A Clopidogrel-Insensitive Inducible Pool of P2Y12 Receptors Contributes to Thrombus Formation: Inhibition by Elinogrel, a Direct-Acting, Reversible P2Y12 Antagonist

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ABSTRACT

It is known that hepatic metabolism limits the antiaggregatory activity of clopidogrel and, as a consequence, its clinical benefits. In this study, we investigated whether other factors exist that could account for clopidogrel’s suboptimal antithrombotic activity. Using an in vivo murine FeCl3 thrombosis model coupled with intravital microscopy, we found that at equivalent, maximal levels of inhibition of ADP-induced platelet aggregation, clopidogrel (50 mg/kg p.o.) failed to reproduce the phenotype associated with P2Y12 deficiency. However, elinogrel (60 mg/kg p.o.), a direct-acting reversible P2Y12 antagonist, achieved maximal levels of inhibition in vivo, and its administration (1 mg/kg i.v.) abolished residual thrombosis associated with clopidogrel dosing. Because elinogrel is constantly present in the plasma, whereas the active metabolite of clopidogrel exists for ~2 h, we evaluated whether an intracellular pool of P2Y12 exists that would be inaccessible to clopidogrel and contribute to its limited antithrombotic activity. Using saturation [3H]2-(methylthio)ADP ([3H]2MeSADP) binding studies, we first demonstrated that platelet stimulation with thrombin and convulxin (mouse) and thrombin receptor activating peptide (TRAP) (human) significantly increased surface expression of P2Y12 relative to that of resting platelets. We next found that clopidogrel dose-dependently inhibited ADP-induced aggregation, signaling (cAMP), and surface P2Y12 on resting mouse platelets, achieving complete inhibition at the highest dose (50 mg/kg), but failed to block this inducible pool. Thus, an inducible pool of P2Y12 exists on platelets that can be exposed upon platelet activation by strong agonists. This inducible pool is not blocked completely by clopidogrel, contributes to thrombosis in vivo, and can be blocked by elinogrel.

Introduction

ADP, one of the two most important mediators of hemostasis and thrombosis, binds to two G protein-coupled receptors on platelets. Interaction of ADP with P2Y1 initiates the aggregation reaction, and that with P2Y12 drives sustained aggregation and secretion processes (Gachet, 2001; Andre et al., 2003a). Clinical studies using the thienopyridine clopidogrel, a prodrug undergoing biotransformation to an active metabolite that irreversibly antagonizes P2Y12 receptors (Savi et al., 2000; Yusuf et al., 2001), have demonstrated a significant clinical benefit in patients with acute coronary syndrome (Mehta et al., 2001; Tantry, 2006). It is interesting to note that the results of the GRAVITAS study (Price et al., 2011) comparing single (75 mg) and double (150 mg) chronic doses of clopidogrel showed similar benefits, possibly due to the fact that hepatic metabolism is still a limitation in patients who are poor responders, even at higher doses. However, because genetic polymorphisms in cytochrome P450 (CYP) and transporters (ABCB1), which are involved in clopidogrel metabolism and disposition, only partially explain the correlation with suboptimal clopidogrel response (Camp et al., 2011), one could speculate that other factors exist that could account for a lack of dose response in clinical outcomes as seen in GRAVITAS.

Inhibition of platelet aggregation by direct-acting and reversible antagonists might be preferable to irreversible prodrug inhibitors because of faster onset and offset, less interpatient variability, and higher levels of platelet inhibition with less impact on hemostasis. Ticagrelor, a direct-acting
and reversible allosteric modulator of P2Y_{12}, demonstrated greater clinical benefit over clopidogrel without a proportional increase in overall bleeding risk but increased noncoronary artery bypass graft bleeding in a recent phase III study (Wallentin et al., 2009). Elinogrel (Oestreich, 2010; Ueno et al., 2010), a potent, selective, and direct-acting reversible antagonist of the P2Y_{12} receptor that completed phase II clinical development (Lieu et al., 2007; Leonardi et al., 2010) has been demonstrated to overcome high platelet reactivity in patients with suboptimal response to clopidogrel (Gurbel et al., 2010).

