Multiple Cytochrome P450 Isoforms Are Involved in the Generation of a Pharmacologically Active Thiol Metabolite, whereas Paraoxonase 1 and Carboxylesterase 1 Catalyze the Formation of a Thiol Metabolite Isomer from Ticlopidine

Min-Jung Kim, Eun Sook Jeong, Jung-Soon Park, Su-Jun Lee, Jong Lyul Ghim, Chang-Soon Choi, Jae-Gook Shin, and Dong-Hyun Kim

Department of Pharmacology and Pharmacogenomics Research Center, College of Medicine (M.-J.K., E.S.J, J.-S.P., S.-J.L., J.L.G, J.-G.S., D.-H.K.), and Department of General Surgery, Busan Paik Hospital (C.-S.C.), Inje University, Busan, South Korea

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ABSTRACT

Ticlopidine is a first-generation thienopyridine antiplatelet drug that prevents adenosine 5'-diphosphate (ADP)-induced platelet aggregation. We identified the enzymes responsible for the two-step metabolic bioactivation of ticlopidine in human liver microsomes and plasma. Formation of 2-oxo-ticlopidine, an intermediate metabolite, was NADPH dependent and cytochrome P450 (CYP) 1A2, 2B6, 2C19, and 2D6 were involved in this reaction. Conversion of 2-oxo-ticlopidine to thiol metabolites was observed in both microsomes (M1 and M2) and plasma (M1). These two metabolites were considered as isomers, and mass spectral analysis suggested that M2 was a thiol metabolite bearing an exocyclic double bond, whereas M1 was an isomer in which the double bond was migrated to an endocyclic position in the piperidine ring. The conversion of 2-oxo-ticlopidine to M1 in plasma was significantly increased by the addition of 1 mM CaCl₂. In contrast, the activity in microsomes was not changed in the presence of CaCl₂. M1 formation in plasma was inhibited by EDTA but not by other esterase inhibitors, whereas this activity in microsomes was substantially inhibited by carboxylesterase (CES) inhibitors such as bis-(p-nitrophenyl)phosphate (BNPP), diisopropylphosphorofluorid (DFP), and clopidogrel. The conversion of 2-oxo-ticlopidine to M1 was further confirmed with recombinant paraoxonase 1 (PON1) and CES1. However, M2 was detected only in NADPH-dependent microsomal incubation, and multiple CYP isoforms were involved in M2 formation with highest contribution of CYP2B6. In vitro platelet aggregation assay demonstrated that M2 was pharmacologically active. These results collectively indicated that the formation of M2 was mediated by CES isoforms whereas M1, an isomer of M2, was generated either by human PON1 in plasma or by CES1 in the human liver.

Introduction

Ticlopidine [5-(2-chlorophenyl)methyl-4,5,6,7-tetrahydrothieno [3,2-c] pyridine] (Fig. 1) was the first thienopyridine antiplatelet agent with potent and long-acting inhibition of platelet aggregation (Noble and Goa, 1996). It inhibits adenosine 5'-diphosphate (ADP)-induced platelet aggregation by irreversible binding of an active thiol metabolite to the 2-methylthio-ADP-binding receptor. Although it is effective in preventing atherothrombotic events in cardiovascular, cerebrovascular, and peripheral vascular disease, administration of ticlopidine results in a relatively high incidence of hematoxicities (Lesese et al., 1994; Love et al., 1998) such as agranulocytosis (Ono et al., 1991), thrombotic thrombocytopenic purpura (Steinhubl et al., 1999), and aplastic anemia (Mataix et al., 1992). Therefore, clopidogrel, a second-generation thienopyridine antiplatelet agent is a safer, better-tolerated alternative to ticlopidine.

A large inter-individual variability of platelet responsiveness was observed in patients receiving a standard regimen of clopidogrel (Gurbel et al., 2003), and this variation was related to CYP2C19 genetic polymorphisms (Shuldiner et al., 2009; Hulot et al., 2011; Yin and Miyata, 2011; Cuisset et al., 2012). Clopidogrel is converted to a biologically active thiol metabolite via a two-step enzymatic activation that involves an initial thiolactone metabolite formation by CYP3A, 2B6, and 2C19, followed by a thiol metabolite formation produced primarily by CYP2C19 (Kazui et al., 2010). Recently, paraoxonase 1 (PON1) was shown to be involved in the generation of a thiol metabolite (Bouman et al., 2011; Dansette et al., 2012a).

Ticlopidine is reported to be a useful alternative therapy for the treatment of patients showing resistance to clopidogrel (Aleil et al., 2007). In addition, the antiplatelet effects of ticlopidine are not affected by CYP2C19 genetic polymorphisms (Farid et al., 2010; Maeda et al., 2011), suggesting that other drug metabolizing enzymes may be involved in the formation of a thiol metabolite from 2-oxo-ticlopidine.

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Even though the initial formation of 2-oxo-ticlopidine from ticlopidine is known to be mediated by multiple CYP isoforms (Farid et al., 2010), the pathways that activate ticlopidine have not been identified. Therefore, the characterization of enzymes involved in the bioactivation of ticlopidine could be useful in understanding in vivo pharmacologic activity and differences in the effects of CYP2C19 polymorphisms among thienopyridine antiplatelet agents.

Our study characterized enzymes responsible for the formation of a thiolactone and an active thiol metabolite in human using in vitro systems. Our results showed that multiple CYP isoforms were involved in the formation of 2-oxo-ticlopidine from ticlopidine and the formation of an active thiol metabolite (exo-form) from 2-oxo-ticlopidine in human liver microsomes. In addition, a ring-opened thiol metabolite isomer (endo-form) was generated mainly by carboxylesterase 1 (CES1) in human liver microsomes and primarily by PON1 in human plasma.

