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Evaluation of the physiological significance of botrocetin/von Willebrand factor *in vitro* signaling

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Summary

Background—A signaling pathway is difficult, if not impossible, to elucidate in platelets using only *in vivo* studies. Likewise, the physiological significance of signaling information obtained exclusively from *in vitro* observations is unknown. Therefore, both *in vitro* and *in vivo* experiments are required to establish the physiological significance of a signaling pathway.

Objective—To evaluate the physiological significance of signaling data obtained from botrocetin (bt)/von Willebrand factor (VWF)-stimulated washed platelets.

Method—Stable thrombus formation in response to $FeCl_3$-induced injury of the mouse carotid artery was used to evaluate the physiological significance of signaling data obtained from bt/VWF-stimulated washed platelets.

Results—Syk, PLCγ2, Gαq and P2Y12, but not LAT, were found either to be required for or to affect stable thrombus formation. Prior *in vitro* studies had demonstrated that LAT is not required for bt/VWF-induced platelet aggregation in the presence of exogenous fibrinogen. These data provide the first demonstration of the *in vivo* role for these signaling molecules in GPIb-dependent/initiated signal transduction and are consistent with the signaling pathway deduced from *in vitro* studies of bt/VWF-stimulated washed platelets using metabolic inhibitors and knockout mice.

Conclusion—The broad agreement between the *in vitro* and the *in vivo* results establish that bt/VWF stimulation of washed platelets can provide physiologically significant glycoprotein Ib-dependent/initiated signaling data.

Keywords

carotid artery; GPIb/IX/V; *in vivo* thrombosis

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

Introduction

Stable thrombus formation in response to $FeCl_3$-induced arterial injury provides an endpoint for evaluating the physiological role of signaling molecules in hemostasis *in vivo* [1,2]. Stable thrombus formation in this system results from at least two processes: (i) glycoprotein Ibα (GPIb)-initiated signaling that activates αIIbβ3; and (ii) the consequences of subsequent αIIbβ3 and contact-dependent outside-in signaling. The GPIb signaling that activates αIIbβ3 is initiated by GPIb binding to subendothelial von Willebrand factor (VWF) exposed by damage to the endothelium. A diverse range of GPIb-dependent *in vivo* signaling events have been implicated as causing activation of αIIbβ3 [3]. Although the versatility of GPIb signaling *in vitro* is interesting, it raises the perplexing question of which of these mechanisms, if any, are utilized by GPIb *in vivo* for stable thrombus formation at sites of vascular injury [4]. This is an important concern because stable arterial thrombus formation associated with endothelial damage underlies many aspects of cardiovascular disease [5].

Stable thrombus formation on the subendothelial matrix is a complex process involving a variety of receptors, including GPIb-IX-V and αIIbβ3, and multiple signaling molecules [3, 6]. Elucidation of the physiological significance of the platelet signaling that underlies this complex process requires a combination of *in vitro* and *in vivo* experimentation, particularly because platelets are anucleate. Elucidation of this signaling can be simplified by characterizing the two distinct component processes of stable thrombus formation independently of each other *in vitro*. Under appropriate conditions botrocetin (bt)/VWF induces a GPIb-dependent biphasic aggregation-like response [7]. The first phase is VWF-mediated platelet agglutination resulting from cross-linking of platelets by VWF binding to GPIbα on adjacent platelets. During agglutination, signaling initiated by GPIb elicits αIIbβ3 activation. The second phase of the biphasic response is platelet aggregation mediated by αIIbβ3. The αIIbβ3-initiated signaling that occurs during the second phase is aggregation dependent *in vitro* [7].

Results obtained using bt/VWF-stimulated washed mouse platelets demonstrate that the GPIb-initiated signaling that activates αIIbβ3 culminates in thromboxane A2(TxA2) production and TxA2-elicited signaling [7,8]. The TxA2-elicited signaling is required for αIIbβ3 activation [7,8]. The GPIb-elicited signaling that results in TxA2 production is initiated by Lyn, enhanced by Src, and propagated through Syk, Src homology 2 (SH2) domain-containing leukocyte protein76 (SLP-76), phosphatidylinositol 3-kinase(PI3K), Bruton tyrosine kinase (Btk), phospholipase Cγ2 (PLCγ2) and protein kinase C (PKC) [4,9] (Fig. 1). These signaling molecules were required for the first phase of the bt/VWF-induced response. Specifically, the GPIb/VWF-mediated platelet agglutination-initiated signaling utilized these signaling molecules to elicit the production of the TxA2 that is required for activation of αIIbβ3.