It has been well established that several platelet receptors, such as P2Y_{1}, thromboxane receptor α (Nurden et al., 2003), and integrin α_{IIb}β_{3} (Nurden et al., 1997), are present on the platelet surface but also on the membranes of α granules, constituting an inducible intracellular pool. Upon stimulation with thrombin or collagen, intracellular receptors redistribute to the surface and increase the total receptor number that can mediate biological responses. One example of the importance of blocking both populations of receptors has been shown in a study where internal integrin α_{IIb}β_{3} receptors that were expressed on the surface after potent agonist stimulation were not occupied by the integrin α_{IIb}β_{3} antagonist abciximab, possibly explaining the incomplete inhibition of platelet aggregation observed in response to thrombin receptor activating peptide (TRAP) (Quinn et al., 2001). A possible mechanism involved in the maintenance of the intracellular receptor pool in platelets was described for the thrombin receptor where tonic internalization, mediated by a different mechanism than agonist-induced receptor endocytosis, might be important in sustaining the intracellular pool (Shapiro et al., 1996). Agonist-induced regulation, trafficking, and cellular distribution of the P2Y_{12} receptor have been studied. Initial studies did not demonstrate desensitization of P2Y_{12}-mediated inhibition of cAMP or colocalization with the recycling vesicle marker transferrin in astrocytoma cells transfected with green fluorescent protein-tagged P2Y_{12} (Baurand et al., 2005). However, subsequent studies showed that P2Y_{12} rapidly desensitizes and internalizes in platelets and, when expressed in the 1321N1 cell line, internalizes mainly via G protein-coupled receptor kinase and protein kinase C (novel)-dependent processes (Hardy et al., 2005). In addition, it has been demonstrated that after internalization P2Y_{12} recycles back to the plasma membrane, allowing for rapid resensitization to occur (Mundell et al., 2008). Although there is intracellular distribution of P2Y_{12} receptors in resting platelets (Baurand et al., 2005), it is not known whether P2Y_{12} redistributes to the platelet surface upon stimulation and whether this inducible pool contributes to functional response.

In this study, we determined whether human and mouse platelets have a functional, inducible (intracellular) pool of P2Y_{12} that is biologically active and could account for clopidogrel’s suboptimal antithrombotic activity.

**Materials and Methods**

**In Vivo Thrombosis Model.** Thrombosis on mouse mesenteric arteries (1000–1300 s$^{-1}$) was performed and recorded as described previously with minor modifications (André et al., 2003b). Platelets were labeled in situ using rhodamine 6G (0.2 mg/ml) administered through the tail vein 10 min before the visualization of the arteries. Vessel-wall injury was induced by a 1 × 1 mm filter paper saturated with a 10% FeCl$_3$ solution. After 5 min, the filter paper was removed, and mesenteric arteries were rinsed with warmed saline (37°C). Platelet-vascular wall interactions were recorded for an additional 40 min or until full occlusion occurred and lasted for more than 40 s. C57BL/6J mice or P2Y$_{12}^{-/−}$ mice (André et al., 2003b) were gavaged orally 48, 24 and 2 h before the injury with either vehicle control (0.5% methylcellulose, wild-type and P2Y$_{12}^{-/−}$ mice), clopidogrel (50 mg/kg q.i.d., wild-type mice), elinogrel (60 mg/kg b.i.d. for 3 consecutive days, wild-type mice) (Ueno et al., 2010), or ticagrelor (100 mg/kg b.i.d. for 3 consecutive days). In addition, in some experiments, elinogrel (1 mg/kg) was injected intravenously in clopidogrel-treated mice (50 mg/kg q.i.d., 3 days) after the initiation of the thrombotic process. All of the procedures conformed to institutional guidelines and to the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, 1996).

**Mouse Platelet-Rich Plasma Aggregation.** Aggregations using platelet-rich plasma (PRP) (3.8% citrated mouse blood centrifuged at 200g for 10 min) were performed at 37°C using an aggregometer (Chrono-Log Corp., Havertown, PA) at a stirring speed of 1200 rpm. PRP (3 × 10$^4$ platelets per milliliter) was prepared from vehicle control and clopidogrel-treated (0.5–50 mg/kg) mice (see above). Platelet poor plasma was used for the calibration. Changes in light transmittance were determined over a time period of 6 min after the addition of either ADP (10 μM) (Sigma-Aldrich, St. Louis, MO), murine TRAP (AYPGKF) (1.6 mM) (Bachem California, Torrance, CA), or collagen (20 μg/ml) (Chrono-Log Corp.).