Materials and Methods

Chemicals and Reagents. Ticlopidine hydrochloride, clopidogrel hydrogen sulfate, coumarin, diethylidithiocarbamate, furafylline, ketoconazole, montelukast, quinidine, sulfaphenazole, thio-TEPA, β-nicotinamide adenine dinucleotide phosphate, loperamide, glutathione (GSH), bis-(p-nitrophenyl)phosphate (BNPP), diisopropylphosphorofluoridate (DFP), phystostigmine sulfate (eserine), phenylmethylsulfonyl fluoride (PMSF), 3'-methoxyphenacyl bromide (MPB), 2-methylthiодenosine diposphate (2MeSADP), fibrinogen, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase were purchased from Sigma-Aldrich (St. Louis, MO). We obtained 2-oxo-ticlopidine, 2-oxo-clopidogrel hydrochloride, clopidogrel carboxylic acid, clopidogrel carboxylic acid-d₆, and S-benzylthriparanol from Toronto Research Chemicals (Toronto, Canada). The solvents were high-performance liquid chromatography (HPLC)-grade (Fisher Scientific Co., Pittsburgh, PA), and the other chemicals were of the highest quality available.

Baculovirus-insect cell expressed CYP1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 3A4 (Supersomes), CES1 (2300 nmol/min/mg for 4-nitrophenyl acetate assay), CES2 (1300 nmol/min/mg for 4-nitrophenyl acetate assay), and control microsomes were purchased from BD Gentest Co. (Woburn, MA). Recombinant human carboxymethylenebutenolidase (CMBL) was kindly donated by Shiseido, Tokyo, Japan (with the peak signal intensity of the methoxy group to those of tetramethylsilane spiked solution/acetoniitle (72/27, v/v) at a flow rate of 1.2 ml/min. The peaks were repeatedly collected, lyophilized, and analyzed by nuclear magnetic resonance (NMR). The concentrations of MPB-thiol metabolite were estimated by comparing the peak area with 2-oxo-ticlopidine standard solution/acetonitrile (72/27, v/v) at a flow rate of 1.2 ml/min. The peaks were repeatedly collected, lyophilized, and analyzed by nuclear magnetic resonance (NMR). The concentrations of MPB-thiol metabolite were estimated by comparing the peak area with 2-oxo-ticlopidine standard solution.

Construction of Recombinant Vectors for PON1 Variants. The wild-type PON1 cDNA cloned in the pCR4-TOPO plasmid (Invitrogen). The transfected cells were cultured for 1 day in FreeStyle 293 medium before expression.

Isolation and Characterization of MPB-Derivatized Ticlopidine Thiol Metabolite. Because a MPB-derivatized ticlopidine metabolite standard was not available, the MPB-derivatized metabolite was separated and fractionized by HPLC. First, the MPB-derivatized ticlopidine active metabolite peak was identified using liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). Samples of microsomes incubated with 200 μM 2-oxo-ticlopidine were subjected to LC-MS/MS, and the [M+H]+ ion at m/z 446 was consistent with an MPB-derivatized ticlopidine metabolite structure (Fig. 3).

Fractionation was performed using an Agilent 1100 series HPLC system (Agilent, Palo Alto, CA) and CAPCELL PAK C18 (UG120) column (4.6 × 250 mm, 5 μm; Shiseido, Tokyo, Japan) with a mobile phase consisting of 0.1% formic acid in water and 0.1% formic acid in acetonitrile. The peaks were repeatedly collected, lyophilized, and analyzed by nuclear magnetic resonance (NMR). The concentrations of MPB-thiol metabolite were estimated by comparing the peak signal intensity of the methoxy group to those of tetramethylsilane spiked solution/acetoniitle (72/27, v/v) at a flow rate of 1.2 ml/min. The peaks were repeatedly collected, lyophilized, and analyzed by nuclear magnetic resonance (NMR). The concentrations of MPB-thiol metabolite were estimated by comparing the peak area with 2-oxo-ticlopidine standard solution/acetonitrile (72/27, v/v) at a flow rate of 1.2 ml/min. The peaks were repeatedly collected, lyophilized, and analyzed by nuclear magnetic resonance (NMR). The concentrations of MPB-thiol metabolite were estimated by comparing the peak area with 2-oxo-ticlopidine standard solution.

Expression and Purification of Human PON1 in a Mammalian Cell Line. FreeStyle 293-F cells were transfected with 30 μg of pcDNA-DEST40 empty vector, pcDNA-DEST40-PON1-His-WT, or pcDNA-DEST40-PON1-His-variants using DNA-293fectin, according to the manufacturer’s instructions (Invitrogen). The transfected cells were cultured for 1 day in FreeStyle 293 medium.
expression medium (Invitrogen). The cells were harvested and resuspended in lysis buffer containing 50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, and 0.05% Tween 20 (pH 8.0). After centrifugation at 10,000g for 10 minutes, the supernatant was mixed with Ni-NTA slurry (Qiagen Inc., Valencia, CA). The overexpressed histidine-tagged proteins were purified by Ni-affinity chromatography with an Econo-Column (cat no. 737-1052; Bio-Rad Laboratories, Hercules, CA.). The elutes were collected and dialyzed against 1 liter of 50 mM Tris- HCl buffer containing 5% glycerol (pH 7.5); the resultant proteins were stored at −80°C until use. Protein concentration was determined by using the method of Bradford with bovine serum albumin as the standard.

