1521-009X/42/1/141–152$25.00
DRUG METABOLISM AND DISPOSITION

http://dx.doi.org/10.1124/dmd.113.053017
Drug Metab Dispos 42:141–152, January 2014

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[S]

Min-Jung Kim, Eun Sook Jeong, Jung-Soon Park, Su-Jun Lee, Jong Lyul Ghim, Chang-Soo 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

Received May 22, 2013; accepted October 29, 2013

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 $CaCl_2$. In contrast, the activity in microsomes was not changed in the presence of $CaCl_2$. 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), diisopropylphosphorofluoride (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 CYP 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 hematologic toxicities (Lesesve 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.

This work was supported by a National Research Foundation of Korea (NRF) grant funded by the Korean Government (MEST) [No. R13-2007-023-00000-0].
dx.doi.org/10.1124/dmd.113.053017.
[S] This article has supplemental material available at dmd.aspetjournals.org.

ABBREVIATIONS: BNPP, bis-(*p*-nitrophenyl)phosphate; CES, carboxylesterase; CL_{int}, intrinsic clearance; CMBL, carboxymethylenebutenolidase; CYP, cytochrome P450; DFP, diisopropylphosphorofluoride; HPLC, high-performance liquid chromatography; LC-MS/MS, liquid chromatography coupled with tandem mass spectrometry; 2MeSADP, 2-methylthioadenosine diphosphate; MPB, 3′-methoxyphenacyl bromide; PCR, polymerase chain reaction; PMSF, phenylmethylsulfonyl fluoride; PON, paraoxonase.

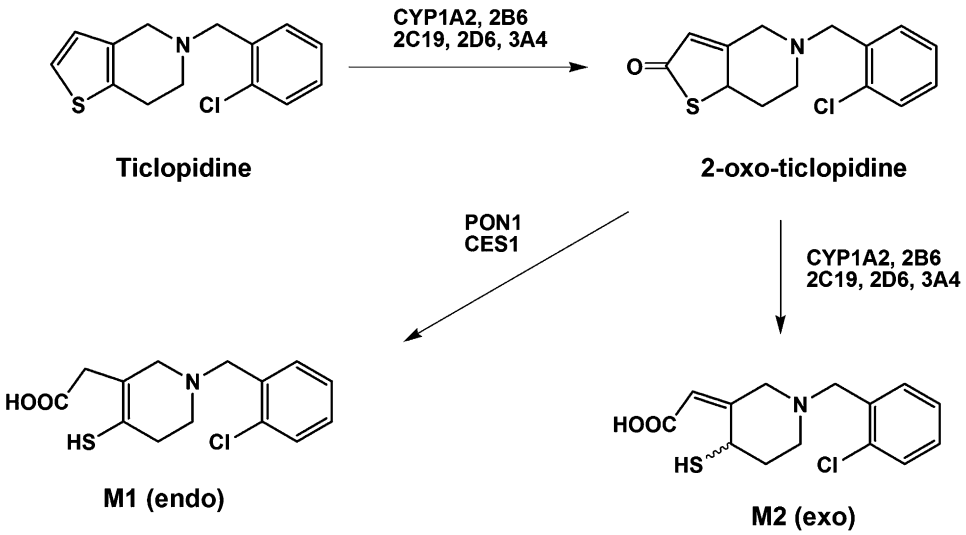

Fig. 1. Postulated pathways for the metabolism of ticlopidine to thiol metabolites.

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, diethyldithiocarbamate, furafylline, ketoconazole, montelukast, quinidine, sulfaphenazole, thio-TEPA, β-nicotinamide adenine dinucleotide phosphate, loperamide, glutathione (GSH), bis-(*p*-nitrophenyl)phosphate (BNPP), diisopropylphosphorofluoride (DFP), physostigmine sulfate (eserine), phenylmethylsulfonyl fluoride (PMSF), 3′-methoxyphenacyl bromide (MPB), 2-methylthioadenosine diphosphate (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-d_4, and *S*-benzylnirvanol 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 Daiichi Sankyo Co. (Tokyo, Japan).

Human Plasma and Liver Microsomes. Human plasma was prepared from fresh blood collected from healthy male subjects (20–30 years old) who had fasted overnight. Blood (20 ml) was taken and transferred to a heparin Vacutainer. After centrifugation at 1000*g* for 10 minutes, plasma was taken and stored at −80°C. Human liver microsomes were individually prepared from 20 male human liver tissue donors, patients undergoing partial hepatectomy for removal of metastatic tumors at the Department of General Surgery, Busan Paik Hospital (Busan, South Korea). The samples were of non-tumor-bearing parenchymal tissue confirmed as histopathologically normal. The liver tissues and their clinical information were obtained from Inje Biobank at the Inje University Busan Paik Hospital. The use of human tissues was approved by the institutional review board of Busan Paik Hospital.

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% formate 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 signal intensity of the methoxy group to those of tetramethylsilane spiked in nuclear magnetic resonance solvent (d_3-methanol).

Construction of Recombinant Vectors for PON1 Variants. The wild-type PON1 cDNA cloned in the pCR4-TOPO plasmid (Thermo Fisher Scientific, Portsmouth, NH) was used as a polymerase chain reaction (PCR) template for amplification of the PON1 open-reading frame with an additional 6×-His tag to the C-terminal region. PCR primers included a forward primer 5′-CACCATGGCGAAGCTGATTGCGCTCACCCTCTTG-3′, and a reverse primer 5′-TCAATGGTGATGGTGATGGTGGAGCTCACAGTAAAGAGCT-3′. The amplified PON1 cDNA was cloned into a TOPO entry vector (Invitrogen, Carlsbad, CA) and subsequently subcloned into a pcDNA-DEST40 (Invitrogen) using the Gateway cloning system according to the manufacturer's protocol. After construction of wild-type PON1 cDNA in the pcDNA-DEST40 vector, site-directed mutagenesis was performed using the QuikChange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA). The PCR primers for the site-directed mutagenesis were as follows: PON1L55MF (5′-CTGGCTCTGAAGAC**A**TGGAGATACTGCCT-3′), PON1L55MR (5′-AGGCAGTATCTCCA**T**GTCTTCAGAGCCAG-3′), PON1Q192RF (5′-CTTGACCCCTACTTAC**G**ATCCTGGGAGATG-3′), and PON1Q192RR (5′-CATCTCCCAGGAT**C**GTAAGTAGGGGTCAAG-3′). The nucleotides marked as bold type are mismatches with the PON1 reference sequence. The entire open-reading frame region was sequenced in both directions, and changes were confirmed before expression.

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

expression medium (Invitrogen). The cells were harvested and resuspended in lysis buffer containing 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, and 0.05% Tween 20 (pH 8.0). After centrifugation at 10,000*g* 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.

Immunoblotting. Human liver microsomes (30 μg), cellular proteins transfected 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 mg/ml pooled microsomes or 20 pmol/ml recombinant CYP, ticlopidine (2 and 20 μM), and an NADPH-generating system (1.3 mM β-nicotinamide adenine dinucleotide phosphate, 3.3 mM glucose 6-phosphate, 3.3 mM $MgCl_2$, and 1.0 U/ml glucose-6-phosphate dehydrogenase) in a total volume of 100 μL phosphate buffer (0.1 M, pH 7.4). The reaction was initiated by the addition of the NADPH-generating system and continued in a water bath at 37°C for 10 minutes. The reaction was terminated by the addition of the same volume of acetonitrile-containing internal standard (1 μM of 2-oxo-clopidogrel). After centrifugation at 16,000*g* for 5 minutes, a 2-μL aliquot of the supernatant was injected directly into the LC-MS/MS system.