**Preparation of Mouse Washed Platelets.** Washed platelets were prepared as described previously (Jantzen et al., 2001). In brief, 0.7 ml of mouse blood was collected by cardiac puncture into 0.14 ml of acid-citrate-dextrose (38 mM citric acid, 75 mM trisodium citrate, 360 mM NaCl, 5.5 mM glucose, 2.9 mM KCl, and 12 mM NaHCO$_3$ (pH 7.4)) containing 1 mM CaCl$_2$, 1 mM MgCl$_2$, and 0.1% bovine serum albumin.

**Determination of cAMP Levels in Mouse Platelets.** Platelet cAMP levels were measured using the cAMP ELISA system (Assay Designs, Ann Arbor, MI) in washed mouse platelets (2.5 × 10$^8$ platelets per milliliter) isolated from mice treated with either vehicle or clopidogrel (1.5–50 mg/kg) for 3 days. After the incubation with 100 μM 3-isobutyl-1-methylxanthine (Sigma-Aldrich), platelets were treated with ADP (0.01–20 μM) in the presence of 1 μM forskolin (Sigma-Aldrich) for 10 min. Reactions were terminated, processed, and quantified according to the manufacturer’s instructions.

**Platelet Activation as Measured by P-Selectin Activation.** Washed platelets (2.5 × 10$^8$ platelets per milliliter) from vehicle control, P2Y$_{12}^{-/−}$ (André et al., 2003a), or clopidogrel-treated mice (0.5–50 mg/kg) were incubated for 5 min at room temperature with either saline, thrombin (0.5 or 5 nM), or convulxin (0.5 μg/ml). To detect surface P-selectin expression, platelets were incubated with fluorescein isothiocyanate-CD62 antibodies (BD Biosciences, San Jose, CA) for 20 min in the dark at room temperature. Analysis of platelet-bound fluorescein isothiocyanate-CD62 was performed using a FACSort flow cytometer after the collection of 10,000 events.

**Radioligand Binding Studies.** Saturation binding studies were performed using a range of [3H]H2-(methylthio) ADP (2MeSADP) (PerkinElmer Life and Analytical Sciences, Waltham, MA) concentrations (0.1–100 nM) on human and mouse platelets. In initial experiments, the number of total binding sites (P2Y$_{12}$ + P2Y$_{1}$) was established using unlabeled 2MeSADP (20 μM), whereas specific P2Y$_{12}$ or P2Y$_{1}$ binding sites were defined by using selective P2Y$_{12}$ (30 μM elinogrel) or P2Y$_{1}$ (100 μM 2’-deoxy-N’-methyl adenosine 3’,5’-diphosphate (MRS2179)) antagonists. Elinogrel is a competitive P2Y$_{12}$ receptor antagonist and can displace [3H]2MeSADP in radioligand binding studies (Supplemental Fig. 1). Specific binding was...
calculated as the difference between total binding determined in the presence of \[^{3}H\]2MeSADP and nonspecific binding measured in the presence of either excess elinogrel (P2Y_{12}), MRS2179 (P2Y_{1}), or 2MeSADP (P2Y_{12} + P2Y_{1}) (Sigma-Aldrich). On mouse platelets, ~82% of sites labeled by \[^{3}H\]2MeSADP represent P2Y_{12} receptors (total binding sites P2Y_{12} + P2Y_{1} = 914 ± 24; P2Y_{12} = 745 ± 50; P2Y_{1} = 160 ± 11; n = 3). On human platelets, ~95% of sites labeled by \[^{3}H\]2MeSADP can be attributed to P2Y_{12} receptors, and the remainder can be attributed to P2Y_{1}. In further experiments, the number of P2Y_{12} receptors on mouse platelets isolated from vehicle- or clopidogrel-treated mice before and after stimulation with convulxin (Pentapharm, Norwalk, CT) (0.5 μg/ml, 5 min at room temperature) or thrombin (Hematologic Technologies, Inc., Essex Junction, VT) (5 nM, 5 min at room temperature) was assessed by saturation binding, where specific P2Y_{12} binding was determined and B_{max} and K_{d} were calculated using Prism software (GraphPad Software, Inc., San Diego, CA). Binding experiments were performed using murine PRP with and without convulxin treatment and using murine washed platelets with and without thrombin treatment. The same approach was used to examine the number of P2Y_{12} receptors on resting and 5 μM TRAP-stimulated human platelets using PRP.