Immuno blotting. Human liver microsomes (30 µg), cellular proteins trans- fected with empty vector (30 µg/ lane), and purified PON1 proteins (1 µg/ lane) were separated by electrophoresis in a NuPAGE 4%–12% Bis-Tris gel (Invitrogen) and transferred to a polyvinylidene difluoride (PVDF) membrane (GE Healthcare Bio-Sciences, Piscataway, NJ). The membranes were blocked with 5% nonfat milk in 10 mM Tris- HCl, pH 7.4, including 0.9% NaCl (TBS). Specific anti-PON1 IgG (Abcam, Cambridge, MA) was used at a 1:2500 dilution as the primary antibody. The membranes were washed thoroughly with TBS plus 0.1% Tween 20 (TBST). Immunoreactive proteins were detected using the enhanced electrochemiluminescence system (GE Healthcare Bio-Sciences).

Formation of 2-Oxo-Ticlopidine in Human Liver Microsomes and cDNA-Expressed CYP. The incubation mixture consisted of 0.2 mM 2-oxo- phosphatase, 3.3 mM glucose 6-phosphate, 3.3 mM MgCl₂, and 1.0 U/ml human liver microsomes (0.2 mg/ml), and an NADPH-generating system in the presence of inhibitors and their concentrations were described elsewhere (Seo et al., 2012).

CYP2C19; 10 µM quinidine for CYP2D6; 10 µM ketoconazole for CYP3A. The CYP isoform selective inhibitors were used as follows: 10 µM thio-TEPA for CYP2B6; 10 µM sulfaphenazole for CYP2C9; 10 µM montelukast for CES1 and CES2 (Preuss and Svensson, 1996; Takahashi et al., 2009), EDTA for PON (Gonzalvo et al., 1997), codiglodip for CES1 (Shi et al., 2006), loperamide for CES2 (Quimney et al., 2005), and PMSF for serine hydrolase (Johnson and Moore, 2000). Each inhibitor was used at 0.01, 0.1, and 1.0 mM. The experimental procedure was similar to those for metabolism in human plasma and liver microsomes. Activities of all inhibitors were compared with those of inhibitor-free controls. CYP isoform selective inhibitors were used as described earlier.

Dialysis. To evaluate the effect of dialysis, human plasma and microsomes were placed in 10,000 molecular-weight-cutoff dialysis tubes (Slide-A-Lyzer MINI Dialysis Unit; Thermo Scientific, Waltham, MA) and dialyzed for 20 hours at 4°C against 2-1.0 M Tris- HCl buffer (pH 8.0). The dialyzed plasma and microsomes were incubated with 2-oxo-ticlopidine in the presence and absence of 1.0 mM CaCl₂ for 5 minutes. After adding MBP and the internal standard, the samples were evaluated using the LC-MS/MS system.

Correlation Experiments. For correlation analysis, active thiol metabolite formation rates from 2-oxo-ticlopidine in 20 human liver microsomes were determined by incubating 2-oxo-ticlopidine (50 µM) with 0.2 mg/ml microsomal protein for 5 minutes. Codiglodip hydrolysis activity as a marker of CES1 was measured by LC/MS/MS, as described elsewhere (Sato et al., 2012). P450 hydrolysis activity was measured as a marker activity of PON1, as described elsewhere (Richter et al., 2009). The correlation coefficients between active thiol metabolite formation rates and those of codiglodip and paraoxon hydrolysis in the microsomes were evaluated using Pearson’s correlation coefficient analysis (SAS version 4.3; SAS Institute Inc., Cary, NC). P < 0.05 was considered statistically significant.

LC-MS/MS Analysis. HPLC was performed using an ACQUITY UPLC system (Waters, Milford, MA). The analytical column was a reverse-phase Kinetex C18 (2.1 × 100 mm i.d., 2.6 µm; Phenomenex, Torrance, CA) equipped with a Turbo ion spray source. Electrospray ionization was performed in the positive ion mode with nitrogen as the drying (700 l/h) and nebulizing (50 l/h) gas. The capillary voltage used was 3500 V and extraction cone voltage 3 V for all compounds. The desolvation temperature was 350°C and source temperature 120°C. Quadrupoles Q1 and Q3 were set on unit resolution.

Multiple reaction monitoring detection was performed using argon as the collision gas. Quantitation of metabolites was performed by monitoring the transitions of m/z 280→125 for 2-oxo-ticlopidine, m/z 338→155 for 2-oxo-codiglodip (internal standard for 2-oxo-ticlopidine), m/z 446→154 for MBP-endo thiol metabolite (M1), m/z 446→266 for MBP-endo-thiol metabolite (M2), and m/z 308→198 for codiglodip carboxylic acid (internal standard for MBP-thiol metabolite). The analytical data were processed using the MassLynx software (version 4.1; Waters).

In Vitro Platelet Aggregation. Washed platelets were prepared from fresh blood taken from healthy male subjects as described elsewhere (Abell and Liu, 2011). Final isolated platelets were responded in modified Tyrode’s buffer containing 2 mM calcium chloride and 1 mg/ml human fibrinogen with cell density between 200,000 and 250,000 platelets/µL. Washed platelets (870 µL) were transferred into Eppendorf tubes. Then, 10 µL of 2-oxo-ticlopidine stock solution was added to the final concentration of 5 and 20 µM, and the contents were mixed by inversion. The reaction was initiated by adding 120 µL of prewarmed microsomal incubation mixture (2.0 mg/ml microsomes, 25 mM glucose-6-phosphate, 1 mM NADP⁺, 1 mM glucose-6-phosphate dehydroge- nase in 0.1 M phosphate buffer, pH 7.4). After 10 minutes of incubation at 37°C, 140 µL of the reaction mixture was pipetted into the wells of a clear-bottomed microtiter plate followed by the immediate addition of 10 µL of 15 mM 2MeSADP to start the aggregation reaction.

