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The influence of $P2Y_{12}$ receptor deficiency on the platelet inhibitory activities of prasugrel in a mouse model: Evidence for specific inhibition of $P2Y_{12}$ receptors by prasugrel

Masami Hashimoto [a], *Atsuhiro Sugidachi* [a], *Takashi Isobe* [a], *Yoichi Niitsu* [a], *Taketoshi Ogawa* [a], *Joseph A. Jakubowski* [b], *Fumitoshi Asai* [a,*]

[a] *Shinagawa R&D Center, Daiichi Sankyo Co. Ltd., Tokyo, Japan*
[b] *Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, IN, USA*

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ABSTRACT

Prasugrel is a novel orally active thienopyridine with faster, higher and more reliable inhibition of platelet aggregation than clopidogrel reflecting its metabolism *in vivo* to an active metabolite with selective $P2Y_{12}$ antagonistic activity. Several lines of evidence support the contention that prasugrel provides selective $P2Y_{12}$ receptor antagonistic activity. To date, however, direct evidence of $P2Y_{12}$ specific action by prasugrel *in vivo* is limited. In the present study, effects of prasugrel on *ex vivo* platelet aggregation were examined in wild type (WT) and $P2Y_{12}^{-/-}$ mice. In WT mice, prasugrel showed platelet inhibition that was 8.2 times more potent than clopidogrel. In $P2Y_{12}^{-/-}$ mice, ADP induced platelet aggregation was minimal, and its extent was similar to that in prasugrel-treated WT mice. In addition, no further inhibition of platelet aggregation was observed after administration of prasugrel to $P2Y_{12}^{-/-}$ mice. Furthermore, prasugrel-treated WT mice showed similar aggregation patterns using collagen- and murine PAR-4 agonist peptide to those of $P2Y_{12}^{-/-}$ mice treated with vehicle or prasugrel. Overall, these results clearly provide additional *in vivo* evidence that prasugrel has selective $P2Y_{12}$ antagonistic activity.

1. Introduction

Thrombus formation at a site of arterial injury is initiated by the adhesion of platelets to the damaged arterial wall, with several agonists such as adenosine 5′-diphosphate (ADP), 5-hydroxytryptamine and thromboxane A_2, released from the activated platelets, promoting additional platelet activation and aggregation [1]. ADP is one of the more important agonists inducing and accelerating platelet activation and those effects are mediated by at least two subtypes of G-protein coupled ADP receptors existing on the platelet membrane, namely $P2Y_1$ and $P2Y_{12}$ [2,3]. Both $P2Y_1$ and $P2Y_{12}$ ADP receptors are reported to be essential for full platelet activation and aggregation [4,5]. Furthermore, several studies have shown that $P2Y_{12}$ receptors are an effective therapeutic target for antithrombotic agents [6]. The $P2Y_{12}$ receptor was cloned by three groups in 2001 [6–8]. In addition, numerous investigators have elucidated the role of $P2Y_{12}$ in mediating platelet aggregation and thrombus formation using selective $P2Y_{12}$ antagonists such as AR-C69931MX, ticlopidine and clopidogrel

* *Corresponding author at*: Biological Research Laboratories II, Daiichi Sankyo Co. Ltd., 1-2-58 Hiromachi, Shinagawa-ku, Tokyo 140-8710, Japan. Tel.: +81 3 3492 3131; fax: +81 3 5436 8566.
E-mail address: asai.fumitoshi.ux@daiichisankyo.co.jp (F. Asai).
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[9–12]. In parallel with these investigations, study of $P2Y_{12}$-deficient (knockout, KO) mice has been pursued by a number of groups, further clarifying the role of $P2Y_{12}$ in thrombus formation [13,14].

Prasugrel (CS-747, LY640315), now under phase 3 evaluation [15], is a novel thienopyridine antiplatelet prodrug with fast onset, high potency and irreversible action in experimental animals [16,17] and humans [18,19]. This activity is believed to be mediated via $P2Y_{12}$ receptor antagonism by its active metabolite [20,21]. Notably, there is evidence both in healthy subjects and stable cardiac patients that the pharmacodynamic response variability noted with clopidogrel is less after a loading dose of 60 mg prasugrel compared to 300 mg clopidogrel [22,23], supporting the potential clinical utility of prasugrel. To date, *in vitro* and *ex vivo* studies of prasugrel have been persued, however, *in vivo* evidence for selective $P2Y_{12}$ inhibition by prasugrel has not been fully described. In addition, the antiplatelet activity of prasugrel in mice has not been explored.