Duplicate binding reactions were carried out in 0.11 ml of platelets (2 × 10^8 platelets per milliliter) for 20 min at room temperature and were terminated by rapid filtration through GF/c glass fiber filters (Whatman, Clifton, NJ) under vacuum using a Micro96 Harvester (Molecular Devices, Sunnyvale, CA). Radioactivity bound to the filters was measured by scintillation counting in a MicroBeta2 counter (PerkinElmer Life and Analytical Sciences). In some experiments, platelets isolated from vehicle control- and clopidogrel-treated mice were fixed after stimulation with strong agonist by continuous rotation for 25 min in the presence of 4% formaldehyde. Platelets were isolated by centrifugation (1000 g, 10 min) and resuspended in binding buffer (20 mM HEPES and 1 mM MgCl₂) to a density of 2 × 10^8 platelets per milliliter, and binding reactions were carried out as described above.

**Results**

The Effects of Clopidogrel on Blocking ADP-Induced Platelet Aggregation, Surface P2Y_{12} Receptors, and P2Y_{12}-Mediated Signaling on Resting Platelets. We studied the effects of different doses of clopidogrel (1.5–50 mg/kg p.o., 3 days) on multiple outcomes mediated by P2Y_{12}. We first examined ex vivo platelet aggregation induced by 10 μM ADP relative to that of vehicle-treated mice. Clopidogrel treatment dose-dependently inhibited aggregation, reaching near complete inhibition (98%) at the highest dose of 50 mg/kg (1.5 mg/kg clopidogrel, p < 0.01 versus vehicle control; 5–50 mg/kg, p < 0.001 versus vehicle control) (Fig. 1). Platelets isolated from 50 mg/kg clopidogrel-treated mice were functional because they aggregated in response to high concentrations of collagen (20 μg/ml) or murine TRAP (1.6 nM) to a similar level as platelets isolated from vehicle control-treated mice (data not shown).

In addition, oral administration of increasing doses of clopidogrel significantly and dose-dependently inhibited binding of \[^{3}H\]2MeSADP to P2Y_{12} receptors (B_{max}), reaching maximal inhibition (100%) of P2Y_{12} at the highest dose (50 mg/kg) in mouse PRP (Fig. 2A) and mouse washed platelets (Fig. 2B). Although the B_{max} value for P2Y_{12} receptors decreased with increasing clopidogrel concentrations, the dissociation constant (K_{d}) of \[^{3}H\]2MeSADP was not affected significantly (vehicle control, K_{d} = 0.57 ± 0.31 nM; 5 mg/kg clopidogrel, K_{d} = 0.35 ± 0.24 nM), as expected for an irreversible antagonist.

Because P2Y_{12} couples through G_{i} and represses cAMP levels, we next assessed the effects of clopidogrel doses on cAMP signaling induced by ADP. In vehicle control-treated mice, significant inhibition of forskolin-stimulated cAMP by ADP was detected (EC_{50} = 0.24 ± 0.09 μM). In clopidogrel-treated mice, at the lower dose (1.5 mg/kg), we observed a rightward shift of the ADP dose-response curve for the inhibition of cAMP (EC_{50} = 3.04 ± 0.9 μM). The ADP-induced inhibitory effect on forskolin-stimulated cAMP was blocked by higher doses of clopidogrel (15 mg/kg, forskolin versus forskolin + ADP, p = 0.06; 50 mg/kg, forskolin versus forskolin + ADP, p = 0.88), confirming maximal blockade of all P2Y_{12} (Fig. 3).

Our results demonstrated maximal inhibition of ADP-mediated platelet function and signaling as a consequence of maximal blockade of surface P2Y_{12} in 50 mg/kg clopidogrel-treated mice. These data are in agreement with previous studies from P2Y_{12}(-/-) mice showing complete inhibition of ADP-induced platelet aggregation and cAMP-mediated signaling (André et al., 2003a).