The second phase of the bt/VWF-induced biphasic aggregation response is dependent on αIIbβ3, signaling through the TxA2 receptor, Tp [10], the ADP receptor P2Y1, the heterotrimeric G protein, Gαq, the ADP receptor P2Y12 and presumably Gi [4,7]. Specifically, the VWF/GPIb-mediated agglutination initiates signaling that culminates in TxA2 production which enables a low level of ADP secretion in a Gαq-independent manner. The ADP secreted in response to agglutination apparently signals through P2Y1 and causes the secretion of more ADP in a Gαq-dependent manner. This secreted ADP signals via P2Y12 causing α-granule secretion [7]. Although the Fc receptor γ-chain (FcRγ-chain) and the linker for activation of T cells (LAT) were required for bt/VWF-induced aggregation of washed platelets in the absence of exogenous fibrinogen, these requirements were eliminated by the presence of exogenous fibrinogen [9]. FcRγ-chain and LAT were also not required for the GPIb-mediated signaling leading to αIIbβ3 activation [9,11]. Although much has been learned about GPIb signaling *in vitro* using this system [4,7,9], evaluation of the physiological significance of bt/VWF-elicited signaling requires *in vivo* analysis [4].

The physiological significance of bt/VWF facilitated, GPIb-elicited Btk and TxA2 signaling was shown by demonstrating the requirement for Btk and TxA2 signaling for stable thrombus formation *in vivo* using the $FeCl_3$ carotid artery injury model [4]. This model was used because the damage elicited by $FeCl_3$ treatment of mouse mesentery has been characterized [12,13] and because stable thrombus formation in this system is GPIb-dependent [4,14,15] and mediated primarily, though not exclusively, by VWF [16]. Thus, the $FeCl_3$ carotid artery injury model is appropriate for evaluating the requirement for specific signaling molecules during GPIb-dependent stable thrombus formation *in vivo*. Although other *in vivo* thrombosis models may be GPIb-dependent, they were deemed inappropriate for this comparison because they are not known to be GPIb-dependent.

In this study, we tested the physiological significance of other signaling molecules found to be required for either GPIb-dependent TxA2 production, and therefore αIIbβ3 activation, or for the subsequent aggregation of bt/VWF-stimulated mouse platelets by asking if the same molecules that are required for bt/VWF-induced platelet aggregation are also required for GPIb-dependent stable thrombus formation in $FeCl_3$-injured murine carotid arteries.

Materials and methods

Materials

Isofluorane was from NLS Animal Health (Oklahoma City, OK,USA). Cacodylate, paraformaldehyde, the glutaraldehyde solution, osmium tetroxide and uranyl acetate were from Electron Microscopy Sciences (Hatfield, PA, USA).

Animals

Mice deficient in PLCγ2 [17], Gαq [18], LAT [19], P2Y12 [20] or IL-4Rα/GPIbα-tg [21] were generated as described. The IL-4Rα/GPIbα-tg mice do not express murine GPIbα; instead they expressed the extracellular domain of the human interleukin-4 (IL-4) receptor fused with the transmembrane and cytoplasmic domains of human GPIbα [21]. Heterozygotes of this strain have been backcrossed with C57Bl/6 for 10 generations. Syk−/− chimeric mice were produced by fetal liver cell transplant as described [22]. The Syk chimaeras were made with Syk+/− mice backcrossed with C57/Bl6 mice for eight generations and their cells were transferred to inbred C57/Bl6 recipients. Wild-type littermate siblings were used as controls unless otherwise stated. All the other mouse strains with the exception of the P2Y12 strain have been inbred in our mouse facility for at least 5 years.

Ferric chloride-induced carotid artery injury

Carotid arteries were separated from other tissue in isofluorane-anesthetized mice. Injury was induced using 10% $FeCl_3$ and blood flow monitored using a laser Doppler system as described [4]. Monitoring of carotid artery blood flow was initiated at the time of $FeCl_3$ treatment. $FeCl_3$ treatment consisted of placement of a 4 × 10-mm strip of Whatman No. 1 filter paper saturated with a 10% $FeCl_3$ solution over the exposed adventitia of the carotid artery for 3 min. The treated tissue was then rinsed three times with a physiologic saline solution. Blood flow was continuously monitored for 45 min. Carotid artery blood flow of < 0.6 blood flow units (BFUs) per minute was scored as occlusion, allowing the time to first occlusion time to be determined. Statistical analyses were performed using Student's *t*-test.