Chemical Inhibition of 2-Oxo-Ticlopidine Formation. We used the following CYP isoform-selective inhibitors: 10 μM furafylline for CYP1A2; 100 μM coumarin for CYP2A6; 5 μM thio-TEPA for CYP2B6; 1 μM montelukast for CYP2C8; 10 μM sulfaphenazole for CYP2C9; 1 μM *S*-benzylnirvanol for CYP2C19; 10 μM quinidine for CYP2D6; 10 μM diethyldithiocarbamate for CYP2E1; and 1 μM ketoconazole for CYP3A. The CYP isoform selective inhibitors and their concentrations were described elsewhere (Seo et al., 2012). Incubation mixtures consisted of ticlopidine (2 and 20 μM), an inhibitor, pooled human liver microsomes (0.2 mg/ml), and an NADPH-generating system in 0.1 ml of 0.1 M potassium phosphate buffer (pH 7.4). The mechanism-based inhibitors, furafylline and diethyldithiocarbamate, were preincubated for 15 minutes with microsomes in the presence of an NADPH-generating system before addition of ticlopidine to initiate the reaction.

Formation of Thiol Metabolite from 2-Oxo-Ticlopidine. The incubation mixture (100 μL final volume) contained 0.1 M Tris-HCl buffer (pH 8.0), 5 mM glutathione, and various enzyme sources (human liver microsomes, 0.2 mg/ml; human plasma, 1 μL/ml; recombinant CYP isoforms, 20 pmol/ml; recombinant PON1 proteins, 0.05 mg/ml; CES1 and CES2, 0.1 mg/ml; CMBL, 0.2 mg/ml). An NADPH-generating system was added to the mixture in microsomes and recombinant CYP reactions. When assessing the effects of $CaCl_2$ on the formation of a thiol metabolite, it was used at the concentration of 1 mM. The mixtures were prewarmed for 5 minutes at 37°C, and the reaction was initiated by adding various concentrations of 2-oxo-ticlopidine (0–1000 μM). After a 5-minute incubation, the reaction was terminated by adding 100 μl acetonitrile containing 4 mM MPB. The mixture was incubated at 37°C for 30 minutes to complete the derivatization reaction. After adding the internal standard (1 μM of clopidogrel carboxylic acid), the mixture was centrifuged at 16,000*g* for 5 minutes, and an aliquot of the supernatant was injected into the LC-MS/MS for determination of the MPB-derivatized thiol metabolites. The reactivity of M1 and M2 toward MPB was different in derivatization reaction, and optimal concentration of MPB for the reaction was determined to be 4 mM (Supplemental Fig. 1).

Chemical Inhibition of Active Thiol Metabolite Formation. Various esterase inhibitors were used to determine the enzymes involved in thiol metabolite formation from 2-oxo-ticlopidine in human plasma and liver microsomes. Reaction mixtures were incubated in the presence and absence of inhibitors, and the thiol metabolite formation rates from 50 μM 2-oxo-ticlopidine measured. The esterase inhibitors used were BNPP and DFP for general CES (Heymann and Krisch, 1967), eserine for choline esterase (ChE), CES1, and CES2 (Preuss and Svensson, 1996; Takahashi et al., 2009), EDTA for PON (Gonzalvo et al., 1997), clopidogrel for CES1 (Shi et al., 2006), loperamide for CES2 (Quinney 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-l 0.1 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 $CaCl_2$ for 5 minutes. After adding MPB 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. Clopidogrel hydrolysis activity as a marker of CES1 was measured by LC/MS/MS, as described elsewhere (Sato et al., 2012). Paraoxon 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 clopidogrel 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 C_{18} (2.1 × 100 mm i.d., 2.6 μm; Phenomenex, Torrance, CA). The mobile phases consisted of 0.1% formic acid solution (A) and 0.1% formic acid in acetonitrile (B). A gradient program was used for HPLC separation with a flow rate of 0.4 ml/min. The initial composition of mobile phase B was 15%, linearly ramped to 45% in 2 minutes, then 95% in 2.5 minutes and maintained for 1.5 minutes, followed by re-equilibration to the initial condition for 1 minute. The total run time was 5 minutes. HPLC was coupled to a Quattro Premier XE LC-MS/MS System (Micromass Ltd., Manchester, UK) 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 were performed by monitoring the transitions of *m/z* 280→125 for 2-oxo-ticlopidine, *m/z* 338→155 for 2-oxo-clopidogrel (internal standard for 2-oxo-ticlopidine), m/z 446→154 for MBP-endo thiol metabolite (M1), *m/z* 446→266 for MBP-exo-thiol metabolite (M2), and *m/z* 308→198 for clopidogrel 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 $NADP^+$, 3 U/ml glucose-6-phosphate dehydrogenase 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 μM 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,

CA) every 10 seconds for 3 minutes with mixing between each reading. The slope for the linear portion of the aggregation time course was used to calculate the rate of plate aggregation. The percentage inhibition of platelet aggregation (%IPA) was calculated using the following equation:

$$\%\text{IPA} = (1 - (R_{\text{reaction}}/R_{\text{control}}) \times 100$$

where R_{reaction} equals the slope of the aggregation assay containing 2-oxo-ticlopidine and microsomal mixture and R_{control} equals the slope of the aggregation assay containing only microsomal mixture.

Data Analysis. The apparent kinetic parameters of 2-oxo ticlopidine metabolism were determined by fitting the unweighted kinetic data from plasma, human liver microsomes, and human esterases to an one-enzyme Michaelis-Menten equation or a Hill equation [$V = V_{\max} \times [S]^n/(K_m + [S]^n)$]. Calculated parameters included the maximum rate of metabolite formation ($V_{\max}$), Michaelis-Menten constant (apparent K_m), the intrinsic clearance ($CL_{\text{int}} = V_{\max}/K_m$), and Hill coefficient (n). The percentages of inhibition were calculated by the ratio of the rate of metabolite formation with and without the specific inhibitor. Calculations were performed using WinNonlin, version 2.1 (Pharsight, Mountain View, CA).

Results

Identification of Enzymes Responsible for the Formation of 2-Oxo-Ticlopidine. Ticlopidine was metabolized to 2-oxo-ticlopidine in human liver microsomes only when NADPH was added to the incubation. To characterize CYP isoforms involved in the formation of 2-oxo-ticlopidine, chemical inhibition with CYP isoform selective inhibitors and metabolism with cDNA-expressed CYP were performed. The formation of 2-oxo-ticlopidine in human liver microsomes was not substantially inhibited by any of CYP-selective inhibitors used at 2 and 20 μM ticlopidine (data not shown). No significant inhibition of 2-oxo-ticlopidine formation by CYP isoform selective inhibitors might be due to the involvement of multiple CYP isoforms. The formation rates of 2-oxo-ticlopidine from ticlopidine (2 and 20 μM) using cDNA-expressed CYP1A2, 2A6, 2B6, 2C8, 2C8, 2C19, 2D6, and 3A4 are shown in Fig. 2. Multiple CYP isoforms mediated 2-oxo-ticlopidine formation. Among them, CYP2D6 showed the highest activity at 2 μM whereas CYP2B6 and 2C19 showed relatively high activity at 20 μM ticlopidine.

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 Cl^{35} and Cl^{37} 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 in the piperidine ring (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.