In the present study, we examined the antiplatelet activity of prasugrel in both WT and $P2Y_{12}$-deficient mice, and report additional *in vivo* evidence that prasugrel specifically inhibits $P2Y_{12}$ receptors.

2. Materials and methods

2.1. Chemicals

Prasugrel hydrochloride ($C_{20}H_{20}FNO_3S{\cdot}HCl$, M.W. 409.90) and clopidogrel hydrogen sulfate ($C_{16}H_{16}ClNO_2S{\cdot}H_2SO_4$, M.W. 419.90) were supplied by Ube Industries, Ltd. (Ube, Yamaguchi, Japan) and stored at −20 °C. Both test compounds were suspended in 5% (w/v) gum arabic (Sigma–Aldrich Co. St. Louis, MO, USA) solution. The vehicle (5% gum arabic solution) or suspensions of prasugrel or clopidogrel were orally administered to mice in a volume of 10 mL/kg. Adenosine 5′-diphosphate (ADP) sodium salt was obtained from Sigma–Aldrich Co. Murine protease activated receptor-4 (mPAR-4) agonist peptide (GYPGKF) was obtained from Sigma Genosys Japan Co. (Ishikari, Hokkaido, Japan). Collagen was obtained from Nycomed Pharma GmbH Co. (Unterschleiβheim, Bayern, Germany). Sodium pentobarbital (Nembutal™) was obtained from Dainippon Pharmaceutical Co. Ltd. (Osaka, Japan).

2.2. Experimental animals

$P2Y_{12}$ receptor-deficient ($P2Y_{12}^{-/-}$) mice were generated according to the report by Foster et al. [13] in the Medicinal Safety Research Laboratories of Sankyo Co. Ltd. To confirm the $P2Y_{12}$-deficient genotype, tail DNA from the putative $P2Y_{12}^{-/-}$ mice was screened for targeted recombination by a PCR strategy, then by Southern blots of restriction enzyme-digested DNA (Fig. 1). The SphI fragment detected by the indicated probe is reduced from 7.6 to 5.5 kb due to a new SphI site in the targeted locus, indicating knockout of $P2Y_{12}$ gene [13]. Age- and gender-matched C57BL/6J × 129S1 control mice which do not differ genetically from $P2Y_{12}^{-/-}$ mice, except at the targeted locus, were used as wild-type (WT) mice. $P2Y_{12}^{-/-}$ mice and their offspring were maintained at Charles River Japan, Inc., and used for the present study at 11–18 weeks of age. The WT mice were also maintained on a mixed C57BL/6J × 129S1 genetic background at Charles River Japan, Inc., and used for the present study at 11–33 weeks of age. The mice were housed in animal quarters set at a constant temperature of 23–24 °C, humidity of 48–68% and a light/dark cycle of 12 h light. They were maintained with free access to water and food (FR-2, Funabashi Farm Co. Ltd., Funabashi, Chiba, Japan) after transporting them to the animal facility of Sankyo Co. Ltd., Tokyo.

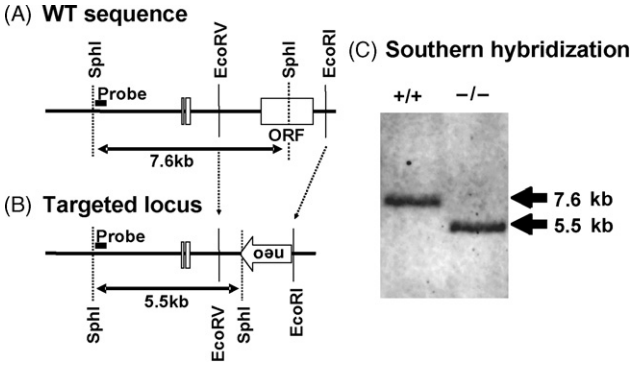

Fig. 1 – (A) Wild type $P2Y_{12}$ gene locus: the exonic sequence is shown by three open rectangles (ORF). (B) Targeted locus: the *neo* gene is inserted. (C) Southern hybridization of SphI-digested genomic DNA from a WT (+/+) and a KO (−/−) mouse. The SphI fragment detected by the indicated probe is reduced from 7.6 to 5.5 kb due to a new SphI site in the targeted locus.

