzed PLD activity and platelet activation on FIPI treatment. Although platelet stimulation with thrombin triggered PLD activity in vehicle-treated platelets, FIPI dose-dependently inhibited PLD activity (Figure 1A). In subsequent in vitro experiments we chose to use 100 nmol/L FIPI because this was the lowest concentration that abolished PLD activity, consistent with results for other cell types. Treatment of platelets with this concentration of FIPI did not alter glycoprotein expression on the platelet surface (data not shown). Integrin activation and P-selectin exposure, as a measurement for α-granule release, were analyzed flow cytometrically in vehicle- and FIPI-treated wild-type and Pld1−/−/Pld2−/− mice to test for potential off-target effects created by FIPI. The results revealed decreased integrin activation and α-granule release in FIPI-treated platelets upon stimulation with an intermediate concentration (3 mU/mL) of thrombin (Figure 1B). Reduction of platelet activation was observed to a similar extent in PLD-deficient mice, and no additional effects of FIPI treatment were seen for PLD1/2-deficient platelets, indicating that the mechanism of action for FIPI in inhibiting platelet activation proceeds through inhibition of PLD. Next, we assessed von Willebrand factor secretion as a second readout for α-granule secretion. Consistent with the reduced P-selectin exposure, FIPI-treated platelets secreted less von Willebrand factor on thrombin stimulation when compared with vehicle-treated platelets (Figure 1C), confirming that FIPI specifically inhibits α-degranulation. Similar results were obtained when repeating flow cytometric assays and ELISAs with platelets treated at a dose of 500 nmol/L, the calculated plasma concentration in FIPI-treated mice upon stimulation with an intermediate concentration of 3 mg/kg FIPI (data not shown).

Although PLD1/2-deficient platelets are able to aggregate normally, we wanted to analyze ex vivo whether FIPI might affect platelet–platelet interaction because of off-target effects by performing aggregation studies. For this purpose, mice received 3 mg/kg FIPI 1 and 13 hours before the start of the
Thrombus Formation In Vivo Without Exerting Obvious Off-Target Effects

To test whether pharmacological inhibition of PLD is a feasible antithrombotic strategy, we assessed the in vivo effects of FIPI. To test the effect of FIPI treatment on thrombus formation in different vascular beds in vivo, 2 well-established arterial thrombosis models were used. In the first model, the right carotid artery was injured by topical application of ferric chloride, and blood flow was monitored with an ultrasonic flow probe. In all but one of the control mice, the injured arteries occluded within 10 minutes, whereas thrombus formation was delayed in FIPI-treated mice and only 3 of 9 vessels occluded during the 30-minute observation period (Figure 2A and 2B). In the second model, mesenteric arterioles were chemically injured and thrombus formation monitored using intravital microscopy. Initial binding of platelets to the vessel wall and early aggregate formation was comparable in vehicle- and FIPI-treated mice (11.5±3.1 versus 11.4±3.0 minutes; Figure I in the online-only Data Supplement). However, although vessels of control mice occluded after 20.7±3.9 minutes, time to occlusion in FIPI-treated mice was significantly prolonged (25.1±7.7 minutes; Figure 2C and 2D) very similar to the results previously obtained in mice lacking both PLD isoforms. To analyze whether the antithrombotic effect can be ascribed to FIPI-mediated suppression of PLD activity, we performed the mesenteric arteriole thrombosis model with vehicle- and FIPI-treated Pld1−/−/Pld2−/− mice. The time-to-first appearance of thrombi of >10 µm was comparable between all groups (Figure I in the online-only Data Supplement). Whereas all control mice occluded within the 40-minute observation period (mean occlusion time, 14.2±3.8 minutes), 4 of the 13 vehicle-treated Pld1−/−/Pld2−/− mice exhibited no vessel occlusion and the time to vessel occlusion for the full set of PLD-deficient mice was increased (mean occlusion time, 19.8±6.4 minutes), confirming previously published data. Similarly, vessel wall injury in FIPI-treated Pld1−/−/Pld2−/− mice led to delayed vessel occlusion (at 22.2±7.6 minutes, on average) and in 2 of 13 vessels no occlusion occurred. These findings suggest that the antithrombotic effect of FIPI is specifically mediated by pharmacological inhibition of PLD.