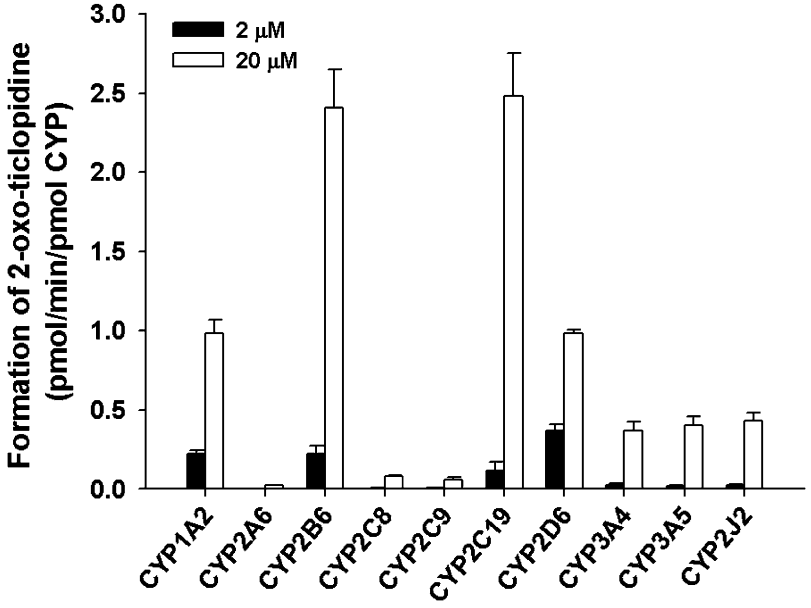

Fig. 2. Formation of 2-oxo-ticlopidine from ticlopidine by human cDNA-expressed CYP isoforms. Ticlopidine at two different concentrations of 2 (■) and 20 μM (□) were incubated with each recombinant CYP isoform (CYP1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 3A4, 3A5, and 2J2) and an NADPH-generating system at 37°C for 5 minutes. Each bar represents the mean ± S.D. of triplicate determinations.

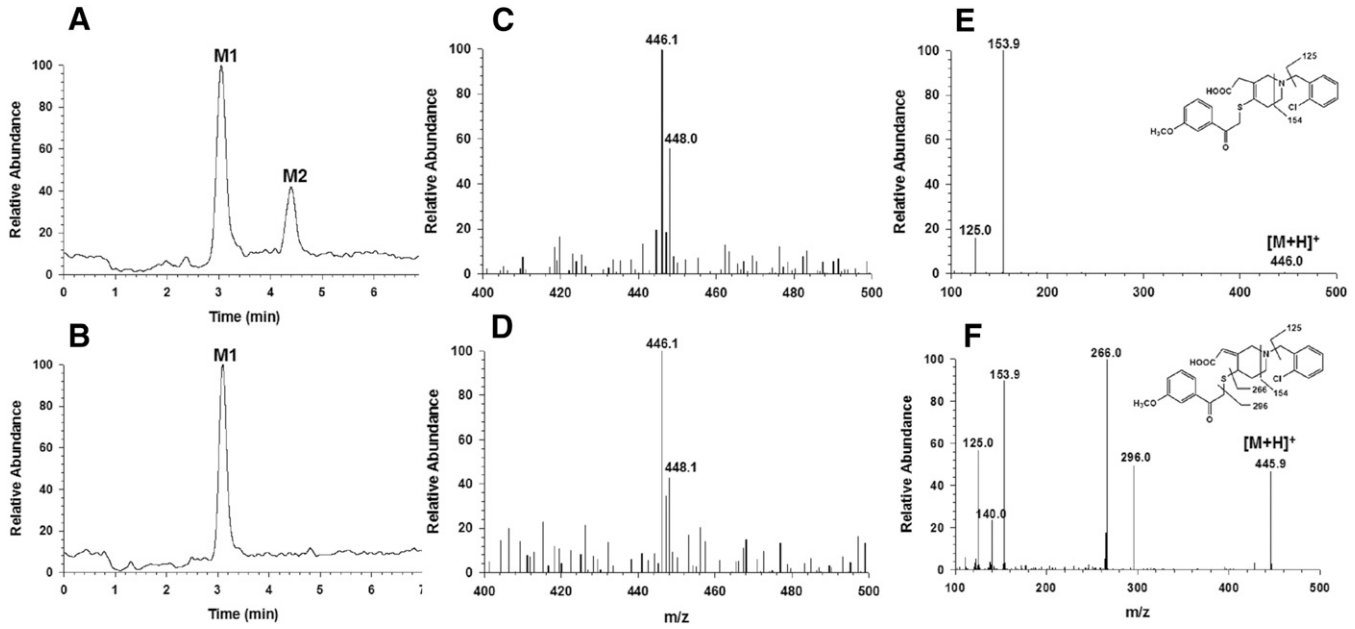

Fig. 3. HPLC chromatograms of incubations of 2-oxo-ticlopidine with human liver microsomes with (A) and without (B) an NADPH-generating system with MS detection at *m/z* 446 after derivatization with 3′-methoxy-phenacyl bromide, mass spectra of M1 (C) and M2 (D), and product ion mass spectra of M1 (E)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

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 $CaCl_2$ 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 $CaCl_2$ to dialyzed plasma restored the activity. In contrast, M1 formation from 2-oxo-ticlopidine in microsomes was not significantly changed in the presence of $CaCl_2$. Dialysis of microsomes for 24 hours resulted in a 37% loss of activity, which was not restored by addition of $CaCl_2$. 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

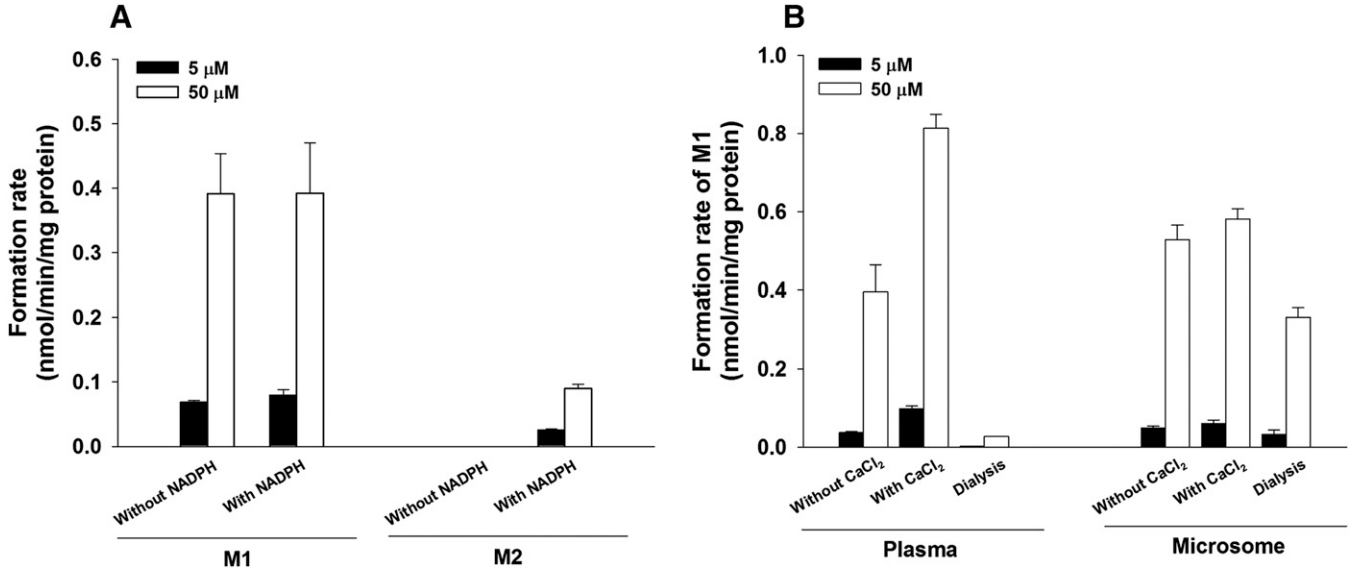

Fig. 4. Formation rates of thiol metabolites after incubation of 2-oxo-ticlopidine with human liver microsomes in the presence and absence of an NADPH-generating system (A), and effects of Ca^{2+} and dialysis on M1 formation rates in human plasma and microsomes (B). Each data point represents the mean ± S.D. of triplicate determinations.