Platelet aggregation was measured by reading the optical density at 595 nm in a preheated (37°C) plate reader (SpectraMax, Molecular Devices, Sunnyvale,
Characterization of Thiol Metabolite Formation in Human Liver Microsomes and Plasma. The identification and quantitation of thiol metabolites of ticlopidine were performed after derivatization with MPB. 2-Oxo-ticlopidine was incubated for 5 minutes at 37°C with human liver microsomes, an NADPH-generating system, and 5 mM glutathione as a reducing agent. Then HPLC analysis was performed after derivatization of the thiol metabolite by MPB treatment. Two thiol metabolites (M1 and M2) were identified in microsomes, but only one thiol metabolite (M1) was detected when incubated without an NADPH-generating system (Fig. 3, A and B). M1 was also detected when 2-oxo-ticlopidine was incubated with human plasma.

Both metabolites showed a protonated ion at m/z 446 and 448 with the ratio expected for the Cl35 and Cl37 isotopes (Fig. 3, C and D). However, M1 and M2 produced different product ion mass spectra, as shown in Fig. 3, E and F. The MS/MS spectrum of protonated M1 showed the fragment ions at m/z 154 and 125. The fragment ions at m/z 154 (chlorobenzyl methyliminium moiety in ticlopidine) and 125 (chlorobenzyl group) were the ions typically observed in ticlopidine (Talakad et al., 2011). On the other hand, the MS/MS spectrum of protonated M2 contained the ions at m/z 296, 266, 154, 140, and 125. The product ion at m/z 296 was generated by the loss of 3'-methoxyphenone moiety (MH+–150), and the ion at m/z 266 was postulated due to the loss of 3'-methoxyphenone containing sulfur moiety (MH+–180).

Previous reports demonstrated that the thiol metabolite bearing exocyclic double bond (exo-form) showed the same fragment pattern, yielding the corresponding product ion at m/z 354 (MH+–150) in clopidogrel (Dansette et al., 2012a) and at m/z 348 (MH+–150) in prasugrel (Dansette et al., 2012b). In addition, the thiol metabolite in which the double bond was migrated to an endocyclic position (endo-form) did not produce these fragment ions. Instead, the ions at m/z 212 (clopidogrel) and 206 (prasugrel), equivalent to the corresponding ion at m/z 154 in ticlopidine, were observed as the most abundant ion in clopidogrel and prasugrel, respectively. Based on MS/MS spectra, M1 and M2 were provisionally assigned to be an endo-form and exo-form of the thiol metabolite, respectively.
Formation of thiol Metabolites in Human Liver Microsomes and Plasma. Conversion of 2-oxo-ticlopidine to M1 was observed regardless of the presence of NADPH in microsomal incubation whereas M2 was generated only in the presence of NADPH (Fig. 4A). In addition, the formation of M1 was not inhibited by 1-aminobenzotriazole, a general CYP inhibitor (data not shown). To assess whether another tissue source was responsible for the catalytic conversion of 2-oxo-ticlopidine to M1, 2-oxo-ticlopidine was incubated with human plasma. M1 formation was also observed in an incubation of 2-oxo-ticlopidine with human plasma. In addition, the formation rate was increased by 2–2.6-fold in the presence of 1 mM CaCl₂ whereas it was abolished almost completely after dialysis of plasma, suggesting that the catalytic conversion of 2-oxo-ticlopidine to M1 might be mediated by PON1 (Fig. 4B). The involvement of PON1 in the formation of the thiol metabolite from 2-oxo-clopidogrel has been reported previously (Bouman et al., 2011; Dansette et al., 2012a). The addition of CaCl₂ to dialyzed plasma restored the activity. In contrast, M1 formation from 2-oxo-ticlopidine in microsomes was not significantly changed in the presence of CaCl₂. Dialysis of microsomes for 24 hours resulted in a 37% loss of activity, which was not restored by addition of CaCl₂. These results collectively indicated that M1 formation was mediated by enzymes other than CYP or PON1.

Kinetic analyses for the formation rates of M1 and M2 from 2-oxo-ticlopidine were conducted using human plasma and liver microsomes (Fig. 5). The parameters of enzyme kinetics are summarized.

Fig. 3. HPLC chromatograms of incubations of 2-oxo-ticlopidine with human liver microsomes with (A) and without (B) an NADPH-generating system with MS detection at m/z 446 after derivatization with 3′-methoxy-phenacyl bromide, mass spectra of M1 (C) and M2 (D), and product ion mass spectra of M1 (E) and M2 (F).

Fig. 4. Formation rates of thiol metabolites after incubation of 2-oxo-ticlopidine with human liver microsomes in the presence and absence of an NADPH-generating system (A), and effects of Ca²⁺ and dialysis on M1 formation rates in human plasma and microsomes (B). Each data point represents the mean ± S.D. of triplicate determinations.
in Table 1. Conversion of 2-oxo-ticlopidine to M1 and M2 followed a single enzyme Michaelis-Menten kinetics. The $K_m$ and $V_{max}$ for M1 formation in plasma were calculated to be 306 $\mu$M and 3.72 nmol/min/mg, respectively, and the kinetic values in microsomes were 382 $\mu$M and 5.81 nmol/min/mg, respectively. Addition of 1 mM CaCl$_2$ caused a 2.3-fold increase in $V_{max}$ value without affecting $K_m$ value in plasma whereas it did not change the $K_m$ and $V_{max}$ values in case of microsomes. The $K_m$ and $V_{max}$ for M2 formation in microsomes were calculated to be 22.8 $\mu$M and 0.13 nmol/min/mg, respectively. In vitro intrinsic clearance (CL$_{int}$) for M1 formation was approximately 3-fold higher than that for M2 formation in microsomes.