Residual Thrombosis In Vivo Observed in 50 mg/kg Clopidogrel-Treated Mice Can Be Blocked by Elinogrel. The antithrombotic activity of clopidogrel (50 mg/kg) next was assessed in vivo using a FeCl_{3}-induced vascular injury model as described previously (André et al., 2003a) and compared directly with the phenotype of P2Y_{12}(-/-) mice. Although clopidogrel (50 mg/kg) prevented vascular occlusion due to an effect on thrombus stability during the 40-min observation period, similar to P2Y_{12}(-/-) mice (André et al., 2011), there were significant differences in the thrombotic profiles in that with clopidogrel-treated animals larger platelet aggregates were observed after clopidogrel treatment (Fig. 4A, right), with the total fluorescence intensity (which reflects the amount of fluorescently labeled platelets at the site of vascular injury accumulated over the 40-min observation period) significantly higher compared with
that of P2Y12(-/-) mice (p = 0.0023) (Fig. 4B). In contrast, elinogrel (60 mg/kg) provided a superior level of inhibition of thrombosis compared with that of clopidogrel (p = 0.02), reproducing the phenotype of P2Y12(-/-) mice (Fig. 4A, right, and 4B). A similar level of inhibition was achieved by ticagrelor (100 mg/kg), another direct-acting P2Y12 antagonist (Fig. 4A, right). Because these data suggested that P2Y12-mediated signaling occurred in vivo through unblocked P2Y12, we next determined whether elinogrel treatment in addition to clopidogrel could provide additive activity. In a second set of experiments, injury was initiated in clopidogrel-treated mice (50 mg/kg p.o., 3 days), and elinogrel was injected subsequently as a 1 mg/kg i.v. bolus via the tail vein. The residual thrombotic process observed in animals treated with clopidogrel was eliminated after intravenous injection of elinogrel (Fig. 4C).

Because 50 mg/kg clopidogrel completely blocked 1) all of the surface P2Y12 receptors on resting platelets, 2) signaling through cAMP, and 3) ADP-induced aggregation to the level observed on platelets from P2Y12(-/-) mice (Andre et al., 2003a), we hypothesized that residual thrombosis observed after the treatment with 50 mg/kg clopidogrel could be mediated by an intracellular pool of P2Y12 exposed on the plate-
A New Pool of \( \text{P2Y}_{12} \) Can Be Expressed on Mouse Platelets upon Strong Agonist Stimulation. To examine whether an inducible (intracellular) pool of \( \text{P2Y}_{12} \) receptors can be exposed on mouse platelets upon activation, we established conditions of maximal platelet activation as assessed by P-selectin expression (an \( \alpha \) granule marker up-regulated after platelet stimulation with strong agonists). Convulxin (0.5 \( \mu \)g/ml) and thrombin (5 nM) induced significant and comparable increases in the expression of P-selectin on mouse platelets (Fig. 5A). Using agonist treatments (convulxin or thrombin) demonstrated to fully activate mouse platelets, we determined the number of surface \( \text{P2Y}_{12} \) receptors present after strong agonist stimulation. A significant increase in \( \text{P2Y}_{12} \) receptor number (\( B_{\text{max}} \)) was observed upon stimulation with convulxin (PRP) or thrombin (washed platelets), with no significant change in \( K_d \) observed in control versus stimulated conditions (control, \( K_d = 1.00 \pm 0.97 \) nM; thrombin-stimulated, \( K_d = 1.17 \pm 1.00 \) nM) (Fig. 5, B and C).

Clopidogrel Does Not Completely Block the Inducible Pool of \( \text{P2Y}_{12} \). We next examined whether an increase in \( \text{P2Y}_{12} \) receptor number could be detected after thrombin stimulation using platelets isolated from clopidogrel-treated mice (5–50 mg/kg). We first established the concentration of thrombin that caused maximal stimulation of the platelets isolated from clopidogrel-treated mice, as was seen previously with the platelets from clopidogrel-naive animals (Table 1) (because 50 mg/kg clopidogrel induced complete inhibition of \( [\text{H}]\text{2MeSADP} \) binding to \( \text{P2Y}_{12} \), we could not determine \( B_{\text{max}} \) for the nontreated condition). We also confirmed this result using formaldehyde-fixed platelets, which minimizes possible internalization of \( \text{P2Y}_{12} \) receptors on the platelets from mice treated with clopidogrel (Mundell et al., 2006). In this method, platelets were fixed after stimulation with the agonist and before measuring the level of P-selectin expression on the platelet surface. Unlike platelets from control mice where both concentrations of thrombin (0.5 and 5 nM) induced comparable increases in P-selectin expression (data not shown), the higher concentration of thrombin (5 nM) was required to achieve the same level of P-selectin expression on the platelets from 50 mg/kg clopidogrel-treated mice. This was confirmed in \( \text{P2Y}_{12/ -/ -} \) mice where only the higher thrombin concentration (5 nM) caused maximal P-selectin expression (data not shown).