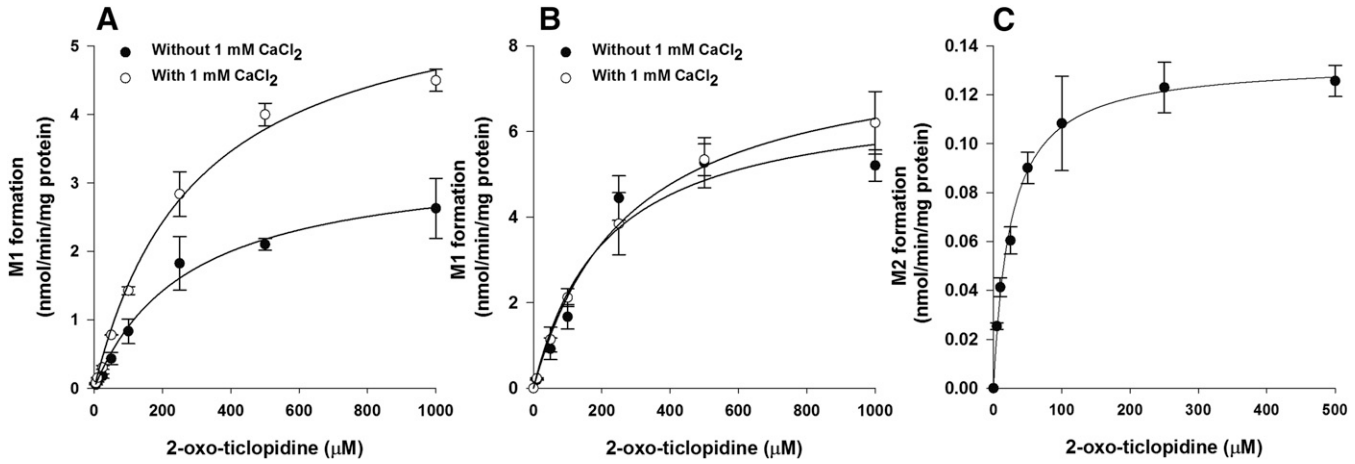

Fig. 5. Kinetics of M1 formation from 2-oxo-ticlopidine in pooled human plasma (A), pooled human liver microsomes (B) in the absence (●) or presence (○) of 1 mM $CaCl_2$, and M2 formation from 2-oxo-ticlopidine in pooled human liver microsomes (C). An increasing concentration of 2-oxo-ticlopidine was incubated with microsomes (0.2 mg/ml) or plasma (1 μL/ml) at 37°C for 5 minutes. 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 μM and 3.72 nmol/min/mg, respectively, and the kinetic values in microsomes were 382 μM 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 μM 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 μM) 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 ~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 ± 0.24 nmol/min/mg) with 50 μM 2-oxo-ticlopidine in the presence of 1 mM $CaCl_2$. The catalytic activities of PON1 192R and 55M 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

Kinetic parameters of M1 and M2 formation by human tissue preparations

Enzyme Source	M1			M2		
	K_m	V_{max}	CL_{int}	K_m	V_{max}	CL_{int}
	μM	*nmol/min/mg protein*	*μl/min/mg protein*	*μM*	*nmol/min/mg protein*	*μl/min/mg protein*
Plasma	274	3.36	12.3	—	—	—
Plasma + $CaCl_2$[a]	297	6.03	20.3	—	—	—
Microsome	210	6.90	32.9	—	—	—
Microsome + $CaCl_2$[a]	274	8.03	29.3	—	—	—
Microsome + NADPH	280	5.32	19.0	22.8	0.13	5.83

[a] Concentration of $CaCl_2$ was 1 mM.

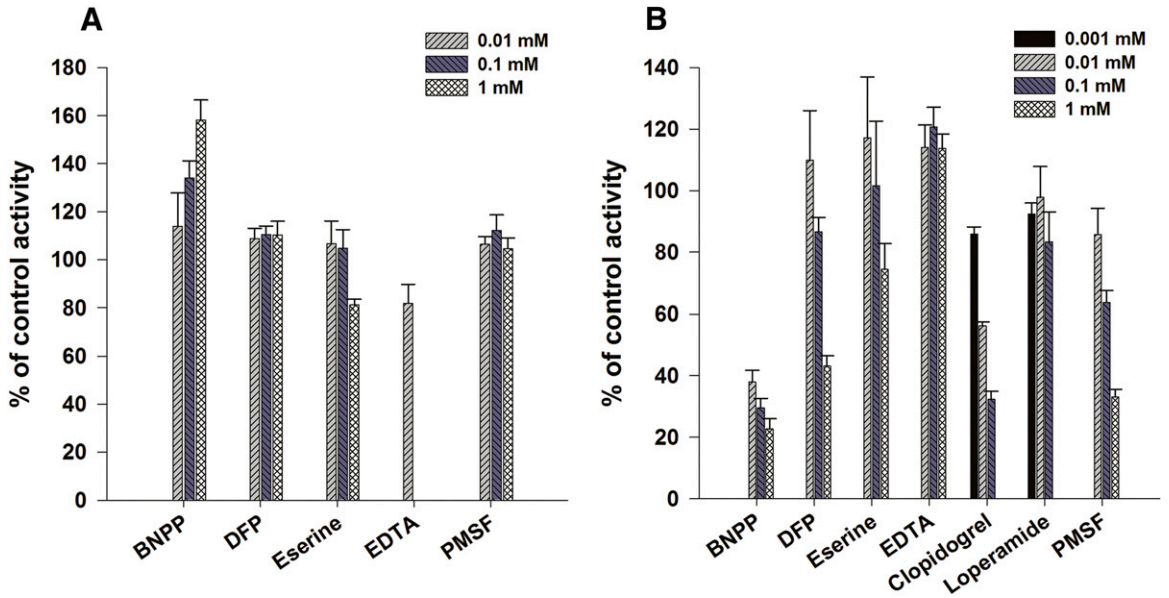

Fig. 6. Effects of carboxyesterase, choline esterase, and paraoxonase 1 inhibitors on M1 formation in human plasma (A) and liver microsomes (B). Pooled microsomes (0.2 mg/ml) or plasma (1 μl/ml) were incubated with 50 μM 2-oxo-ticlopidine in the absence or presence of various chemical inhibitors at 37°C for 5 minutes. Each data point represents the mean ± S.D. of triplicate determinations.

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 μM and 2.16 nmol/min/mg, respectively, resulting in CL_{int} value of 7.8 μL/min/mg. In contrast, the K_m and V_{max} values of PON1 192Q were 338 μM 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