Histological and scanning electron microscope studies

For histological studies, the isolated vessel segment was fixed in 16% paraformaldehyde for 24 h at 4 °C. The tissue was processed at room temperature with increasing concentrations of ethanol (70% for 30 min, 95%, 3× for 30 min, and absolute ethanol, 3× for 30 min), cleared

with xylene, 3× for30 min, infiltrated with paraffin for 1 h, and then embedded in paraffin blocks. Sections of the tissue (5 μm thickness) were stained with Diffquick reagent.

For preparation of the scanning electron microscope images, the carotid artery tissue was fixed by immersing the tissue in aqueous 0.1 M cacodylate, pH 7.35, containing 2.5% glutaraldehyde for at least 2 h at room temperature. The tissue was then rinsed three times for 5 min in aqueous 0.1 M cacodylate, pH 7.35, and fixed in 2% osmium tetroxide. After being sequentially rinsed with 0.1 M cacodylate and deionized water, the tissue was stained with 2% aqueous uranyl acetate for 2 h. The stained tissue was rinsed with deionized water and dehydrated with a graded series of ethyl alcohol (10% through absolute). The tissue was dried using a Critical Point Dryer in absolute ethyl alcohol and then in liquid carbon dioxide. The longitudinal incision segment of the carotid artery was sputter coated with gold-palladium, mounted and analyzed using a Philips Environmental Scanning Electron Microscope (SEM).

Results

GPIba is necessary for thrombus formation in response to arterial injury induced by $FeCl_3$

As shown by others [23], histological and SEM images of sections obtained from the occluded carotid arteries demonstrated that occlusion resulted from thrombus formation (Fig. 2). After total occlusion, if blood flow did not increase during the observation period, occlusion was scored as resulting from stable thrombus formation. It is also unlikely that the occlusion characterized here resulted from vasospasm because occlusion was GPIb-dependent (Table 1), and there is no obvious relationship between vasospasm and GPIb. If blood flow increased after occlusion occurred, it was assumed that embolization occurred.

Syk plays a role in stable thrombus formation in response to $FeCl_3$-induced injury

Syk is required for GPIb-dependent TxA2 production in bt/VWF-stimulated washed mouse platelets [9]. Therefore, we examined wild-type (either +/− or +/+ mice) and Syk-deficient chimeric mice in the $FeCl_3$-induced carotid artery thrombosis model. The average time to first occlusion for the Syk−/− chimeric mice was 8.04 min, in contrast to 5.25 min ($P < 0.01$) in the control mice (Table 2, line A). Also, substantial embolization occurred in four out of five Syk −/− chimeras, but in only one of the four control mice, and that was minor. The injured carotid artery of only one of the five Syk−/− chimeras remained patent; none of the injured carotid arteries of the control chimeras remained patent (Fig. 3A). These results demonstrate that Syk plays a role in GPIb-dependent stable thrombus formation. The diminished, but distinct blood flow characteristic of the Syk−/− chimeras in response to arterial injury is discussed below.

PLCγ2 is required for stable thrombus formation in response to arterial injury induced by $FeCl_3$

PLCγ2 is required for GPIb-dependent signaling in general [24–26] and for TxA2 production in bt/VWF-stimulated washed mouse platelets in particular [9]. Accordingly, we found that PLCγ2 was required for stable thrombus formation in $FeCl_3$-injured carotid arteries (Table 2, line B and Fig. 3B). The time to first partial occlusion in the PLCγ2−/− mice (11.30 ± 4.82, $P < 0.05$) was greater than the time to first occlusion in wild-type carotid arteries (5.00 ± 0.79), and in contrast to injured wild-type carotid arteries, none of which remained patent, all of the injured carotid arteries in the PLCγ2−/− mice remained patent. These results demonstrate that PLCγ2 is required for GPIb-dependent stable thrombus formation.

The physiological significance of Syk and PLCγ2 in $FeCl_3$-induced stable thrombus formation was tested because those signaling molecules had been shown to be required for GPIb-induced αIIbβ3 activation in response to platelet treatment with bt/VWF [4,9], the first phase of bt/VWF-induced platelet aggregation.