To determine whether clopidogrel blocked this inducible pool of \( \text{P2Y}_{12} \) receptors, we determined the number of \( \text{P2Y}_{12} \) receptors on the platelets from mice treated with clopidogrel (using saturation binding studies with \( [\text{H}]\text{2MeSADP} \) and unlabeled 30 \( \mu \)g/ml elinogrel) performed on untreated (control) platelets and platelets stimulated with convulxin (0.5 \( \mu \)g/ml) or thrombin (5 nM). Values are expressed as the mean ± S.E.M.; a representative experiment of three experiments is shown. B, representative saturation binding experiment measuring \( [\text{H}]\text{2MeSADP} \) binding to untreated (control) platelets and platelets stimulated with convulxin (0.5 \( \mu \)g/ml) or thrombin (5 nM). C, significant increase in the surface \( \text{P2Y}_{12} \) receptor number was observed upon platelet stimulation with convulxin (untreated, 798 ± 56; convulxin-stimulated, 1014 ± 67, \( p = 0.03; n = 3 \)) or thrombin (untreated, 872 ± 87; thrombin-stimulated, 1162 ± 98, \( p = 0.001; n = 5 \)). Values are expressed as mean ± S.E.M.

**TABLE 1**

Increase in \( \text{P2Y}_{12} \) receptor number on mouse platelets upon stimulation with thrombin.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Platelet ( \pm ) S.E.M.</th>
<th>( n )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle Control</td>
<td>872 ± 87</td>
<td>5</td>
</tr>
<tr>
<td>Clopidogrel, 1.5 mg/kg</td>
<td>1162 ± 98</td>
<td>5</td>
</tr>
<tr>
<td>Clopidogrel, 5 mg/kg</td>
<td>1202 ± 101</td>
<td>5</td>
</tr>
<tr>
<td>Clopidogrel, 10 mg/kg</td>
<td>1342 ± 132</td>
<td>5</td>
</tr>
<tr>
<td>Clopidogrel, 50 mg/kg</td>
<td>1432 ± 153</td>
<td>5</td>
</tr>
</tbody>
</table>

\( B_{\text{max}} \) could not be calculated for the nontreated condition due to complete inhibition of \( [\text{H}]\text{2MeSADP} \) binding to \( \text{P2Y}_{12} \); see text under Results.
performing radioligand binding studies using a single [3H]2MeSADP concentration (50 nM) and conditions allowing assessment of P2Y12-specific binding. Using fixed conditions, we also observed an increase in surface expression of P2Y12 in the platelets from vehicle control- and clopidogrel-treated mice (5, 15, and 50 mg/kg) (Fig. 6) (**, p < 0.05; ***, p < 0.01, thrombin-stimulated versus corresponding unstimulated), demonstrating that clopidogrel does not completely block this inducible pool (Fig. 6).

**Human Platelets Express an Inducible Pool of P2Y12 upon Strong Agonist Stimulation.** We next assessed whether an inducible pool of P2Y12 exists in human platelets. Stimulation of human platelets with 5 μM or 20 μM TRAP peptide induced a comparable and significant increase in the expression of P-selectin measured by flow cytometry (Fig. 7A). We then addressed whether newly exposed P2Y12 receptors can be detected using saturation radioligand binding to determine P2Y12 receptor number on unstimulated (control) platelets versus those stimulated with TRAP (5 μM). A representative saturation binding experiment with and without treatment with 5 μM TRAP is shown in Fig. 7B. The increase in P2Y12 receptor number observed upon TRAP stimulation was significant (untreated, 423 ± 28; TRAP-stimulated, 529 ± 28 p = 0.02; n = 3) (Fig. 7C), with no significant change in Kd observed in control versus stimulated conditions (control, Kd = 3.6 ± 1.7 nM; TRAP-stimulated, Kd = 5 ± 1.5 nM). These data confirm that, similar to mouse platelets, an inducible pool of P2Y12 receptors exists on human platelets and that these internal receptors can be mobilized to the platelet surface after strong agonist stimulation.