2.3. Platelet preparation

We performed two separate series of experiments, for Fig. 2 and Figs. 3–6, using individual platelet-rich plasma (PRP) and

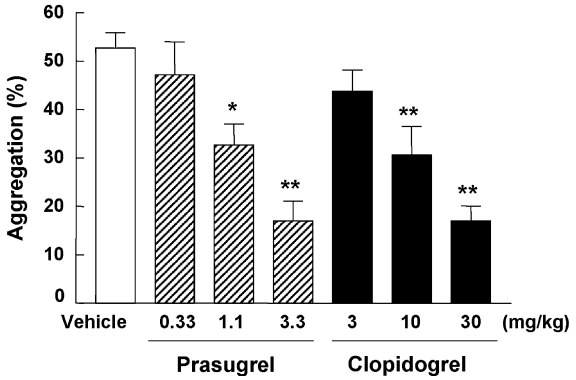

Fig. 2 – *Ex vivo* effects of single oral administration of prasugrel and clopidogrel on ADP induced platelet aggregation in mice. Platelet aggregation induced by ADP (3 μM) was measured 4 h after the dosing of prasugrel (0.33, 1.1 and 3.3 mg/kg) and clopidogrel (3, 10 and 30 mg/kg). Results are expressed as the mean ± S.E. (n = 5). Comparison between the test compound-treated groups and the vehicle-treated control group was carried out using the Dunnett test; *P < 0.05, **P < 0.01 vs. vehicle-treated control group.

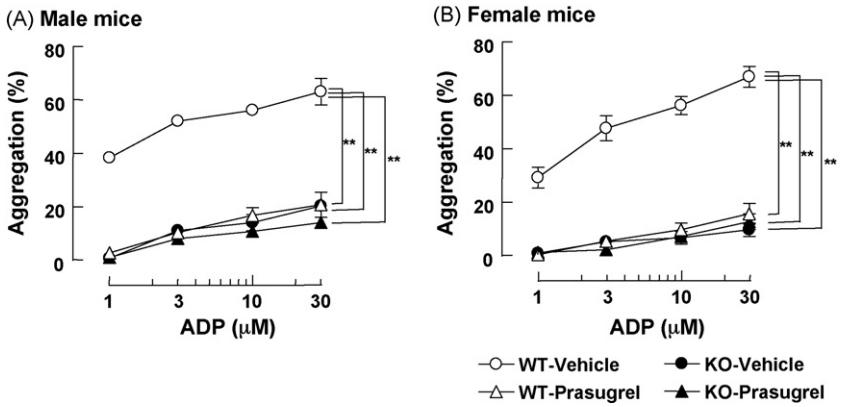

Fig. 3 – *Ex vivo* effects of single oral administration of prasugrel on ADP induced platelet aggregation in wild type (WT) and $P2Y_{12}$-deficient (KO) mice. Prasugrel (3 mg/kg) was orally administered to male (A) and female (B) mice and platelet aggregation induced by ADP (1–30 μM) was measured 4 h after the dosing. Results are expressed as the mean ± S.E. (n = 3–4). Two-way ANOVA followed by Tukey's post hoc test revealed a statistical significance ($P < 0.01$) between the vehicle-treated WT group and the other three groups, but no significance between three groups of prasugrel-treated $P2Y_{12}$-deficient mice, prasugrel-treated WT mice, and vehicle-treated $P2Y_{12}$-deficient mice.**

pooled PRP, respectively. Blood (0.72 mL) was collected from each mouse anesthetized with sodium pentobarbital (100 mg/kg, i.p.) using 1/9 (v/v) volume (0.08 mL) of 3.8% sodium citrate solution as the anticoagulant 4 h after the administration of prasugrel, clopidogrel or vehicle. In the experiments using pooled PRP, the blood, approximately 6 mL, was pooled from 7 to 9 mice and PRP was prepared by centrifuging the blood at 230 × g for 15 min at room temperature (himac CF8DL, Hitachi Koki Co. Ltd.). In the experiment using individual PRP, PRP was prepared by centrifuging the individual blood samples, approximately 0.8 mL at 72 × g for 15 min at room temperature. Platelet-poor plasma (PPP) was obtained by subsequent centrifugation of the remaining blood at 1800 × g for 10 min at room temperature. Platelet counts in PRP were determined by an automated hematology analyzer (KX-21N, Sysmex Corporation) and adjusted to 3×10^8 platelets/mL by adding PPP.