FIPI Treatment Exerts Protection From Ischemic Stroke Without Impairing Hemostasis

Mice lacking PLD1 or both PLD isoforms are protected from infarct progression in a model of ischemic stroke. To test whether PLD inhibition can reproduce this protective effect, FIPI-treated mice were subjected to 60-minute transient middle cerebral artery occlusion (MCAO). The infarct volumes

Figure 2. 5-Fluoro-2-indolyl des-chlorohalopemide (FIPI) treatment results in impaired thrombus formation in vivo without exerting obvious off-target effects. Phospholipase D (PLD) activity in platelets was blocked by intraperitoneal injection of 3 mg/kg FIPI 13 and 1 hour before the experiment. A and B, Carotid arteries were topically injured with a filter paper saturated with 15% FeCl₃ for 1.5 minutes and blood flow was measured. Time to stable occlusion (A) and a representative blood flow chart (B) are depicted. C to F, Thrombus formation in small mesenteric arterioles of vehicle- and FIPI-treated wild-type and Pld1−/−/Pld2−/− mice was induced by topical application of 20% FeCl₃. Time to occlusion (C and E) and representative pictures (D and F) are shown. White asterisks indicate vessel occlusion. Each symbol represents 1 individual. *P<0.05; and **P<0.01.
of FIPI-treated mice 24 hours after infarction, as assessed by 2,3,5-triphenyltetrazoliumchloride staining, were reduced to \( \approx 60\% \) of infarct volumes in control mice (108.7±35.4 versus 65.5±19.5 mm\(^3\); Figure 3A). Accordingly, these mice had significantly better motor and global neurological function, which was determined by the grip test and Bederson score, respectively (Figure 3B and 3C). Serial MRI on a separate group of mice confirmed that ischemic lesions were significantly smaller in FIPI-treated mice (151.4±20.0 versus 90.5±21.8 mm\(^3\); Figure 3D). Hemorrhagic transformations, which would be visible as hyper-intense areas within the infarcted brains, were present in neither the control nor the FIPI-treated mice (Figure 3D). This argues against a major effect of FIPI treatment on vascular integrity in the setting of acute ischemic stroke. Furthermore, mice treated with FIPI at a dose of 3 mg/kg displayed unaltered tail bleeding times compared with control mice (5.1±2.4 versus 3.7±2.7 minutes; Figure 3E), demonstrating that PLD inhibition did not impair normal hemostasis. We also treated mice with 6 mg/kg FIPI, which is the highest possible concentration still soluble in 4% dimethyl sulfoxide in saline and performed tail bleeding assays. Bleeding in these mice similarly ceased at 5.7±1.9 minutes, demonstrating that even higher FIPI concentrations do not have an impact on tail bleeding times (Figure 3E). Although there is a lack of correlation between bleeding time and risk of clinical hemorrhages,\(^{15}\) our data suggest that pharmacological PLD inhibition is a safe and effective treatment for the inhibition of thrombotic activity and for the prevention of ischemic stroke.

**Therapeutic FIPI Treatment Does Not Protect Mice From Ischemic Stroke**

In another transient MCAO experiment, we investigated whether FIPI provides therapeutic benefit in the setting of acute stroke by treating the mice with 3 mg/kg FIPI directly after MCAO and 8 hours later. In this setting, no protective effect of FIPI treatment was observed. Infarct volumes of FIPI-treated mice (120.0±24.6 mm\(^3\)) were comparable with those of vehicle-treated mice (116.5±26.1 mm\(^3\); Figure 4A). Consequently, the motorical and global neurological outcomes of the mice were diminished to the same extent (Figure 4B and 4C). In addition, we performed transient MCAO of mice treated with 6 mg/kg FIPI to assess whether higher amount of FIPI might exert therapeutic effects. In fact, with the doubled amount of FIPI, which is also the highest applicable concentration, we did observe a tendency toward decreased infarct volumes (86.9±45.0 mm\(^3\)) and a slightly better neurological outcome 24 hours after transient MCAO (Figure 4).