**Chemical Inhibition of Formation of M1 in Human Liver Microsomes and Plasma.** To further characterize the enzymes involved in the formation of M1 from 2-oxo-ticlopidine, chemical inhibition studies were performed using representative esterase inhibitors; BNPP and DFP as CES inhibitors; eserine as an inhibitor of ChE, CES1, and CES2; EDTA as a PON inhibitor; clopidogrel as a CES1 inhibitor; loperamide as a CES2 inhibitor; and PMSF as a serine hydrolase inhibitor. Concentrations of each esterase inhibitor were 0.01, 0.1, and 1.0 mM. Catalytic activity was completely inhibited by 0.1 mM EDTA but not by other esterase inhibitors in plasma (Fig. 6A). However, M1 formation in microsomes was substantially inhibited by CES inhibitors such as BNPP, DFP, clopidogrel, and PMSF in a concentration-dependent manner. In addition, the conversion of 2-oxo-ticlopidine to M1 in microsomes was mildly inhibited by eserine whereas no inhibition was observed by EDTA and loperamide (Fig. 6B). The chemical inhibition results indicated the possible involvement of CES, especially CES1, in M1 formation in human liver microsomes.

**Identification of the Human Esterases Responsible for M1 Formation.** The enzymes involved in the formation of M1 from 2-oxo-ticlopidine were further confirmed using various cDNA-expressed enzymes including PON1 (wild-type, Q192R, and L55M), CES1, CES2, and CMBL. M1 formation rates from 2-oxo-ticlopidine (50 $\mu$M) using PON1, CES1, CES2 and CMBL are shown in Fig. 7. PON1 catalyzed the conversion of 2-oxo-ticlopidine to M1 at the highest rate. CES1 also showed considerable catalytic activity with $\approx 15\%$ of the formation rate of M1 by PON1. In contrast, CES2 and CMBL showed negligible catalytic conversion of 2-oxo-ticlopidine to M1.

To determine whether a PON1 polymorphism affects M1 formation from 2-oxo-ticlopidine, M1 formation rates were measured after the incubation with cDNA-expressed PON1 variants (wild-type, Q192R, and L55M). The PON1 gene has a common polymorphism within coding region causing amino acid substitution that results in a change in catalytic activity (Mackness et al., 1998; Bhattacharyya et al., 2008). Expression of PON1 proteins was confirmed by immunoblotting with a monoclonal anti-PON1 antibody (Fig. 8A). PON1 192Q showed the highest catalytic activity (5.06 $\pm$ 0.24 nmol/min/mg) with 50 $\mu$M 2-oxo-ticlopidine in the presence of 1 mM CaCl$_2$. The catalytic activities of PON1 192R and L55M were 85% and 50%, respectively, that of PON1 192Q (Fig. 8B). These results suggested that the genetic polymorphisms of PON1 Q192R and L55M might affect the formation of M1 from 2-oxo-ticlopidine.

### Table 1

<table>
<thead>
<tr>
<th>Enzyme Source</th>
<th>$K_m$ ($\mu$M)</th>
<th>$V_{max}$ (nmol/min/mg)</th>
<th>$CL_{int}$ ($\mu$L/min/mg protein)</th>
<th>$K_m$ ($\mu$M)</th>
<th>$V_{max}$ (nmol/min/mg)</th>
<th>$CL_{int}$ ($\mu$L/min/mg protein)</th>
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<tr>
<td>Plasma</td>
<td>274</td>
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<td>12.3</td>
<td>382</td>
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<td>32.9</td>
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<tr>
<td>Plasma + CaCl$_2$</td>
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<td>20.3</td>
<td>22.8</td>
<td>0.13</td>
<td>5.83</td>
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<tr>
<td>Microsome</td>
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<td>29.3</td>
<td>22.8</td>
<td>0.13</td>
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<tr>
<td>Microsome + CaCl$_2$</td>
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<td>5.32</td>
<td>19.0</td>
<td>22.8</td>
<td>0.13</td>
<td>5.83</td>
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* Concentration of CaCl$_2$ was 1 mM.
We performed kinetic analyses for M1 formation by cDNA-expressed PON1 variants and CES1 to determine the substrate affinity and intrinsic clearance of PON1 and CES1 (Fig. 7B and Fig. 8C). The parameters of enzyme kinetics are summarized in Table 2. M1 formation in recombinant PON1 variants (wild-type, Q192R, and L55M) and CES1 also exhibited single-enzyme Michaelis-Menten kinetics. The $K_m$ and $V_{max}$ values of CES1 were 277 $\mu$M and 2.16 nmol/min/mg, respectively, resulting in $CL_{int}$ value of 7.8 L/min/mg. In contrast, the $K_m$ and $V_{max}$ values of PON1 192Q were 338 $\mu$M and 145 nmol/min/mg, respectively. The $K_m$ values of PON1 192R and 55M were similar to that of PON1 192Q, but $V_{max}$ values were decreased by 38% and 71%, respectively. Kinetic analyses showed that genetic polymorphisms in PON1 gene did not affect substrate binding affinity but decreased the catalytic conversion rates of 2-oxo-ticlopidine to M1.