**Discussion**

Dosing of mice with clopidogrel (50 mg/kg) completely inhibited ADP-induced ex vivo platelet aggregation and signaling (cAMP) and blocked all of the surface P2Y12 receptors on resting platelets. It is interesting to note that the same high dose of clopidogrel (50 mg/kg) prevented

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**Fig. 6.** P2Y12 Receptor up-regulation on platelets isolated from clopidogrel-treated mice (5–50 mg/kg p.o., 3 days) upon thrombin stimulation. Radioligand binding was performed (50 nM [3H]2MeSADP radioligand; unlabeled 30 μM elinogrel) in formaldehyde-fixed mouse platelets. An increase in specific P2Y12 binding was observed on platelets from vehicle control- and clopidogrel-treated mice after treatment with 5 nM thrombin. Data are expressed as mean ± S.E.M. (n = 3–6) (thrombin-stimulated versus respective unstimulated controls; *, p < 0.05; **, p < 0.01; ***, p < 0.001).

**Fig. 7.** New P2Y12 receptors are exposed on human platelets after stimulation with TRAP. A, P-selectin expression on human platelets was measured by flow cytometry analysis of fluorescein isothiocyanate-CD62 antibody binding to resting (control) or TRAP-stimulated (5 and 20 μM) platelets. Values are expressed as mean fluorescence ± S.E.M.; a representative experiment is shown. B, representative experiments of saturation binding (0.01–100 nM [3H]2MeSADP radioligand; competition with unlabeled 30 μM elinogrel) performed on untreated (control) or TRAP-stimulated (5 μM) platelets. C, significant increase in the surface P2Y12 receptor number was observed upon platelet stimulation with TRAP (p = 0.02, n = 3).
vascular occlusion but did not achieve the thrombotic profile associated with P2Y12 deficiency, whereas the direct-acting inhibitor elinogrel did recapitulate the profile of P2Y12(-/-) mice. Addition of elinogrel (1 mg/kg i.v.) to this clopidogrel regimen (50 mg/kg, 3 days) completely blocked the residual thrombosis, confirming that this process was mediated by P2Y12 receptors. On the basis of this data, we hypothesized that residual thrombosis observed in clopidogrel-treated mice might be due to an unblocked (intracellular) pool of P2Y12 exposed on the platelet surface after platelet activation in vivo. In this study, we demonstrated that an inducible pool of P2Y12 receptors exists in human and mouse platelets and becomes exposed after the activation with strong platelet agonists (e.g., TRAP peptide, convulxin, or thrombin). This inducible pool of P2Y12 that contributed to the thrombosis could be blocked by elinogrel but not by clopidogrel.