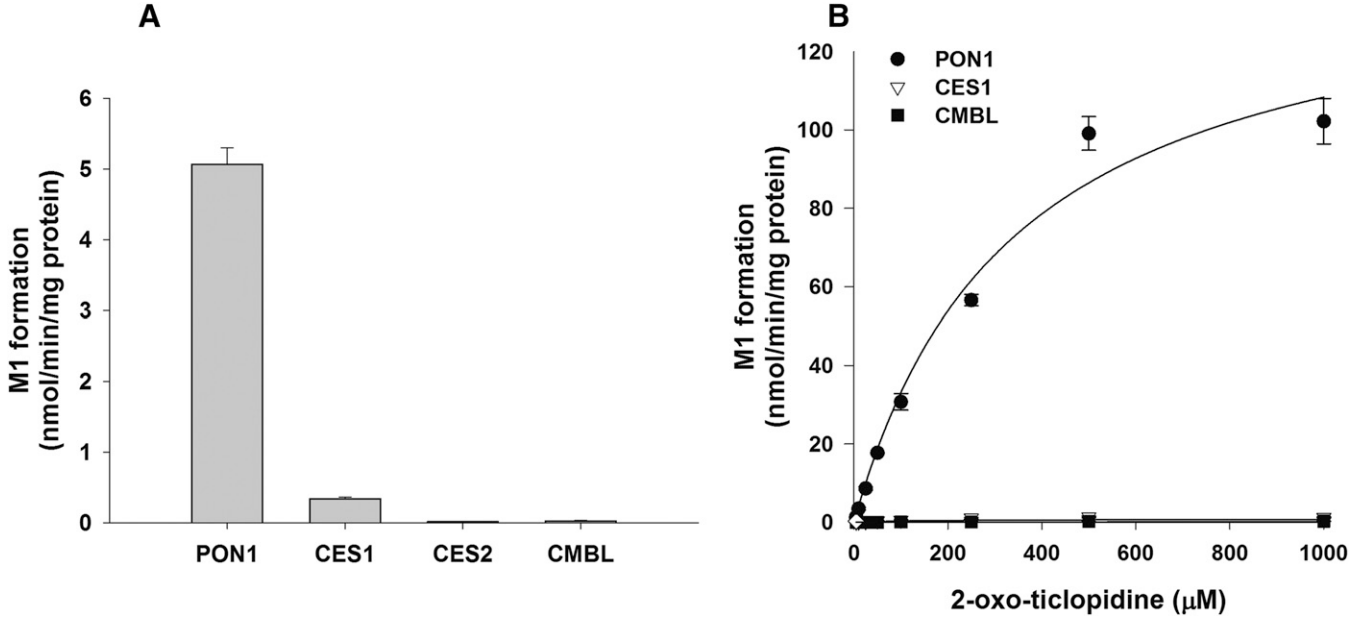

Fig. 7. M1 formation rates (A), and kinetics of M1 formation from 2-oxo-ticlopidine (B) by human cDNA-expressed PON1, CES1, CES2, and CMBL. An increasing concentration of 2-oxo-ticlopidine was incubated with recombinant human PON1 (0.05 mg/ml), CES1, CES2 (0.1 mg/ml), and CMBL (0.2 mg/ml) at 37°C for 5 minutes. Each data point represents the mean ± S.D. of triplicate determinations.

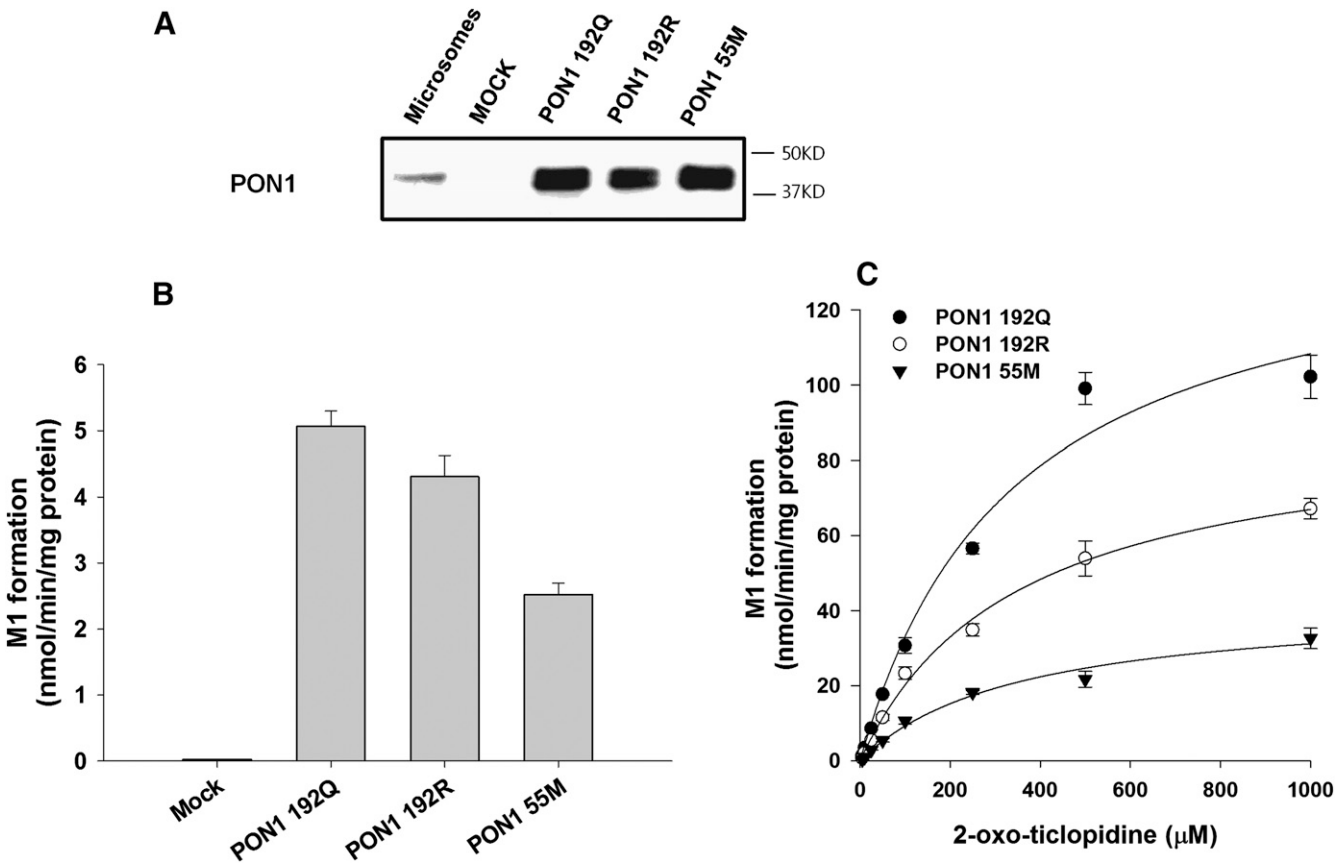

Fig. 8. The effects of *PON1* polymorphisms (Q192R and L55M) on M1 formation from 2-oxo-ticlopidine. Immunoblotting of recombinant PON1 192Q, 192R, and 55M expressed in Free Style 293-F cells (A). Formation rates of M1 from 2-oxo-ticlopidine by mock, recombinant human PON1 192Q, 192R, and 55M. The proteins were incubated with 50 μM 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.

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 μM 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 μM) 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 μM 2-oxo-ticlopidine.

TABLE 2

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

Enzyme Source	K_m	V_{max}	CL_{int}
	μM	*nmol/min/mg protein*	*μl/min/mg protein*
PON1 wild type	388	145	374
PON1 Q192R	346	90.1	260
PON1 L55M	355	42.2	119
CES1	277	2.16	7.80

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_{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_{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