Gαq, P2Y12, but not LAT, are required for stable thrombus formation in response to arterial injury induced by FeCl3

The second stage of thrombus formation was also investigated. We investigated the role of three signaling molecules that are required for bt/VWF-induced, GPIb-elicited αIIbβ3-dependent aggregation, but not for αIIbβ3 activation: Gαq, P2Y12 and LAT [7,9]. Gαq, P2Y12 and LAT are required *in vitro* in response to bt/VWF stimulation for the αIIbβ3 signaling that results in fibrinogen secretion and platelet aggregation [7,9]. Despite these similarities, there was an important difference in the behavior of the LAT−/− platelets relative to the Gαq−/− platelets and wild-type platelets treated with a P2Y12 antagonist, AR-C69931MX. Stimulation of all these platelets with bt/VWF resulted in agglutination and the agglutination driven levels of TxA2, but not aggregation and the aggregation driven levels of TxA2. However, aggregation of the LAT−/− washed platelets enabled by exogenous fibrinogen restored TxA2 production to almost 80% of the level induced by aggregation in wild-type platelets; in contrast, aggregation of washed Gαq−/− and the AR-C69931MX-treated platelets enabled by exogenous fibrinogen did not enhance TxA2 production [7,9]. Accordingly, both Gαq (Fig. 3C) and P2Y12 (Fig. 3D), but not LAT (Fig. 3E) were required for stable thrombus formation. $FeCl_3$ treatment failed to cause occlusion of blood flow in Gαq−/−mice and blood flow remained patent. Likewise, stable occlusion of blood flow did not occur in the $FeCl_3$-treated P2Y12−/− mice [Fig. 3D, mean time to first partial occlusion 17.40 ± 16.59, $P < 0.0001$ (Table 2)] and blood flow remained patent. These data demonstrate that Gαq and P2Y12, but not LAT, are required for GPIb-dependent stable thrombus formation *in vivo* in the $FeCl_3$-induced arterial injury model and are consistent with the importance of TxA2-induced signaling in this process because in the absence of either of those signaling molecules, TxA2 is not produced and platelet aggregation does not occur [4,7].

Discussion

The results presented here demonstrate the requirement for a variety of signaling molecules for GPIb-dependent stable thrombus formation in response to $FeCl_3$-treated mouse carotid arteries. Stable thrombus formation was shown to be GPIb-dependent by injecting VCL (a recombinant protein functionally equivalent to a monomeric formof the A1 domain of VWF [27], which contains the VWF GPIb binding site) into tail veins of mice containing $FeCl_3$-treated carotid arteries [4]. In addition, two studies using mice that did not express the extracellular domain of GPIb (IL-4Rα/GPIbα-tg mice) have demonstrated that GPIb is essential for stable thrombus formation in $FeCl_3$-treated carotid arteries [14] and mesenteric arterioles [15]. The data in Table 1 confirm that the $FeCl_3$- induced platelet signaling characterized in our experiments was also GPIb dependent.

The results obtained using Syk−/− chimeric mice (Fig. 3A) demonstrate a requirement for Syk in stable thrombus formation in this system. The results were confounded by the findings that the injured carotid arteries in four of the five Syk deficient chimeras did not remain patent and that blood flow was greatly diminished in the other Syk-deficient chimera, presumably as a result of the presence of 1–2% remanant circulating wild-type platelets in the Syk−/− chimeras. That is, the circulating wild-type platelets in the Syk−/− chimeras would accumulate as thrombi at the sites of $FeCl_3$-induced injury, but the limited availability of wild-type platelets would preclude extensive thrombus formation. Although only 1–2% of the platelets in each of the Syk−/− chimeric mice usually are wild type, each mouse could easily have $1–2 \times 10^7$ wild-type platelets in circulation (based on 1.0×10^9 platelets/mouse). The presence of a small percentage of wild-type platelets in the Syk−/− chimeric mice results from the method used to construct the chimeras. Although the chimeras were produced by infusing fetal Syk−/− stem cells into lethally irradiated Syk+/+ mice, the irradiation does not kill all the platelet generating stem cells in the recipient mice. However, other factors may be responsible for the response

of the Syk−/− chimeric mice to $FeCl_3$ injury. For example, Syk may not be the only non-receptor tyrosine kinase that can contribute to PLCγ2 activation in response to GPIb signaling *in vivo* as some level of PLCγ2 activation may be mediated directly by Src-family kinases. Further work is required to resolve this issue.

As expected, PLCγ2 was found to be required for stable thrombus formation in this system (Fig. 3B). Although all the carotid arteries of the PLCγ2−/− mice remained patent, blood flow was diminished relative to flow in the uninjured artery. Possibly another phospholipase C (PLC) isoform present in low amount in platelets [28] accounts for the formation of unstable thrombi in the PLCγ2-deficient mice and/or thrombin may compensate in part for the absence of PLCγ2 [13,29]. Nonetheless, the data presented here demonstrate that PLCγ2 is required for GPIb-dependent stable thrombus formation *in vivo* and thereby establish the physiological significance of a variety of *in vitro* data.

