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PDK1 governs thromboxane generation and thrombosis in platelets by regulating activation of Raf1 in the MAPK pathway: comment

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PDK1 has gained interest in the field as a potential target for antithrombotic therapies [1]; however, the role of PDK1 downstream of the $P2Y_{1/12}$ receptors remained unknown. We therefore read with great interest a recent report published in the *Journal of Thrombosis and Haemostasis*, by *Manne et al*. [2] describing the role of PDK1 in regulating platelet activation and TxA_2 generation downstream of the $P2Y_{1/12}$ receptors. In their study, *Manne et al*. used both pharmacological and genetic methods to conclusively show that targeting PDK1 can significantly attenuate pulmonary thromboembolism-induced mortality in an *in vivo* murine model. They further show that PDK1 regulates activation of Raf1 in the MAPK pathway induced by 2MeSADP.

Cytoplasmic phospholipase A_2 ($cPLA_2$) is recognized as one of the main PLA_2 that is responsible for the release of AA from membrane phospholipids and subsequent TxA_2 generation in platelets. Phosphorylation of $cPLA_2$ at Ser^{505} increases its activity [3]. While initial studies of $cPLA_2$ in cell free kinase assays showed that $cPLA_2$ could be phosphorylated directly by either ERK or p38 [4]; follow-up studies using p38 inhibitors suggested that it was p38 and not ERK1/2, which is responsible for phosphorylating $cPLA_2$-Ser^{505} in platelets [5]. Unfortunately, the identity of the kinase directly responsible for phosphorylation of $cPLA_2$-Ser^{505} remained in limbo for over a decade. However, recent reports using knockout mice may have solved this conundrum. Recently, we found that Ask1 null platelets showed a loss of p38 phosphorylation, but enhanced activation of ERK1/2 when stimulated with thrombin, collagen, or ADP. Interestingly, we observed a complete absence of $cPLA_2$ phosphorylation despite augmented ERK1/2 activity [6]. These data suggested that it was in fact p38, and not ERK, which is responsible for $cPLA_2$-Ser^{505} phosphorylation. This result was further supported by a contemporary report by Shi et al., who found that p38α null platelets also showed an absence of $cPLA_2$-Ser^{505} phosphorylation despite ERK1/2 being hyperphosphorylated [7].

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Addendum

P. Patel designed the research, performed experiments, analyzed results and wrote the manuscript. K. Golla performed experiments. U. Naik designed the research, analyzed results and wrote the manuscript.

Disclosure of Conflicts of Interest

The authors state that they have no conflict of interest.

In their report, *Manne et al.* described a mechanism of $cPLA_2$ phosphorylation in platelets induced by 2MeSADP. *Manne et al.* report that: (1) the $P2Y_{1/12}$ agonist 2MeSADP induces $cPLA_2$-Ser^{505} phosphorylation in an ERK1/2 and p38 dependent manner, as the PDK1 inhibitor BX-795 was able to block ERK1/2 and $cPLA_2$ phosphorylation, while the p38 inhibitor SB203580 also blocked $cPLA_2$ phosphorylation. (2) p38 can be activated downstream of $P2Y_{1/12}$ independently of ASK1 as the ASK1 inhibitor MSC2032964A failed to block 2MeSADP-induced p38 activation [2]. However, previously published data from our group and others suggests that ERK1/2 is not the main kinase responsible for directly phosphorylating $cPLA_2$ in platelets [6, 7], and that agonist-induced p38 phosphorylation is exclusively dependent on ASK1 signaling in platelets [6]. The difference in these studies is that we and Shi et al., used ADP and not 2MeSADP as used by *Manne et al.*

To resolve this discrepancy, we performed a series of experiments using platelets isolated from *Ask1*$^{-/-}$ mice. In agreement with our previous report, when Ask1 null platelets were stimulated with 2MeSADP, we found that p38 activation was completely ablated while Erk1/2 activation was augmented (Fig. 1A–B). At a low dose, 2MeSADP (2.5nM) rapidly (within 1min) phosphorylated $cPla_2$ in WT platelets but failed to cause any $cPla_2$ phosphorylation in Ask1 null platelets, even after 3 minutes. However, when the dose was increased to 50nM we found that $cPla_2$ was phosphorylated, albeit at a later time point (3 minutes post stimulation) (Fig. 1A–B). While our data indicates that $cPla_2$ can be phosphorylated in the absence of p38 activity, this phosphorylation appears to occur downstream of outside-in signaling. In fact, $cPLA_2$ has been shown to be associated with $\alpha_{IIb}\beta_3$ and is activated downstream of outside-in signaling [8]. Additionally, it has been shown that ADP-induced TxA_2 generation requires integrin outside-in signaling [9]. In addition, this data suggests, unlike *Manne et al.*, that p38 is exclusively activated downstream of ASK1 in platelets.