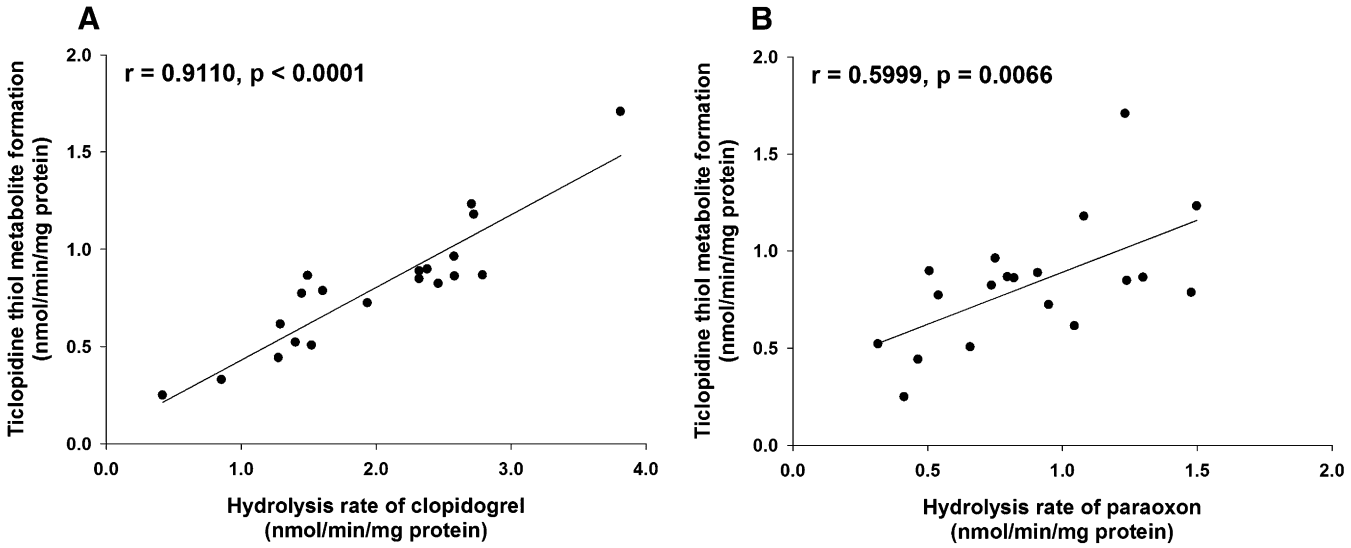

Fig. 9. Correlation analysis of the rates of thiol metabolite formation from 2-oxo-ticlopidine and the hydrolysis rates of clopidogrel (A) and paraoxon (B) in 20 human liver microsomes. The concentrations of 2-oxo-ticlopidine, clopidogrel, and paraoxon were 50, 5, and 3000 μM, respectively.

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).

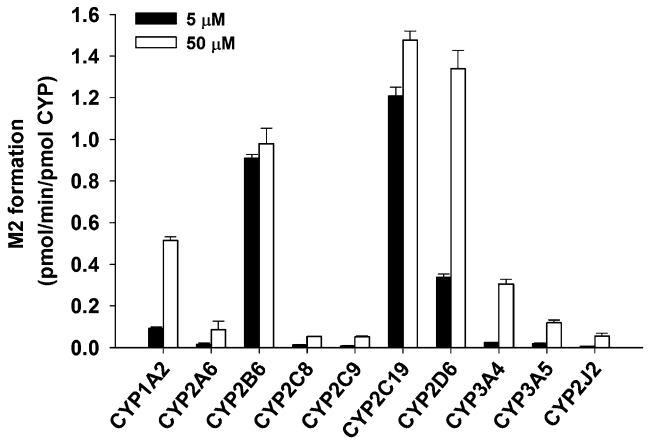

Fig. 10. Formation of M2 from 2-oxo-ticlopidine by human cDNA-expressed CYP isoforms. 2-Oxo-ticlopidine at two different concentrations of 5 (■) and 50 μM (□) were incubated with each recombinant CYP isoform (CYP1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 3A4, 3A5, and 2J2). Each bar represents the mean ± SD of triplicate determinations.

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-clopdogrel, 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 $CaCl_2$. In addition, dialysis of plasma completely abolished the catalytic activity and addition of 1 mM $CaCl_2$ to dialyzed plasma restored the activity to the original level, indicating that M1 formation in plasma is dependent on Ca^{2+}. Second, plasma activity was completely inhibited by EDTA but not by other esterase inhibitors such as BNPP, eserine, and clopidogrel. Finally, cDNA-expressed

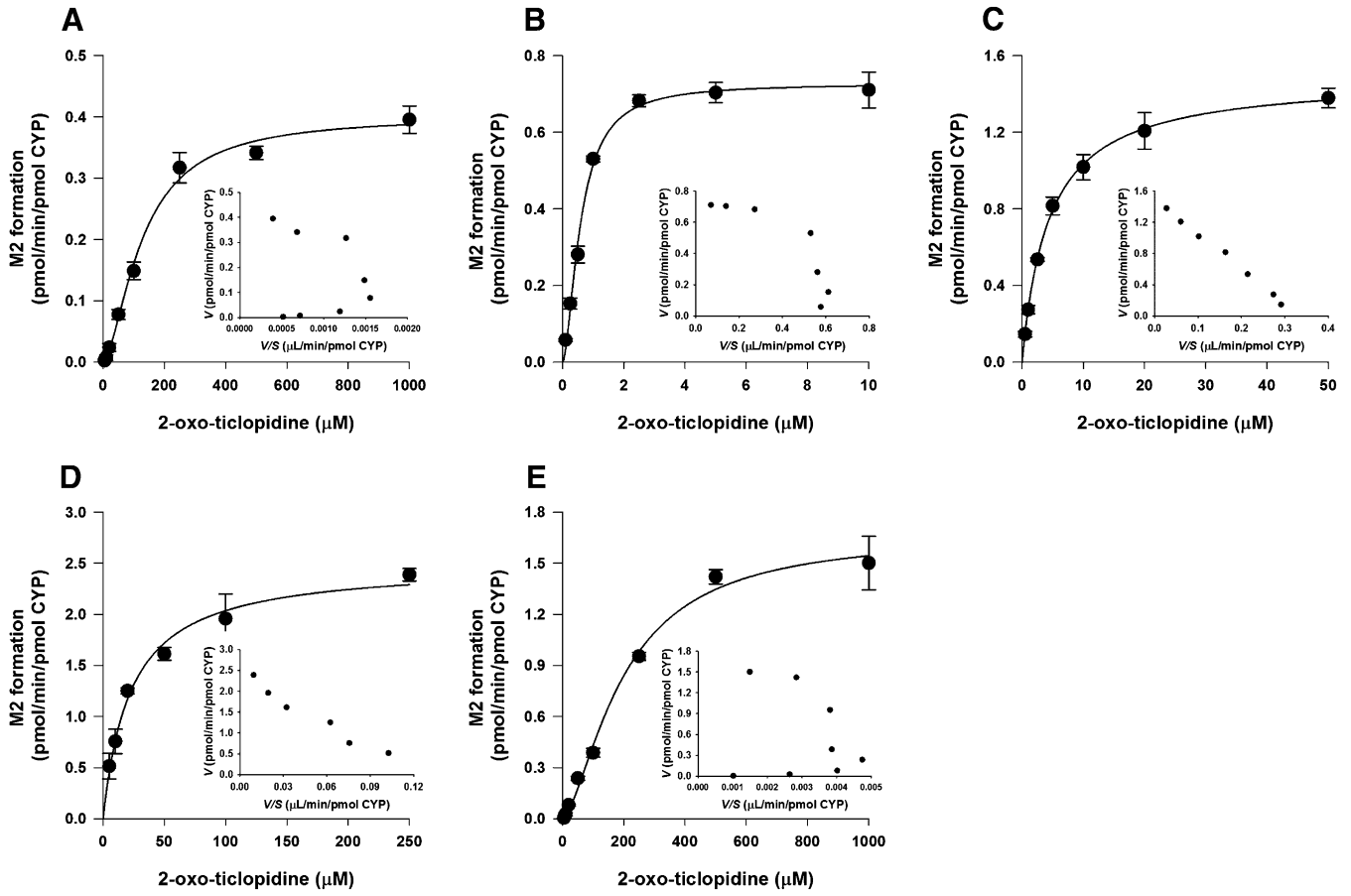

Fig. 11. Kinetics for the formation of M2 from 2-oxo-ticlopidine in human cDNA-expressed CYP 1A2 (A), 2B6 (B), 2C19 (C), 2D6 (D), and 3A4 (E). An increasing concentration of 2-oxo-ticlopidine was incubated with each recombinant CYP (20 pmol/ml) and an NADPH-generating system at 37°C for 5 minutes. The 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.