To further characterize the role of CES1 in M1 formation in human liver microsomes, M1 formation rates from 2-oxo-ticlopidine were compared with clopidogrel hydrolysis activities, a known marker activity of CES1 (Sato et al., 2012), in 20 human liver microsomes (Fig. 9). M1 formation rates were statistically significantly correlated with the rates of clopidogrel hydrolysis ($r = 0.9110, P < 0.0001$). M1 formation rates were also considerably correlated with paraoxon...
hydrolysis rates, but a lesser degree of correlation was observed ($r = 0.5999$, $P = 0.0066$).

Identification of CYP Isoforms Responsible for M2 Formation. M2 formation was observed in human liver microsomes only when an NADPH-generating system was added. To characterize CYP isoforms involved in the formation of M2, chemical inhibition with CYP isoform selective inhibitors and metabolism with cDNA-expressed CYP were performed. The formation of M2 in microsomes was not substantially inhibited by any of CYP-selective inhibitors used at 5 and 50 $\mu$M 2-oxo-ticlopidine (data not shown). No significant inhibition of M2 formation by CYP isoform selective inhibitors might be due to the involvement of multiple CYP isoforms. The formation rates of M2 from 2-oxo-ticlopidine (5 and 50 $\mu$M) by CYP1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, and 3A4 are shown in Fig. 10. CYP2B6 and 2C19 catalyzed the conversion of 2-oxo-ticlopidine to M2 with similar rates at both concentrations. CYP1A2 and 2D6 also showed considerable activity, but the M2 formation rates were 8% and 28% of CYP2C19 catalytic activity, respectively. However, relative formation rates of M2 by CYP1A2, 2D6, and 3A4 compared with that by CYP2C19 were increased while CYP2B6-mediated rate was not much changed at a 50 $\mu$M 2-oxo-ticlopidine.

Kinetic analyses for the formation of M2 from 2-oxo-ticlopidine were performed using cDNA-expressed CYP1A2, 2B6, 2C19, 2D6, and 3A4 (Fig. 11 and Table 3). Conversion of 2-oxo-ticlopidine to M2 by CYP2C19 and 2D6 followed a single enzyme Michaelis-Menten kinetics. On the other hand, the reaction mediated by CYP1A2, 2B6, and 3A4 was best fitted by a Hill equation. Eadie-Hofstee plots of M2 formation by CYP1A2, 2B6, and 3A4 showed the convex relationship, indicating positive cooperativity ($n > 1$). The saturation of CYP2B6- and 2C19-mediated M2 formation at relatively low concentrations of 2-oxo-ticlopidine might be due to metabolism-dependent inactivation of these enzymes by 2-oxo-ticlopidine. The metabolism-dependent inactivation of CYP2B6 and CYP2C19 by ticlopidine is well characterized (Nishiya et al., 2009a,b). Although the $V_{\text{max}}$ values for M2 formation by CYP2C19, CYP2D6, and CYP3A4 were greater (2.0-, 3.4- and 2.3-fold, respectively) compared with that of CYP2B6, the $K_{m}$ values obtained from CYP2C19, CYP2D6, and CYP3A4 were much larger (7.4-, 38.3- and 337-fold, respectively) than that of CYP2B6. Consequently, in vitro $CL_{\text{int}}$ for CYP2B6-catalyzed M2 formation was 3.6-, 11.3-, and 155-fold faster compared with CYP2C19, CYP2D6, and CYP3A4, respectively.

In Vitro Inhibition of Platelet Aggregation. To clarify the pharmacologic activity of M1 and M2, in vitro platelet aggregation

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**TABLE 2**

Kinetic parameters of M1 formation from 2-oxo-ticlopidine by human cDNA-expressed esterases

<table>
<thead>
<tr>
<th>Enzyme Source</th>
<th>$K_{m}$ (mM)</th>
<th>$V_{\text{max}}$ (nmol/min/mg protein)</th>
<th>$CL_{\text{int}}$ (mL/min/mg protein)</th>
</tr>
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<tbody>
<tr>
<td>PON1 wild type</td>
<td>388</td>
<td>145</td>
<td>374</td>
</tr>
<tr>
<td>PON1 Q192R</td>
<td>346</td>
<td>90.1</td>
<td>260</td>
</tr>
<tr>
<td>PON1 L55M</td>
<td>355</td>
<td>42.2</td>
<td>119</td>
</tr>
<tr>
<td>CES1</td>
<td>277</td>
<td>2.16</td>
<td>7.80</td>
</tr>
</tbody>
</table>

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Fig. 8. The effects of PON1 polymorphisms (Q192R and L55M) on M1 formation from 2-oxo-ticlopidine. Immunoblotting of recombinant PON1 192Q, 192R, and 55M expressed in Free Style 293-F cells (A). Formation rates of M1 from 2-oxo-ticlopidine by mock, recombinant human PON1 192Q, 192R, and 55M. The proteins were incubated with 50 $\mu$M 2-oxo-ticlopidine (B). Kinetics of M1 formation from 2-oxo-ticlopidine by PON1 192Q (○), 192R (□), and 55M (▲) (C). Each data point represents the mean ± S.D. of triplicate determinations.
assay was performed. Because M2 was generated by CYP isoforms and M1 was formed by NADPH-independent esterases in microsomes, washed platelets were incubated with 2-oxo-ticlopidine and microsomes in the presence and absence of an NADPH-generating system. Inhibition of 2MeSADP-induced platelet aggregation was observed when 2-oxo-ticlopidine was incubated with microsomes in the presence of an NADPH-generating system (24 and 38% IPA in 5 and 20 μM, respectively) whereas no substantial inhibition was noted in the incubation without an NADPH-generating system (Fig. 12).