A number of platelet receptors have been shown to be distributed as an extracellular pool on the plasma membrane and a second intracellular pool on the α granule membrane and/or in the open canalicular system (Nurden et al., 1997, 2003). The existence of receptors in two populations, extracellular and intracellular, needs to be taken into account when evaluating drug efficacy, because this could lead to improper dosing (Quinn et al., 2001). Although the P2Y12 receptor has been shown to be present at the plasma membrane and intracellular localization was detected in resting platelets (Baurand et al., 2005), the precise intracellular distribution and possible redistribution to the plasma membrane have not been studied. Agonist-induced regulation of P2Y12 and postendocytic trafficking in platelets has been studied recently. These studies demonstrated that P2Y12 is desensitized in platelets by a G protein-coupled receptor kinase-dependent mechanism and undergoes agonist-induced phosphorylation and internalization (Mundell et al., 2006). The same authors showed that P2Y12 recycles back to the surface after agonist-induced internalization and that any disruption in P2Y12 trafficking (either internalization or recycling) blocks resensitization of P2Y12 (Mundell et al., 2008). In the present study, we detected an increase in the number of P2Y12 receptors expressed on human platelets in response to TRAP stimulation (25%) and on mouse platelets after stimulation with convulxin (27%) or thrombin (33%). This is the first demonstration of P2Y12 up-regulation, suggesting the existence of an inducible (intracellular) pool of P2Y12. In a previously published study (Judge et al., 2008), an intracellular pool of P2Y12 receptors was not detected. A possible explanation for this discrepancy might be due to differences in the methodologies between the two studies. In the study by Judge et al. (2008), there was a greater time interval between platelet stimulation and radioligand binding studies (due to a centrifugation step), which may have resulted in receptor trafficking (internalization) that possibly prevented detection of the newly exposed pool of P2Y12. To avoid receptor trafficking, formaldehyde-fixed platelets can be used, an approach described previously (Mundell et al., 2006). One important observation in our study is the incomplete blockage of the inducible pool of P2Y12 by clopidogrel. Thrombin induced the expression of intracellular P2Y12 receptors despite treatment with clopidogrel (50 mg/kg) at a dose that completely blocked surface-expressed P2Y12 on resting platelets. In addition, these data also demonstrate that the inducible pool of P2Y12 can be exposed on platelets isolated from mice treated with suboptimal doses of clopidogrel. This observation might be important in the context of clopidogrel treatment in human patients where different levels of P2Y12 receptor occupancy and variability in clopidogrel-induced inhibition of ADP-mediated aggregation have been demonstrated (Bal Dit Sollier et al., 2009). It is interesting to note that our data presented herein demonstrated a discrepancy between inhibition of the P2Y12 receptor and platelet aggregation. Indeed, although sub-maximal doses of clopidogrel (0.5–5 mg/kg) inhibited P2Y12 receptor binding by 65 to 86% on resting platelets, it only inhibited ADP-mediated aggregation by 32 to 57%. Thus, a limited number of functional receptors is sufficient to provide a sustained aggregation response, suggesting that exposure of a new pool of P2Y12 (25–35% increase) could have a significant impact on thrombosis, as shown in our in vivo experiments. Our in vivo data in mice also demonstrated that, when the all of the surface P2Y12 is blocked after chronic clopidogrel treatment, there is an inducible pool of P2Y12 expressed after platelet stimulation that is not inhibited by the clopidogrel active metabolite but is fully functional and capable of mediating platelet thrombosis. This inducible pool seems to preferentially affect initial growth of the thrombus triggered by highly thrombogenic materials exposed and/or released at the site of vascular injury, as illustrated by the greater slope of thrombus growth immediately after the injury in clopidogrel-treated animals relative to that in elinogrel-treated animals (Fig. 4A).

Data presented here may have significant implications in the clinical setting. After daily oral dosing of clopidogrel in humans, platelets are exposed transiently to a "pulse" (1–2 h) of active metabolite, which inactivates a percentage of the total platelet population. If a plaque rupture occurs and initiates thrombosis after this pulse of active metabolite has passed, then the newly mobilized P2Y12 receptors on the platelet surface that are unblocked by clopidogrel would be "active" and could contribute to the thrombotic process. Hence, intraday repetitive dosing of clopidogrel could be warranted for optimal protection. Another alternative is the use of a direct-acting P2Y12 antagonist that does not require metabolic conversion to an active metabolite, because this class of inhibitor is present throughout the daily dosing cycle and may contribute to superior clinical outcomes.

In conclusion, results from this study show that 1) an inducible pool of P2Y12 exists on the platelets and can be exposed upon platelet activation by strong agonists, 2) this inducible pool is not completely blocked by clopidogrel, and 3) this pool contributes to thrombosis in vivo. In addition, different properties and mechanisms of action between irreversibly prodrugs (thienopyridines) and direct-acting, reversible P2Y12 antagonists may provide insight into the differences observed in clinical trials between these two classes of P2Y12 antagonists.

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Clonidogrel-Insensitive Pool of P2Y12 Receptors

Authorship Contributions

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References


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A clopidogrel-insensitive inducible pool of P2Y<sub>12</sub> receptors contributes to thrombus formation: Inhibition by elinogrel, a direct-acting reversible P2Y<sub>12</sub> antagonist

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Supplemental Figure 1
Competitive binding experiments on mouse washed platelets. Data are expressed as % of total binding. Elinogrel dose-dependently inhibited 10 nM [H]2MeSADP binding to platelets P2Y<sub>12</sub> receptors with the IC<sub>50</sub> value of 52nM. Data are expressed as mean ± sem; n=2.