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 $CaCl_2$.

TABLE 3

Kinetic parameters of M2 formation from 2-oxo-ticlopidine by human cDNA-expressed CYP isoforms

CYP Isoform	K_m	V_{max}	CL_{int}	n[a]
	μM	*pmol/min/pmol CYP*	*μL/min/pmol CYP*	
CYP1A2	128	0.40	0.003	1.61
CYP2B6	0.59	0.73	1.24	1.69
CYP2C19	4.39	1.49	0.34	—
CYP2D6	22.6	2.50	0.11	—
CYP3A4	199	1.67	0.008	1.54

[a] Hill coefficient.

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

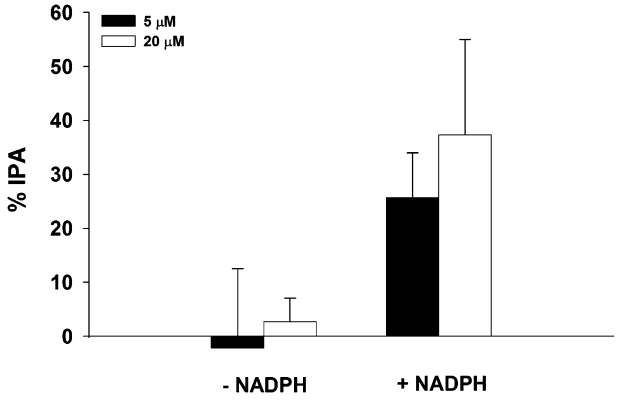

Fig. 12. Inhibition of in vitro 2MeSADP-induced aggregation of washed human platelets by 2-oxo-ticlopidine. Washed platelets were incubated with 5 μM (■) and 20 μM (□) 2-oxo-ticlopidine and microsomes in the presence and absence of an NADPH-generating system. Each data point represents the mean ± S.D. 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; Fukami 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., phenylacetate) (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 differently to the polymorphic *PON1* than pilocarpine and olmesartan medoxomil.

Unlike to 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 μM) 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 faster 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 an active thiol metabolite (exo-form) to its isomer (endo-form) in human liver microsomes were 8.6 and 3.9 times for clopidogrel and prasugrel, respectively (Dansette et al., 2012a,b). However, a reverse phenomenon was noted in ticlopidine, and our results showed that the formation rate of M2 was 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 (Tuffal et al., 2011), we assessed the pharmacologic activity of M1 and M2 using in vitro platelet aggregation assay. Inhibition of 2MeSADP-induced platelet aggregation by 2-oxo-ticlopidine was observed in microsomal 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.

Authorship Contributions

Participated in research design: Lee, Shin, D.-H. Kim.
Conducted experiments: M.-J. Kim, Jeong, Park.
Performed data analysis: M.-J. Kim, Jeong, Choi, Ghim, Shin, D.-H. Kim.
Wrote or contributed to the writing of the manuscript: M.-J. Kim, D.-H. Kim.

References

Abell LM and Liu EC (2011) Dissecting the activation of thienopyridines by cytochromes P450 using a pharmacodynamic assay in vitro. *J Pharmacol Exp Ther* **339**:589–596.

Aleil B, Rochoux G, Monassier JP, Cazenave JP, and Gachet C (2007) Ticlopidine could be an alternative therapy in the case of pharmacological resistance to clopidogrel: a report of three cases. *J Thromb Haemost* **5**:879–881.

Bhattacharyya T, Nicholls SJ, Topol EJ, Zhang R, Yang X, Schmitt D, Fu X, Shao M, Brennan DM, and Ellis SG, et al. (2008) Relationship of paraoxonase 1 (PON1) gene polymorphisms and functional activity with systemic oxidative stress and cardiovascular risk. *JAMA* **299**:1265–1276.

Billecke S, Draganov D, Counsell R, Stetson P, Watson C, Hsu C, and La Du BN (2000) Human serum paraoxonase (PON1) isozymes Q and R hydrolyze lactones and cyclic carbonate esters. *Drug Metab Dispos* **28**:1335–1342.

Borowczyk K, Shih DM, and Jakubowski H (2012) Metabolism and neurotoxicity of homocysteine thiolactone in mice: evidence for a protective role of paraoxonase 1. *J Alzheimers Dis* **30**:225–231.

Bouman HJ, Schömig E, van Werkum JW, Velder J, Hackeng CM, Hirschhäuser C, Waldmann C, Schmalz HG, ten Berg JM, and Taubert D (2011) Paraoxonase-1 is a major determinant of clopidogrel efficacy. *Nat Med* **17**:110–116.

Cuisset T, Morange PE, and Alessi MC (2012) Recent advances in the pharmacogenetics of clopidogrel. *Hum Genet* **131**:653–664.

Dansette PM, Rosi J, Bertho G, and Mansuy D (2012a) Cytochromes P450 catalyze both steps of the major pathway of clopidogrel bioactivation, whereas paraoxonase catalyzes the formation of a minor thiol metabolite isomer. *Chem Res Toxicol* **25**:348–356.

Dansette PM, Rosi J, Debernardi J, Bertho G, and Mansuy D (2012b) Metabolic activation of prasugrel: nature of the two competitive pathways resulting in the opening of its thiophene ring. *Chem Res Toxicol* **25**:1058–1065.

Farid NA, Kurihara A, and Wrighton SA (2010) Metabolism and disposition of the thienopyridine antiplatelet drugs ticlopidine, clopidogrel, and prasugrel in humans. *J Clin Pharmacol* **50**: 126–142.

Fleming CD, Bencharit S, Edwards CC, Hyatt JL, Tsurkan L, Bai F, Fraga C, Morton CL, Howard-Williams EL, and Potter PM, et al. (2005) Structural insights into drug processing by human carboxylesterase 1: tamoxifen, mevastatin, and inhibition by benzil. *J Mol Biol* **352**:165–177.

Fukami T, Takahashi S, Nakagawa N, Maruichi T, Nakajima M, and Yokoi T (2010) In vitro evaluation of inhibitory effects of antidiabetic and antihyperlipidemic drugs on human carboxylesterase activities. *Drug Metab Dispos* **38**:2173–2178.

Gonzalvo MC, Gil F, Hernández AF, Villanueva E, and Pla A (1997) Inhibition of paraoxonase activity in human liver microsomes by exposure to EDTA, metals and mercurials. *Chem Biol Interact* **105**:169–179.

Gonzalvo MC, Gil F, Hernandez AF, Rodrigo L, Villanueva E, and Pla A (1998) Human liver paraoxonase (PON1): subcellular distribution and characterization. *J Biochem Mol Toxicol* **12**: 61–69.

Gurbel PA, Bliden KP, Hiatt BL, and O'Connor CM (2003) Clopidogrel for coronary stenting: response variability, drug resistance, and the effect of pretreatment platelet reactivity. *Circulation* **107**:2908–2913.

Hagihara K, Kazui M, Kurihara A, Yoshiike M, Honda K, Okazaki O, Farid NA, and Ikeda T (2009) A possible mechanism for the differences in efficiency and variability of active metabolite formation from thienopyridine antiplatelet agents, prasugrel and clopidogrel. *Drug Metab Dispos* **37**:2145–2152.

Heymann E and Krisch K (1967) Phosphoric acid-bis-(p-nitro-phenylester), a new inhibitor of microsomal carboxylesterases [article in German]. *Hoppe Seylers Z Physiol Chem* **348**: 609–619.