2.4. *Platelet aggregation*

Platelet aggregation was measured with a platelet aggregometer (MCM HEMA TRACER 313M, MC Medical, Inc.). A cuvette with 115 μL of PRP was placed in the aggregometer,

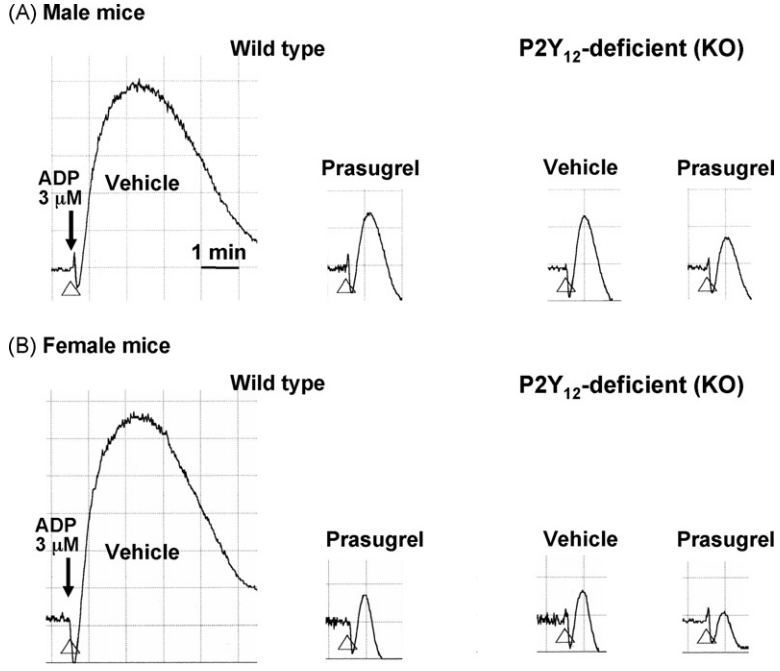

Fig. 4 – Representative tracings of platelet aggregation induced by ADP in male wild type (WT) and $P2Y_{12}$-deficient (KO) mice (A), and female WT and KO mice (B). Prasugrel (3 mg/kg) was orally administered to mice and platelet aggregation induced by ADP (3 μM) was measured 4 h after dosing.

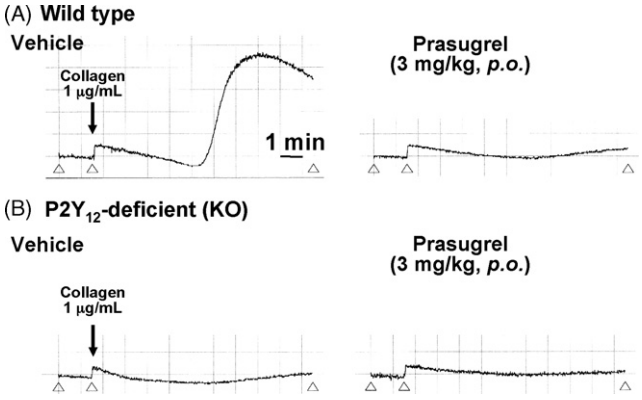

Fig. 5 – Representative tracings of platelet aggregation induced by collagen in male wild type (WT) and $P2Y_{12}$-deficient (KO) mice. Prasugrel (3 mg/kg) was orally administered to mice and platelet aggregation induced by collagen (1 μg/mL) was measured 4 h after dosing.

preincubated at 37 °C for 1.5 min, and then stimulated with a 10 μL solution of ADP (final concentration of 0.3–30 μM), collagen (1 μg/mL) or mPAR-4 agonist peptide (0.75 mM). The concentration of each agonist was selected based on pilot studies that identified minimal concentrations that consistently resulted in approximately 70% aggregation of mouse platelets (data not shown). Platelet aggregation was measured for 2.5–10 min and maximal aggregation (%) recorded.