The role of PLCγ2 has also been studied *in vivo* using the laser-induced mesenteric arteriole injury model in mice [26]. Those results are not discussed here because their relevance to those reported here is not obvious. Unlike the $FeCl_3$ carotid artery injury model, thrombus formation in the laser-induced injury model has not been shown to be GPIb-dependent and platelet activation in that system has been shown to be initiated by thrombin and independent of VWF even although VWF enhances the size of the thrombus [30].

Analyses of the results obtained using $FeCl_3$-injured carotid arteries of Gαq−/− mice and P2Y12−/− mice revealed that both Gαq and P2Y12 are required for GPIb-dependent stable thrombus formation *in vivo* (Fig. 3C,D). It is also clear from these results that G_{13} cannot compensate for the absence of Gαq [31]. The requirement for P2Y12 for stable thrombus formation has been shown previously, but not with carotid arteries [32]. Results obtained *in vitro* demonstrated that Gαq, P2Y1 and P2Y12 are required for signaling events subsequent to activation of αIIbβ3, including α-granule secretion, but not for GPIb-induced αIIbβ3 activation [7]. Accordingly, bt/VWF stimulated Gαq−/− platelets aggregated in the presence, but not in the absence of exogenous fibrinogen [7]. Likewise, A3P5P (a selective P2Y1 antagonist)-treated and AR-C69931MX (a selective P2Y12 antagonist)-treated, bt/VWF-stimulated washed platelets aggregated in the presence, but not in the absence of exogenous fibrinogen [7]. This *in vitro* signaling work demonstrated that P2Y1 is required for α-granule secretion, presumably by supplying ADP for P2Y12/Gi-mediated signaling [7]. Previous work has demonstrated a P2Y1 requirement for stable thrombus formation in response to $FeCl_3$-induced injury of the carotid artery [33]. Taken together, these results appear to demonstrate the importance of α-granule secretion in stabilizing thrombus formation as fibrinogen is not limiting in the KO mice after $FeCl_3$-induced injury. Because Gαq, P2Y1 and P2Y12 are not required for αIIbβ3 activation in response to GPIb signaling, these molecules are likely utilized during αIIbβ3-dependent outside-in signaling for stabilization of the thrombus.

As with PLCγ2−/− mice, the requirement for Gαq for thrombus formation was evaluated in the mesenteric arteriole laser-induced injury model in mice [26]. Consistent with our data, the Gαq-deficient mice failed to form thrombi that were stable during the brief observation period even in response to severe laser-induced injury. So, stable thrombus formation in these very different arterial injury models requires Gαq. Presumably, the agreement between the results obtained using the Gαq−/− mice in the two systems reflects a common Gαq requirement for aggregation (thrombus formation) in both the VWF-independent (laser treatment) [30] and the GPIb-dependent (FeCl3 treatment) systems [4,14,15].

The data presented here do not provide any insight into which ITAM (immunoreceptor tyrosine-based activation motif) containing signaling molecule facilitates Syk activation [34].However, data published elsewhere have shown the FcRγ-chain is not required for GPIb-

dependent activation of αIIbβ3 in mice [9,11]. Therefore, another ITAM containing signaling molecule apparently functions to facilitate activation of Syk.

In summary and conclusion, the data presented here demonstrate that all of the signaling molecules shown to be required *in vitro* for bt/VWF induced GPIb-dependent activation of αIIbβ3 and subsequent platelet aggregation [4,7,9] tested here were found to be required for GPIb-dependent stable thrombus formation in response to $FeCl_3$ induced injury of mouse carotid arteries. These are the first results demonstrating that Syk, PLCγ2, Gαq and P2Y12 are required *in vivo* for GPIb-dependent stable thrombus formation in response to carotid artery injury induced by $FeCl_3$. They also demonstrate that G_{13}, although coupled [31] to Tp [10] (the TxA2 receptor), is unable to compensate for the absence of Gαq for the response to $FeCl_3$-induced injury to mouse carotid arteries. Analyses of the *in vitro* and *in vivo* data reveals that α-granule secretionmay be required for stable thrombus formation *in vivo* and that Gi appears to be required for α-granule secretion. The high concordance between the *in vitro* and *in vivo* results suggests that these signaling molecules play similar roles in both models. It is unlikely that α2β1, rather than GPIb and αIIbβ3, initiated the activation of these signaling molecules because resting α2β1 does not support the adhesion of platelets to immobilized collagen [35] and therefore presumably cannot signal until activated. This is an important point because activation of α2β1 requires the activation of αIIbβ3 [36] and ADP [35,37]. If these *in vitro* observations are relevant to the *in vivo* situation characterized here, α2β1 signaling, if it occurs, is downstream of and dependent on GPIb and αIIbβ3 signaling. Finally, although the genetic deficiencies of knockout mice used here were shown to affect GPIb-dependent platelet signaling *in vitro* in the absence of vascular tissue, we cannot exclude the possibility those genetic deficiencies also affect vascular function. It seems unlikely that vascular deficiencies affected the results presented here because enhanced thrombosis was not observed and because the *in vivo* results were congruent with the platelet *in vitro* results. This latter point is significant because it is not uncommon that the results of *in vitro* studies based on the use of knockout mice differ substantially from those of the corresponding *in vivo* studies [38].