**Discussion**

Our results demonstrate that FIPI, as a model inhibitor of PLD activity, induces potent protection of mice from thrombotic events and ischemic stroke without any detectable effects on hemostasis. Our findings are in accordance with the data obtained in \( \text{Pld}^{1−/−}/\text{Pld}^{2−/−} \) mice\(^{11,12} \) and with a previous report showing that pharmacological PLD inhibition phenocopies genetic \( \text{Pld}^{1} \) ablation in a mouse cancer model, suggesting potential employment of FIPI as a cancer therapeutic.\(^{21} \) By in vitro and in vivo analysis of FIPI-treated \( \text{Pld}^{1−/−}/\text{Pld}^{2−/−} \) mice, we were able to show that FIPI specifically inhibits PLD-mediated integrin activation and α-granule release, resulting in an antithrombotic effect in vivo.

Furthermore, our findings demonstrate that prophylactic FIPI treatment markedly protects mice from infarct progression in the setting of acute stroke. It is of particular interest to develop effective and safe treatments for acute stroke and secondary stroke prevention because conventional therapies with platelet aggregation inhibitors are associated with an increased bleeding risk. The paucity of effective therapy makes stroke one of the leading causes of death and disability worldwide.\(^{1,26} \) Platelet inhibition, for example, by blocking GPIIb/IIa, protects from arterial thrombosis but also leads to increased intracerebral hemorrhages in the setting of acute stroke.\(^{4,7,27} \) Notably, FIPI treatment, like GPIIb-blockade,\(^{1} \) had
no obvious effect on intracranial hemostasis. This further supports our previous studies that suggested interfering with the GPIb-von Willebrand factor axis and downstream signaling molecules as a promising strategy to inhibit thrombotic activity and prevent secondary infarction.3,7

However, therapeutic treatment with FIPI, even at high doses, provided only a tendency toward protective effects in ischemic stroke. This stands in contrast to the strong therapeutic effect of GPIb blockade which is not explained at present. We speculate that this might be because of limited bioavailability of FIPI and potentially an insufficient rate of drug delivery from the intraperitoneal site of injection. Further studies with optimized PLD inhibitors or improved drug delivery methods (eg, intravenous) are required to judge the efficacy of PLD inhibition in the setting of acute stroke.

Analogs of FIPI that are also PLD1/PLD2 dual inhibitors offers new options for PLD inhibition in vivo.28 Nevertheless, prophyllactic blockade of PLD activity may help to decrease the risk of vessel reocclusion after thrombolytic therapy in secondary stroke prevention.29,30

Markedly, pharmacologically and genetically induced PLD inhibition had no obvious side effects, as already described by Chen et al.,21 and did not induce intracranial hemorrhages, although PLD activity was affected in all cells. Accordingly, such a safe therapy might be particularly advantageous in clinical practice. For this reason, new PLD inhibitors need to be screened and analyzed to gain optimized drug delivery, greater potency, and bioavailability. Nevertheless, our study provides a proof-of-principle that small molecule inhibition of PLD1 or both isoforms constitutes a potent antiatherothrombotic approach and suggests that FIPI could serve as a good lead structure for drug optimization.

In summary, our findings demonstrate for the first time that pharmacological inhibition of PLD activity results in efficient and safe protection from arterial thrombosis and ischemic stroke without affecting hemostasis.

Acknowledgments
We thank Juliana Goldmann for excellent technical assistance and Virgil Michels for the magnetic resonance measurements.

Sources of Funding
This work was supported by the Deutsche Forschungsgemeinschaft (grant NI556/8-1 to Bernhard Nieswandt and SFB 688 to Bernhard Nieszwandt and Guido Stoll).

Disclosures
None.