In addition to murine platelets, when we stimulated human platelets with 100nM 2MeSADP we found that ASK1 and p38 were activated, albeit weekly. We found that pretreating platelets with an ASK1 specific inhibitor, GS-4997, significantly attenuated ASK1, p38, and $cPLA_2$ phosphorylation while having minimal effect on ERK1/2 activation (Fig. 1C–D), suggesting again that p38 is solely activated downstream of ASK1 in human and murine platelets and that ASK1/p38 signaling regulates $cPLA_2$ phosphorylation.

To determine if our findings are specific to $P2Y_{1/12}$ signaling, or can be attributed to a more generalized signaling mechanism, we examined if ASK1 regulated p38 activation and $cPLA_2$ phosphorylation downstream of convulxin. When washed human platelets stimulated with 50ng/mL of convulxin, we observed robust activation of ASK1, p38, $cPLA_2$, and ERK1/2. Interestingly, except for ERK1/2, phosphorylation of ASK1, p38, and $cPLA_2$ was dose dependently attenuated by GS-4997 (Fig. 1E–F), suggesting that it is p38, and not ERK1/2, which is responsible for phosphorylating $cPLA_2$ in platelets.

Taken together, our data suggests that: (1) p38 is exclusively phosphorylated downstream of ASK1 in platelets and (2) that phosphorylation of $cPLA_2$ at Ser^{505} is primarily mediated by p38 in platelets. Given that both Pdk1 null murine platelets and human platelets pretreated

with BX-795 show a dramatic reduction in the level of $cPLA_2$ phosphorylation and TxA_2 generation [2], it is reasonable to conclude that PDK1 is involved in the regulation of $cPLA_2$ in platelets. However, *Manne et al.*'s findings showing that both SB203580 and BX-795 can completely abolish $cPLA_2$ phosphorylation, suggests that both p38 and PDK1 regulate $cPLA_2$ phosphorylation albeit through distinct mechanisms as depicted in Figure 1G. It is possible that, in platelets, p38 is the kinase that phosphorylates $cPLA_2$ and PDK1 regulates $cPLA_2$ phosphorylation via ERK1/2 by negatively regulating a $cPLA_2$-Ser^{505} phosphatase [10].