2.5. Statistical analysis

For Figs. 2 and 3, the mean and standard error (S.E.) for each group were calculated. For Fig. 3, the dose response data were analyzed by two-way ANOVA with data grouped according to ADP concentration (μM) and aggregation (%), followed by Tukey's post hoc test using the SAS System Release 8.2 (SAS Institute Inc.). For Fig. 2, comparison between the test compound-treated groups and vehicle-treated control group was carried out using the Dunnett test (SAS System Release 8.2). A *P*-value of less than 0.05 was considered to be statistically significant. The ED_{50} value, the dose required to inhibit relative platelet aggregation by 50%, was estimated by linear regression analysis using two common logarithmic transformed doses in which the mean relative aggregation included the 50% value.

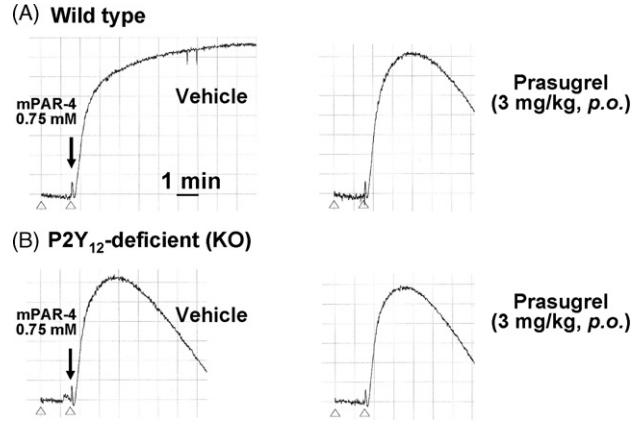

Fig. 6 – Representative tracings of platelet aggregation induced by murine protease-activated receptor-4 (mPAR-4) agonist peptide in male wild type (WT) and $P2Y_{12}$-deficient (KO) mice. Prasugrel (3 mg/kg) was orally administered to mice and platelet aggregation induced by mPAR-4 (0.75 mM) was measured 4 h after dosing.

3. Results

3.1. Generation of WT and $P2Y_{12}^{-/-}$ mice

Fig. 1C shows the result of Southern hybridization of SphI-digested genomic DNA from a WT and a $P2Y_{12}^{-/-}$ mouse and confirmed successful generation of $P2Y_{12}$-deficient mice.

3.2. Effects of prasugrel and clopidogrel on ADP induced platelet aggregation in WT mice

Our preliminary experiments showed that 0.3–30 μM ADP induced mouse platelet aggregation in a concentration-related manner, and near maximal aggregation was observed at 3 μM (data not shown). *Ex vivo* effects of prasugrel on platelet aggregation induced by 3 μM ADP were compared with those of clopidogrel 4 h after oral doses of both compounds in WT mice. Both prasugrel (0.33–3.3 mg/kg, p.o.) and clopidogrel (3–30 mg/kg, p.o.) inhibited platelet aggregation. Significant inhibition compared to vehicle-control was observed at 1.1 mg/kg ($n = 5$, $P < 0.05$) and 3.3 mg/kg ($n = 5$, $P < 0.01$) of prasugrel, and 10 mg/kg ($n = 5$, $P < 0.01$) and 30 mg/kg ($n = 5$, $P < 0.01$) of clopidogrel (Fig. 2). The ED_{50} values of prasugrel and clopidogrel were 1.7 mg/kg (p.o.) and 14 mg/kg (p.o.), respectively. These results showed that in mice prasugrel has 8.2 times more potent inhibitory effects than clopidogrel on ADP induced *ex vivo* platelet aggregation.