References
Platelet inhibition is a major strategy to prevent acute ischemic cardiovascular and cerebrovascular events, which is, however, often associated with an increased bleeding risk. Phospholipase (PL) D1, which catalyzes the hydrolysis of phosphatidycholine to phosphatidic acid, is a promising target for antithrombotic therapy because mice lacking PLD are protected from arterial thrombosis and ischemic stroke, whereas hemostasis is unaffected. However, in vivo evidence in support of this concept has been lacking. In the current study, we used the PLD inhibitor 5-fluoro-2-indolyl des-chlorohalopemide (FIPI) as prototype anti-PLD agent and found that FIPI treatment results in protection from arterial thrombosis. Similarly, FIPI treatment reduced brain infarct sizes and improved motor and neurological function after transient middle cerebral artery occlusion. This protective effect was not associated with intracerebral hemorrhages or prolonged tail bleeding times. Hence, these results provide the first direct evidence that pharmacological PLD inhibition might be a safe therapeutic strategy to prevent arterial thrombosis and ischemic stroke.
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Arterioscler Thromb Vasc Biol. 2013;33:2212-2217; originally published online July 18, 2013; doi: 10.1161/ATVBAHA.113.302030

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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MATERIAL AND METHODS

Mice
6-10-week-old C57BL/6 or Plld1−/−/Plld2−/− mice were used for all experiments. Animal studies were approved by the district government of Lower Franconia (Bezirksregierung Unterfranken).

Pharmacological inhibition of PLD with FIP1
For in vitro experiments washed platelets were incubated with 100nM, 500nM or the indicated concentrations of FIP1 for 5 min at 37°C. For prophylactic treatment mice received intraperitoneal injections of FIP1 (Sigma, Schnelldorf, Germany) at a dose of 3 mg/kg or 6 mg/kg body weight in 4% DMSO/96% saline 13 h and 1 h before start of the in vivo experiment.2 For therapeutic treatment of mice in the tMCAO model mice received 3 mg/kg or 6 mg/kg FIP1 after reperfusion and 8 h later. Control mice were treated accordingly with vehicle.

Platelet preparation and PLD activity measurements
Washed platelets were prepared as described.3 To determine PLD activity the formation of [3H] phosphatidylethanol ([3H] Ptd-EtOH) was measured using standard protocols.4, 5 Washed platelets were adjusted to a final concentration of 3 x 10⁸ platelets/ml and were labelled with 3.7 kBp/ml [ 3H]-palmitic acid (Perkin Elmer) at 37°C for 1 h. Aliquots of 80 μl were pre-incubated with 0.5% ethanol for 10 min and then stimulated with thrombin (0.1 U/ml) or CRP (10 µg/ml) in the presence of 2 mM CaCl₂. Reactions were stopped by adding 500 μl of ice-cold chloroform/methanol and placing on ice. 500 μl ice-cold chloroform and 350 µl water were added to extract the lipids, which were collected in the organic phase and separated by thin layer chromatography. [ 3H] Ptd-EtOH bands were identified through co-migration with standards and quantified by scintillation. PLD activity is depicted as percentage of phosphatidylethanol of total [3H]-labelled phospholipids.

Platelet aggregation and flow cytometry
Platelet rich plasma was stimulated with indicated agonists and aggregation was measured by recording transmission on a four-channel aggregometer (Fibrintimer; APACT) for 10 min. Aggregation was expressed in arbitrary units, with buffer representing 100% transmission. For flow cytometry, heparinized whole blood was diluted 1:20, and incubated with the appropriate fluorophore-conjugated monoclonal antibodies for 15 min at room temperature and analysed on a FACSCalibur (Becton Dickinson).

Measurement of vWF secretion
Washed platelets were adjusted to a final concentration of 0.5 x 10⁶ platelets/μl and activated in the presence of 1.4 μM indometacin and 2 U/ml apyrase with indicated agonists for 15 min at 37°C. Platelets were immediately centrifuged. VWF in the supernatant was quantified by ELISA using unconjugated and horseradish-peroxidase (HRP) conjugated anti-hvWF antibodies (DAKO).

Carotid artery thrombosis model
Mice were anesthetized and the right carotid artery was exposed through a vertical midline incision in the neck. An ultrasonic flow probe (0.5PSB699; Transonic System) was placed around the vessel and thrombosis was induced by topical application of a 0.5 mm by 1 mm
filter paper saturated with 15% FeCl₃ for 1 min and 30 s. Blood flow was monitored until full occlusion of the vessel occurred or for 30 min.