Discussion

Our results suggest that ticlopidine is metabolized to thiol metabolites via two-step enzymatic reactions. Multiple CYP isoforms were involved in the conversion of ticlopidine to 2-oxo-ticlopidine, which was further metabolized to ring-opened thiol metabolites either by PON-1 and CES1 (M1 formation), or by CYP2B6, 2D6, and 2C19 (M2 formation) (Fig. 1).

It is generally accepted that clopidogrel, a structural analog of ticlopidine, is converted to a thiol metabolite by two-step enzymatic conversion and that CYP isoforms are responsible for both steps of the enzymatic reaction (Kazui et al., 2010). CYP2C19 is the principal enzyme responsible for the second step enzymatic conversion of 2-oxo-clopidogrel to the thiol metabolite. Resistance to clopidogrel therapy is due primarily to CYP2C19 polymorphisms (Hulot et al., 2011; Yin and Miyata, 2011; Cuisset et al., 2012). Recently, PON1 was reported to be a major enzyme responsible for the formation of a thiol metabolite of clopidogrel (Bouman et al., 2011). However, the contribution of PON1 to thiol metabolite formation was limited to the hydrolysis of the endo-form of 2-oxo-clopidogrel while CYP are primarily responsible for the hydrolysis of the exo-form of 2-oxo-clopidogrel, leading to the generation of two cis diastereomers (Dansette et al., 2012a). 2-Oxo-ticlopidine can also exist as two rotamers (endo- and exo-forms), and at least two ticlopidine thiol metabolite isomers could be expected in in vitro incubation.

Two ring-opened thiol metabolites were identified in our system, and their structures were characterized on the basis of their product ion mass spectra and the fragmentation pattern of thiol metabolites of clopidogrel and prasugrel, structural analogs of ticlopidine, as presented elsewhere (Dansette et al., 2012a,b). M1 observed in plasma and microsomal incubation was characterized as a thiol metabolite in which the double bond was migrated to an endocyclic position in the piperidine ring (endo-form). On the other hand, M2 generated only in microsomal incubation in the presence of an NADPH-generating system was identified as a thiol metabolite bearing an exocyclic double bond (exo-form).

PON1 is the major enzyme responsible for the metabolic conversion of 2-oxo-ticlopidine to M1 in plasma, and CES1 is the primary mediator of the reaction in human liver microsomes. Involvement of plasma PON1 was verified using several approaches. First, the M1 formation rate in plasma was increased in the presence of 1 mM CaCl2. In addition, dialysis of plasma completely abolished the catalytic activity and addition of 1 mM CaCl2 to dialyzed plasma restored the activity to the original level, indicating that M1 formation in plasma is dependent on Ca2+. Second, plasma activity was completely inhibited by EDTA but not by other esterase inhibitors such as BNPP, eserine, and clopidogrel. Finally, cDNA-expressed
PON1 showed strong activity toward the formation of M1. These results collectively indicated that PON1 was primarily involved in M1 formation from 2-oxo-ticlopidine. PON1 is known to be involved in the hydrolysis of lactone or thiolactone derivatives such as pilocarpine (Billecke et al., 2000; Hioki et al., 2011) and homocysteine thiolactone (Borowczyk et al., 2012).

The primary contribution of CES1 to thiol metabolite formation in liver microsomes was somewhat unexpected when considering the distribution of PON1 in the liver and the structure of 2-oxo-ticlopidine. PON1 is known to be distributed in liver microsomes and in the cytosol (Gonzalvo et al., 1998), and considerable paraoxon hydrolysis activity was detected in our system. However, microsomal conversion of 2-oxo-ticlopidine to M1 was not affected by the addition of CaCl₂. The activity in microsomes was also not inhibited by 1 mM EDTA, a concentration that resulted in complete loss of the activity in human plasma. The involvement of CES1 in M1 formation was further demonstrated using esterase inhibitors such as BNPP, eserine, and clopidogrel. Clopidogrel is hydrolyzed primarily by CES1 (Hagihara et al., 2009; Farid et al., 2010) and eserine is a more selective inhibitor.

**Table 3**

<table>
<thead>
<tr>
<th>CYP Isoform</th>
<th>( K_m ) (( \mu M ))</th>
<th>( V_{max} ) (pmol/min/pmol CYP)</th>
<th>( CL_{int} ) (( \mu L/min/pmol CYP ))</th>
<th>hill coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A2</td>
<td>128</td>
<td>0.40</td>
<td>0.003</td>
<td>1.61</td>
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<tr>
<td>CYP2B6</td>
<td>0.59</td>
<td>0.73</td>
<td>1.24</td>
<td>1.69</td>
</tr>
<tr>
<td>CYP2C19</td>
<td>4.39</td>
<td>1.49</td>
<td>0.34</td>
<td>—</td>
</tr>
<tr>
<td>CYP2D6</td>
<td>22.6</td>
<td>2.50</td>
<td>0.11</td>
<td>—</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>199</td>
<td>1.67</td>
<td>0.008</td>
<td>1.54</td>
</tr>
</tbody>
</table>

* Hill coefficient.

Fig. 11. Kinetics for the formation of M2 from 2-oxo-ticlopidine in human cDNA-expressed CYP 1A2 (A), 2B6 (B), 2C19 (C), 2D6 (D), and 3A4 (E). An increasing concentration of 2-oxo-ticlopidine was incubated with each recombinant CYP (20 pmol/ml) and an NADPH-generating system at 37°C for 5 minutes. The corresponding Eadie-Hofstee plots are shown in the insert, respectively. The kinetic data were fitted by a Hill equation. Each data point represents the mean ± S.D. of triplicate determinations.