3.3. Effects of prasugrel on ADP induced platelet aggregation in WT and $P2Y_{12}^{-/-}$ mice

The effects of prasugrel at 3 mg/kg (p.o.) on ADP (1–30 μM)-induced platelet aggregation were examined in male WT and $P2Y_{12}^{-/-}$ mice. In male WT mice platelet aggregation induced by ADP was inhibited by oral treatment with prasugrel. In male $P2Y_{12}^{-/-}$ (KO) mice, platelet aggregation was minimal compared to vehicle-treated WT mice, and ADP induced aggregation in prasugrel-treated $P2Y_{12}^{-/-}$ mice was similar to that in prasugrel-treated WT male mice. No further inhibition of platelet aggregation was observed by oral dosing of prasugrel (3 mg/kg, p.o.) to $P2Y_{12}^{-/-}$ male mice compared to vehicle-treated $P2Y_{12}^{-/-}$ male mice (Figs. 3A and 4A). The shape change and subsequent minimal aggregation mediated via $P2Y_1$ receptors [4] were observed both in prasugrel-treated WT mice and vehicle- or prasugrel-treated $P2Y_{12}^{-/-}$ mice (Fig. 4A). Similar results were obtained for female WT and $P2Y_{12}^{-/-}$ mice (Figs. 3B and 4B), suggesting that there were no differences in the activity of prasugrel in male versus female mice. These

results are consistent with selective inhibition of $P2Y_{12}$ receptors following the administration of prasugrel.

3.4. *Effects of prasugrel on collagen and mPAR-4 agonist peptide-induced platelet aggregation in WT and $P2Y_{12}^{-/-}$ mice*

We further investigated collagen (1 μg/mL)- and mPAR-4 agonist peptide (0.75 mM)-induced platelet aggregation using male WT and $P2Y_{12}^{-/-}$ mice. Prasugrel at 3 mg/kg (p.o.) almost completely inhibited collagen-induced response in WT mice, but had no effects on mPAR-4 agonist peptide-induced maximal aggregation although an increase in disaggregation was observed (Figs. 5 and 6). In $P2Y_{12}^{-/-}$ mice treated with vehicle or prasugrel, similar platelet responses were observed compared to prasugrel-treated WT mice. When using these alternative platelet activators, platelet aggregation in prasugrel-treated WT mice was similar to that in vehicle-treated and prasugrel-treated $P2Y_{12}^{-/-}$ mice, further supporting the contention that prasugrel is a selective $P2Y_{12}$ receptor inhibitor *in vivo*.

4. Discussion

Antiplatelet activity of prasugrel and prasugrel's active metabolite in several species including human have been reported previously [16–18]. However, to date, antiplatelet activity of prasugrel in mice has not been reported. Accordingly it was necessary for us to first study the activity of prasugrel in WT mice before proceeding to studies in $P2Y_{12}^{-/-}$ mice. In WT mice we found that prasugrel dose-relatedly inhibited ADP induced *ex vivo* mouse platelet aggregation with an ED_{50} value of 1.7 mg/kg (p.o.). Our previous report showed that the ED_{50} value after single oral administration of prasugrel in rats was 1.2 mg/kg [16], indicating the sensitivity of mice to prasugrel's antiplatelet activity is similar to that of rats. We also examined the activity of prasugrel compared to that of clopidogrel in mice. We found that prasugrel was approximately eight times more potent than clopidogrel in accordance with our previous reports using rats, which showed about 13 times more potent effects compared to clopidogrel [16,17]. In addition, clinical studies in healthy human subjects have documented greater inhibition of platelet aggregation by prasugrel than clopidogrel [24,25], although on a mg/kg basis platelet inhibitory doses for both agents are less in man than in mice. Taken together, these results confirm the consistently potent antiplatelet activity of prasugrel across species. These results also supported further experimentation with prasugrel in $P2Y_{12}^{-/-}$ mice.

The first $P2Y_{12}^{-/-}$ mouse model was reported by Foster et al. [13]. André et al. [14] generated $P2Y_{12}^{-/-}$ mice utilizing another gene targeting strategy. Recently, several studies addressing the role of $P2Y_{12}$ in several thrombotic models have been reported using selective $P2Y_{12}$ antagonists [12,26]. However, there have been limited reports using $P2Y_{12}^{-/-}$ mice which addressed critical role of $P2Y_{12}$ on thrombosis. In the present study, we generated $P2Y_{12}^{-/-}$ mouse according to the method by Foster et al. [13], and confirmed $P2Y_{12}$ deficiency by Southern blot analysis. Functional studies showed that ADP induced platelet aggregation in $P2Y_{12}^{-/-}$ mice was minimal (<20%) in response to 30 μM ADP, the highest concentration used. In contrast, collagen and mPAR-4 agonist peptide at relatively high concentrations induced substantial platelet aggregation in $P2Y_{12}^{-/-}$ mice. The effects on platelet aggregation induced by these alternative agonists are consistent with two previous reports [13,14]. In addition, as reported by Foster et al. [13], our pilot studies showed prolongation of tail-transection bleeding time in $P2Y_{12}^{-/-}$ mice compared to WT mice (data not shown). Overall, our genotyping and phenotyping results indicated effective $P2Y_{12}$ deficiency in the mouse model generated for the present studies.