**Anesthetic drugs**
Medetomidine (Pfizer), midazolam (Roche), fentanyl (Janssen-Cilag), and antagonists – atipamezol (Pfizer), flumazenil and naloxon (Delta Select) – were used according to the regulations of the local authorities.

**Intravital microscopy of thrombus formation in FeCl₃-injured mesenteric arterioles**
Four week old mice were anesthetized, and the mesentery was exteriorized through a midline abdominal incision. Arterioles were visualized with a Zeiss Axiovert 200 inverted microscope (10x/0.3 NA objective, Carl Zeiss) equipped with a 100-W HBO fluorescent lamp source, and a CoolSNAP-EZ camera (Visitron). Digital images were recorded and analysed off-line using Metavue software. Injury was induced by topical application of a 3-mm² filter paper saturated with FeCl₃ (20%). Adhesion and aggregation of fluorescently labelled platelets (Dylight-488–conjugated anti-GPIX Ig derivative) in arterioles were monitored for 40 min or until complete occlusion occurred (blood flow ceased for >1 min).

**tMCA occlusion model**
Experiments were conducted on 8-10 weeks-old mice according to previously published recommendations for research in mechanism-driven basic stroke studies. tMCAO was induced under inhalation anesthesia using the intraluminal filament (Doccol Company) technique. After 60 min, the filament was withdrawn to allow reperfusion. For measurements of ischemic brain volume, animals were sacrificed 24 h after induction of tMCAO, and brain sections were stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC, Sigma-Aldrich). Brain infarct volumes were calculated and corrected for edema. Neurological function and motor function were assessed by two independent and blinded investigators 24 h after tMCAO, as previously described. The experiments were conducted according to the recommendations for research in experimental stroke studies and the current ARRIVE guidelines (http://www.nc3rs.org/ARRIVE).

**Assessment of infarction and hemorrhage by MRI**
MRI was performed 24 hours after transient ischemia on a 1.5 T unit (Vision; Siemens) under inhalation anesthesia. A custom-made dual-channel surface coil was used for all measurements (A063HACG; Rapid Biomedical). The MR protocol included a coronal T2-weighted sequence (slice thickness, 2 mm) and a coronal T2-weighted gradient-echo constructed interference in steady state (CISS) sequence (slice thickness, 1 mm). MR images were transferred to an external workstation (Leonardo; Siemens) for data processing. The visual analysis of infarct morphology and the search for eventual intracerebral hemorrhage were performed in a blinded manner. Infarct volumes were calculated by planimetry of hyperintense areas on high-resolution CISS images.

**Bleeding time**
Mice were anesthetized and a 2 mm segment of the tail tip was removed with a scalpel. Tail bleeding was monitored by gently absorbing blood with a filter paper at 20 s intervals without making contact with the wound site. When no blood was observed on the paper, bleeding was determined to have ceased. Experiments were stopped after 20 min.
Statistics
Results are presented as means ± SD or scatter blots. Data were tested for Gaussian distribution with the D’Agostino and Pearson omnibus normality test and then analysed using the Welch’s test. Non-parametric data was assessed by Mann Whitney U test. Differences between more than two groups were analyzed by one-way analysis of variance (ANOVA) with Dunnett’s T3 as post-hoc test or with the Kruskal Wallis test. For statistical analysis SPSS Statistics 20 and PrismGraph 4.0 software (GraphPad Software, Inc.) was used. P < 0.05 was considered as statistically significant.

Supplemental References
Supplemental Figure I. FIPI treatment does not alter initiation of thrombus growth in vivo. PLD activity in platelets was blocked by intraperitoneal injection of 3 mg/kg FIPI 13 and 1 h before the experiment. (A,B) Time to first appearance of thrombi >10µm in small mesenteric arterioles of vehicle- and FIPI-treated wild-type and Pld1−/−/Pld2−/− mice was assessed upon damaging the vessel wall by topical application of 20% FeCl₃. Each symbol represents one individual. *P<0.05, **P<0.01.