Acknowledgments

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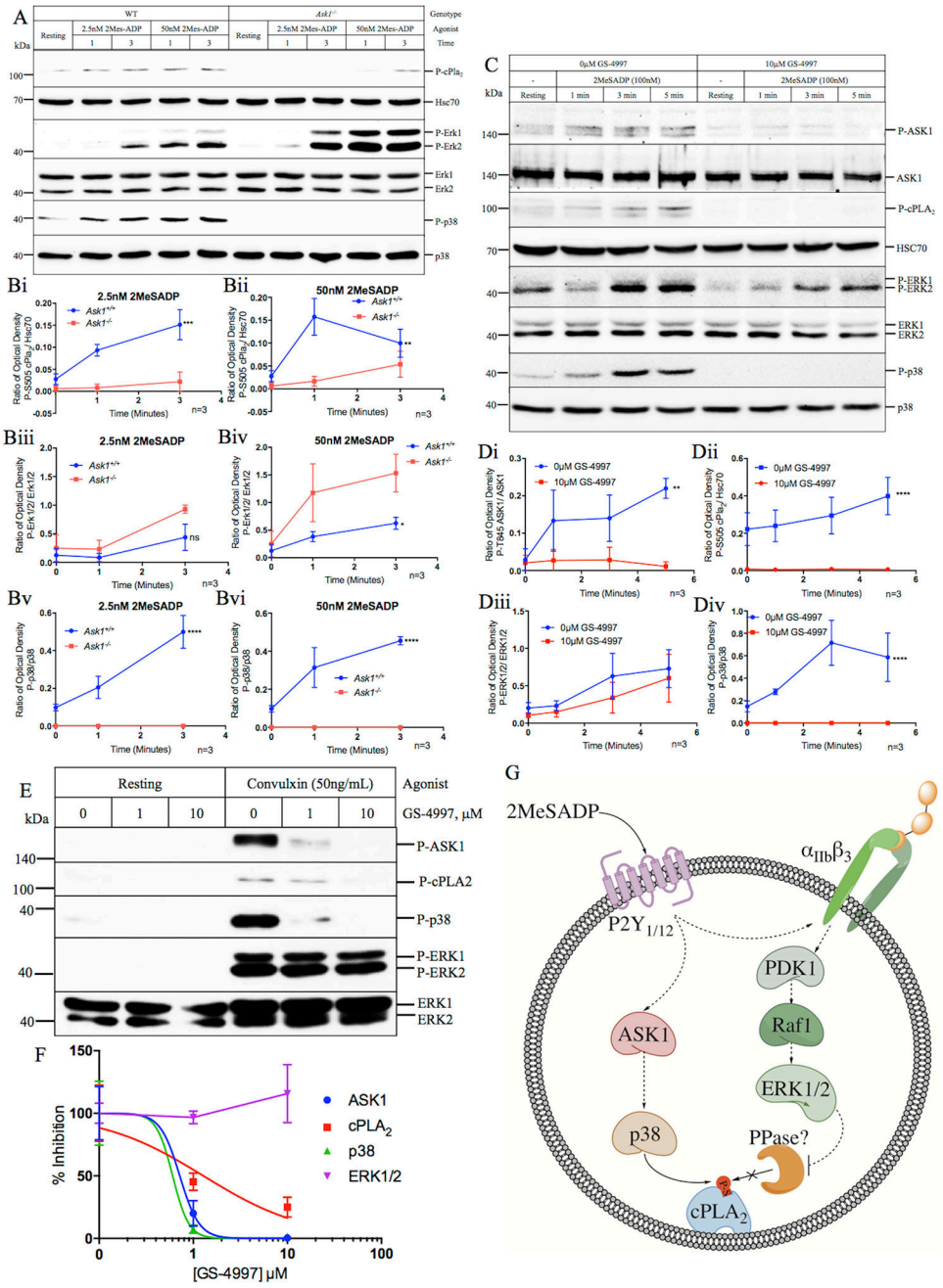

Figure 1. PDK1 and ASK1 regulate ADP-induced cPLA$_2$ phosphorylation and TxA$_2$ generation through two distinct mechanisms

(A) Western blot of lysates of washed murine platelets (2×10^8 platelets/mL) isolated from WT or *Ask1*$^{-/-}$ mice and stimulated with 2MeSADP (2.5 or 50nM). Blots were probed with phospho-specific antibodies against cPla2-Ser^{505}, Erk1/2, and p38 as indicated. Blots were reprobed with anti-Hsc70, anti-p38, and anti-Erk1/2 to ensure equal loading. (B) Quantitation of optical density of bands from A for cPla$_2$-Ser^{505} (Bi–ii), Erk1/2 (Biii–iv), and p38 (Bv–vi) when stimulated with either 2.5nM (Bi, iii, v) or 50nM (Bii, iv, vi) 2MeSADP. (C) Western blot of lysates of washed human platelets (4×10^8 platelets/mL) pretreated with GS-4997 for 20 minutes and stimulated with 2MeSADP (100nM) for 1, 3,

and 5 minutes. Blots were processed as in A. (D) Quantitation of optical density of bands from C for ASK1-Thr^{845} (Di), cPLA$_2$-Ser^{505} (Dii), ERK1/2 (Diii), and p38 (Div). (E) Western blot of human platelet lysate (4×10^8 platelets/mL) pretreated with GS-4997 and stimulated with convulxin (50ng/mL) for 3 minutes. (F) Quantitation of band intensity was expressed as percent inhibition from E. (G) Schematic representation of the signaling mechanism (based on our observations and those of *Manne et al.*,) describing the relationship between ASK1/p38 and PDK1/ERK1/2 as it relates to cPLA$_2$-Ser^{505} phosphorylation. Two-way ANOVA (B & D) and liner regression analysis (F) were performed using Graphpad Prism Software. Representative blots are shown in panel (A, C, and E). Results are from 3 independent experiments.