Prasugrel is a potent, orally active thienopyridine prodrug that is metabolized *in vivo* to an active metabolite, which *in vitro* demonstrates selective $P2Y_{12}$ inhibitory activity [17]. Several lines of evidence support the contention that prasugrel provides selective $P2Y_{12}$ receptor inhibition [20,21], but this evidence has mainly been obtained by *in vitro* active metabolite studies. In the present study, we compared the effect of prasugrel at 3 mg/kg (p.o.) on ADP induced platelet aggregation in WT mice with that in $P2Y_{12}^{-/-}$ mice. In WT mice, ADP induced platelet aggregation was largely inhibited by oral administration of prasugrel. In $P2Y_{12}^{-/-}$ mice, ADP induced platelet aggregation was similar to that in prasugrel-treated WT mice. In addition, no further statistically significant inhibition of platelet aggregation was observed after treatment of $P2Y_{12}^{-/-}$ mice with prasugrel. It is well established that the $P2Y_1$ ADP receptor is involved in shape change and in the initiation of platelet aggregation through calcium mobilization [4,27–30]. ADP induced platelet shape change and subsequent minimal aggregation were observed both in prasugrel-treated WT mice and vehicle- or prasugrel-treated $P2Y_{12}^{-/-}$ mice, indicating no effect of prasugrel on $P2Y_1$ supporting its selective *in vivo* inhibition of $P2Y_{12}$ ADP receptors. In addition, there were no apparent differences between male and female mice in these experiments. Taken together, these results clearly provide additional *in vivo* evidence that prasugrel has selective $P2Y_{12}$ antagonistic activity.

We further investigated collagen- and murine protease activated receptor-4 (mPAR-4) agonist peptide-induced platelet aggregation. In WT mice treated with vehicle, significant platelet aggregation induced by collagen (1 μg/mL) was observed, however, both prasugrel-treated WT and vehicle-treated $P2Y_{12}^{-/-}$ mice showed no obvious platelet aggregation induced by collagen. These results are concordant with previous reports that collagen-induced platelet aggregation in rodents is highly dependent on ADP released from activated platelets [31]. Therefore, one would expect no discrepancy between $P2Y_{12}$ selective antagonism in WT mice and inhibition of collagen-induced response in $P2Y_{12}^{-/-}$ mice. Our result further showed that maximum aggregations induced by mPAR-4 agonist peptide were not inhibited in either prasugrel-treated WT or vehicle-treated $P2Y_{12}^{-/-}$ mice compared to vehicle-treated WT mice, however, disaggregation of platelets was enhanced in both prasugrel-treated WT and vehicle-treated $P2Y_{12}^{-/-}$ mice compared to vehicle-treated WT mice. Recently, Kamae et al. [10] indicated that the continuous interaction between released ADP and $P2Y_{12}$ is critical for the maintenance of $\alpha_{IIb}\beta_3$ activation on platelets stimulated with thrombin. Therefore, the enhancement of platelet disaggregation

observed in prasugrel-treated mice in the present study would provide additional evidence for the crucial role of $P2Y_{12}$ on the maintenance of $\alpha_{IIb}\beta_3$ activation. Moreover, in both collagen- and mPAR-4-induced platelet aggregations, prasugrel did not show any further activity in $P2Y_{12}^{-/-}$ mice, suggesting $P2Y_{12}^{-/-}$ selective *in vivo* action of prasugrel.

In conclusion, overall, these results support the contention that prasugrel is a potent and selective $P2Y_{12}$ receptor inhibitor *in vivo*.

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