Hioki T, Fukami T, Nakajima M, and Yokoi T (2011) Human paraoxonase 1 is the enzyme responsible for pilocarpine hydrolysis. *Drug Metab Dispos* **39**:1345–1352.
Hulot JS, Collet JP, Cayla G, Silvain J, Allanic F, Bellemain-Appaix A, Scott SA, and Montalescot G (2011) CYP2C19 but not PON1 genetic variants influence clopidogrel pharmacokinetics, pharmacodynamics, and clinical efficacy in post-myocardial infarction patients. *Circ Cardiovasc Interv* **4**:422–428.
Ishizuka T, Fujimori I, Kato M, Noji-Sakikawa C, Saito M, Yoshigae Y, Kubota K, Kurihara A, Izumi T, and Ikeda T, et al. (2010) Human carboxymethylenebutenolidase as a bioactivating hydrolase of olmesartan medoxomil in liver and intestine. *J Biol Chem* **285**:11892–11902.
Johnson G and Moore SW (2000) Cholinesterase-like catalytic antibodies: reaction with substrates and inhibitors. *Mol Immunol* **37**:707–719.
Kazui M, Nishiya Y, Ishizuka T, Hagihara K, Farid NA, Okazaki O, Ikeda T, and Kurihara A (2010) Identification of the human cytochrome P450 enzymes involved in the two oxidative steps in the bioactivation of clopidogrel to its pharmacologically active metabolite. *Drug Metab Dispos* **38**:92–99.
Lesesve JF, Callat MP, Lenormand B, Monconduit M, Noblet C, Moore N, Caron F, Humbert G, Stamatoullas A, and Tilly H (1994) Hematological toxicity of ticlopidine. *Am J Hematol* **47**:149–150.
Love BB, Biller J, and Gent M (1998) Adverse haematological effects of ticlopidine. Prevention, recognition and management. *Drug Saf* **19**:89–98.
Mackness B, Mackness MI, Arrol S, Turkie W, Julier K, Abuasha B, Miller JE, Boulton AJ, and Durrington PN (1998) Serum paraoxonase (PON1) 55 and 192 polymorphism and paraoxonase activity and concentration in non-insulin dependent diabetes mellitus. *Atherosclerosis* **139**:341–349.
Maeda A, Ando H, Asai T, Ishiguro H, Umemoto N, Ohta M, Morishima M, Sumida A, Kobayashi T, and Hosohata K, et al. (2011) Differential impacts of CYP2C19 gene polymorphisms on the antiplatelet effects of clopidogrel and ticlopidine. *Clin Pharmacol Ther* **89**:229–233.
Mataix R, Ojeda E, Perez MC, and Jimenez S (1992) Ticlopidine and severe aplastic anaemia. *Br J Haematol* **80**:125–126.
Nishiya Y, Hagihara K, Kurihara A, Okudaira N, Farid NA, Okazaki O, and Ikeda T (2009a) Comparison of mechanism-based inhibition of human cytochrome P450 2C19 by ticlopidine, clopidogrel, and prasugrel. *Xenobiotica* **39**:836–843.
Nishiya Y, Hagihara K, Ito T, Tajima M, Miura S, Kurihara A, Farid NA, and Ikeda T (2009b) Mechanism-based inhibition of human cytochrome P450 2B6 by ticlopidine, clopidogrel, and the thiolactone metabolite of prasugrel. *Drug Metab Dispos* **37**:589–593.
Noble S and Goa KL (1996) Ticlopidine. A review of its pharmacology, clinical efficacy and tolerability in the prevention of cerebral ischaemia and stroke. *Drugs Aging* **8**:214–232.
Ono K, Kurohara K, Yoshihara M, Shimamoto Y, and Yamaguchi M (1991) Agranulocytosis caused by ticlopidine and its mechanism. *Am J Hematol* **37**:239–242.
Preuss CV and Svensson CK (1996) Arylacetamide deacetylase activity towards monoacetyldapsone. Species comparison, factors that influence activity, and comparison with 2-acetylaminofluorene and p-nitrophenyl acetate hydrolysis. *Biochem Pharmacol* **51**:1661–1668.
Quinney SK, Sanghani SP, Davis WI, Hurley TD, Sun Z, Murry DJ, and Bosron WF (2005) Hydrolysis of capecitabine to 5′-deoxy-5-fluorocytidine by human carboxylesterases and inhibition by loperamide. *J Pharmacol Exp Ther* **313**:1011–1016.
Richter RJ, Jarvik GP, and Furlong CE (2009) Paraoxonase 1 (PON1) status and substrate hydrolysis. *Toxicol Appl Pharmacol* **235**:1–9.
Sato Y, Miyashita A, Iwatsubo T, and Usui T (2012) Conclusive identification of the oxybutynin-hydrolyzing enzyme in human liver. *Drug Metab Dispos* **40**:902–906.
Seo KA, Lee SJ, Kim KB, Bae SK, Liu KH, Kim DH, and Shin JG (2012) Ilaprazole, a new proton pump inhibitor, is primarily metabolized to ilaprazole sulfone by CYP3A4 and 3A5. *Xenobiotica* **42**:278–284.
Shi D, Yang J, Yang D, LeCluyse EL, Black C, You L, Akhlaghi F, and Yan B (2006) Anti-influenza prodrug oseltamivir is activated by carboxylesterase human carboxylesterase 1, and the activation is inhibited by antiplatelet agent clopidogrel. *J Pharmacol Exp Ther* **319**:1477–1484.
Shuldiner AR, O'Connell JR, Bliden KP, Gandhi A, Ryan K, Horenstein RB, Damcott CM, Pakyz R, Tantry US, and Gibson Q, et al. (2009) Association of cytochrome P450 2C19 genotype with the antiplatelet effect and clinical efficacy of clopidogrel therapy. *JAMA* **302**:849–857.
Steinhubl SR, Tan WA, Foody JM, and Topol EJ (1999) Incidence and clinical course of thrombotic thrombocytopenic purpura due to ticlopidine following coronary stenting. EPISTENT Investigators. Evaluation of platelet IIb/IIIa inhibitor for stenting. *JAMA* **281**:806–810.
Takahashi S, Katoh M, Saitoh T, Nakajima M, and Yokoi T (2009) Different inhibitory effects in rat and human carboxylesterases. *Drug Metab Dispos* **37**:956–961.
Talakad JC, Shah MB, Walker GS, Xiang C, Halpert JR, and Dalvie D (2011) Comparison of in vitro metabolism of ticlopidine by human cytochrome P450 2B6 and rabbit cytochrome P450 2B4. *Drug Metab Dispos* **39**:539–550.
Tougou K, Nakamura A, Watanabe S, Okuyama Y, and Morino A (1998) Paraoxonase has a major role in the hydrolysis of prulifloxacin (NM441), a prodrug of a new antibacterial agent. *Drug Metab Dispos* **26**:355–359.
Tuffal G, Roy S, Lavisse M, Brasseur D, Schofield J, Delesque Touchard N, Savi P, Bremond N, Rouchon MC, and Hurbin F, et al. (2011) An improved method for specific and quantitative determination of the clopidogrel active metabolite isomers in human plasma. *Thromb Haemost* **105**:696–705.
Williams FM (1985) Clinical significance of esterases in man. *Clin Pharmacokinet* **10**:392–403.
Yin T and Miyata T (2011) Pharmacogenomics of clopidogrel: evidence and perspectives. *Thromb Res* **128**:307–316.

Address correspondence to: Dr. Dong-Hyun Kim, Department of Pharmacology and PharmacoGenomics Research Center, Inje University College of Medicine, #633-165 Gaegum-Dong, Jin-Gu, Busan 614-735, South Korea. E-mail: dhkim@inje.ac.kr