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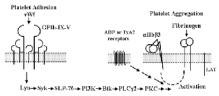

Fig. 1.

The signaling pathway mobilized by GPIbα in response to botrocetin (bt)/ von Willebrand factor (VWF) treatment of suspension washed mouse platelets. The signaling pathway is not comprehensive, but provides background information for this *in vivo* study [4,7,9].

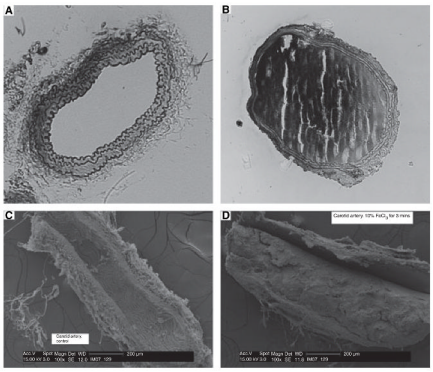

Fig. 2.

$FeCl_3$-treatment of carotid arteries causes stable thrombus formation. Light microscope images of sections of saline-treated (A) or $FeCl_3$-treated (B) wild-type mouse carotid arteries. Scanning electron microscope (SEM) images of saline-treated (C) or $FeCl_3$-treated (D) wild-type mouse carotid arteries.

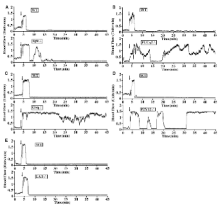

Fig. 3.

Traces of blood flow in $FeCl_3$-treated carotid arteries of wild type and knockout strains of mice. (A) The tracings in this figure are representative of blood flow in $FeCl_3$-treated carotid arteries of wild-type and Syk−/− chimeric mice. Blood flow was monitored for 45 min, including a 3-min treatment starting at zero time done with a strip of filter paper soaked in 10% $FeCl_3$. The arrow indicates the time of removal of the $FeCl_3$ containing strip of filter paper. (B) Representative data of blood flow in $FeCl_3$-treated exposed carotid arteries of control and PLCγ2−/− mice. Blood flow was monitored as described above. Representative data of blood flow in $FeCl_3$-treated exposed carotid arteries of wild-type, Gαq−/− (C), P2Y12−/− (D) and LAT−/− (E) mice.

Table 1

GPIb is essential for stable thrombus formation in $FeCl_3$-treated carotid arteries

Type of mice	Time of occlusion (mean ± SD)	No. of mice
WT	5.81 ± 1.37	8
IL-4R	No occlusion at the end of 45 min*	8

WT, wild type.

*$P < 0.0001$.

Table 2

Summary of the $FeCl_3$ treatment data

	Type of mice	Time of occlusion (Mean ± SD)	No. of mice
A	WT	5.25 ± 0.65	4
	Syk−/−	One out of five CAs remained patent 8.04 ± 0.93*	5
B	WT	5.00 ± 0.79	5
	PLCγ2−/−	No occlusion at the end of 45 min 11.30 ± 4.82**	5
C	WT	5.95 ± 0.82	5
	Gαq−/−	No occlusion at the end of 45 min***	4
D	WT	5.16 ± 0.79	5
	P2Y12−/−	No occlusion at the end of 45 min 17.40 ± 16.59****	5
E	WT	5.63 ± 1.25	5
	LAT−/−	5.75 ± 0.96	5

WT, wild type; LAT, linker for activation of T cells.

* $P < 0.01$,

** $P < 0.05$,

*** $P < 0.0001$,

**** $P < 0.05$.