Fig. 12. Inhibition of in vitro 2MeSADP-induced aggregation of washed human platelets by 2-oxo-ticlopidine. Washed platelets were incubated with 5 \( \mu M \) and 20 \( \mu M \) 2-oxo-ticlopidine and microsomes in the presence and absence of an NADPH-generating system. Each data point represents the mean ± S.D. of triplicate determinations.
of CES1 than CES2 (Takahashi et al., 2009; Sato et al., 2012). Furthermore, recombinant CES1 showed marked activity in terms of M1 formation, whereas recombinant CES2 did not. A significant correlation \((r = 0.9110, P < 0.0001)\) between clopidogrel hydrolysis rates and M1 formation rates in a panel of human liver microsomes additionally supported the role of CES1 in microsomes. M1 formation rates were also correlated with paraoxon hydrolysis rates \((r = 0.5999, P = 0.0066)\) but the degree of correlation was less than that with clopidogrel hydrolysis rates. Our results collectively demonstrated that CES1 was primarily responsible for the conversion of 2-oxo-ticlopidine to M1 in human liver microsomes. Considering chemical inhibition data, other esterases such as PON-1 might also be involved in the formation of M1 from 2-oxo-ticlopidine but their contribution seemed to be much smaller compared with CES-1. It is generally known that drugs containing ester and thioester linkage can be served as good substrates for CES (Williams, 1985). Hydrolysis of cyclic lactone or thiolactone by CES1 has not been reported to our best knowledge, although lactone ring-containing statins such as simvastatin, lovastatin, and mevastatin can inhibit the activities of recombinant CES1. The presence of a lactone ring in the statin backbone is essential for inhibition of CES1 (Fleming et al., 2005; Fukushima et al., 2010).

PON1 has two common polymorphisms, Q192R and L55M. The Q192R polymorphism affects the hydrolysis of various substrates as the kinetics of some substrates are accelerated (e.g., paraoxon) whereas others are slowed down (e.g., soman and diazoxon) or unaffected (e.g., phencylacetate) (Billecke et al., 2000). Olmesartan medoxomil and prulifloxacin, which are converted to an active metabolite by PON1, are known to be more efficiently bioactivated by PON1 192R (Tougou et al., 1998; Ishizuka et al., 2010). In addition, a lactone derivative pilocarpine is also hydrolyzed more rapidly by PON1 192R (Hioki et al., 2011). However, our results showed that the \(CL_{int}\) value of the 2-oxo-ticlopidine hydrolyase activity by recombinant PON1 192Q was 1.65- and 3.62-fold greater than that of recombinant PON1 192R and PON1 55M, respectively. 2-Oxo-ticlopidine, which contains a thiolactone moiety, may bind differentially to the polymorphic PON1 than pilocarpine and olmesartan medoxomil.

Unlike the formation of M1 from 2-oxo-ticlopidine, M2 was exclusively generated by CYP isoforms. Experiments with cDNA-expressed CYP isoforms showed that multiple CYP isoforms were involved in the formation of M2 although their catalytic activities varied. Kinetic analyses showed that the substrate binding affinity of CYP2B6 (\(K_m\) value of 0.59 \(\mu\)M) was 7.4- to 337-fold greater than those of other CYP isoforms. In addition, in vitro \(CL_{int}\) for CYP2B6-catalyzed M2 formation from 2-oxo-ticlopidine was 413-, 3.6-, 11.3-, and 155-fold greater than the corresponding values for CYP1A2, 2C19, 2D6, and 3A4, respectively (Table 3). These results indicate that CYP2B6 plays a major role in the conversion of 2-oxo-ticlopidine to M2. CYP2C19 and CYP2D6 also contribute to the formation of M2 to a considerable extent, but the role of CYP1A2 and 3A4 seems to be negligible in human liver microsomes. The ratios of formation rate of M2 were 4-fold higher than that of M1 in microsomes. The physiologic role of endo-metabolites in thienopyridine antiplatelet agents may need to be further clarified.

Because only the clopidogrel thiol metabolite that possesses the cis configuration with the double bond outside the pyridine ring is known to be pharmacologically active (Tufufl et al., 2011), we assessed the pharmacologic activity of M1 and M2 using in vitro platelet aggregation assay. Inhibition of 2-MeSADP-induced platelet aggregation by 2-oxo-ticlopidine was observed in micromolar incubation only in the presence of an NADPH-generating system, suggesting that M2 was mainly responsible for the inhibition of platelet aggregation like other thienopyridine antiplatelet agents. Therefore, contribution of CYP2B6 to the formation of M2 along with CYP2C19 and 2D6 may explain how ticlopidine is effective in patients resistant to clopidogrel therapy, as evidenced by Aleil et al. (2007), and the antiplatelet effects of ticlopidine are not affected by CYP2C19 genetic polymorphisms (Farid et al., 2010; Maeda et al., 2011).

In conclusion, our results have demonstrated that multiple CYP isoforms are involved in the formation of 2-oxo-ticlopidine from ticlopidine and the formation of a pharmacologically active thiol metabolite (exo-form) from 2-oxo-ticlopidine in human liver microsomes. In addition, a ring-opened thiol metabolite isomer (endo-form) is generated mainly by CES1 in human liver microsomes and primarily by PON1 in human plasma. These results support that ticlopidine could be an alternative therapy in case of pharmacologic resistance to clopidogrel, showing that its antiplatelet effects are affected by CYP2C19 genetic polymorphisms.


