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Prilocaine- and Lidocaine-Induced Methemoglobinemia Is Caused by Human Carboxylesterase-, CYP2E1-, and CYP3A4-Mediated Metabolic Activation[S]

Ryota Higuchi, Tatsuki Fukami, Miki Nakajima, and Tsuyoshi Yokoi

Drug Metabolism and Toxicology, Faculty of Pharmaceutical Sciences, Kanazawa University, Kakuma-machi, Kanazawa, Japan

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ABSTRACT

Prilocaine and lidocaine are classified as amide-type local anesthetics for which serious adverse effects include methemoglobinemia. Although the hydrolyzed metabolites of prilocaine (*o*-toluidine) and lidocaine (2,6-xylidine) have been suspected to induce methemoglobinemia, the metabolic enzymes that are involved remain uncharacterized. In the present study, we aimed to identify the human enzymes that are responsible for prilocaine- and lidocaine-induced methemoglobinemia. Our experiments revealed that prilocaine was hydrolyzed by recombinant human carboxylesterase (CES) 1A and CES2, whereas lidocaine was hydrolyzed by only human CES1A. When the parent compounds (prilocaine and lidocaine) were incubated with human liver microsomes (HLM), methemoglobin (Met-Hb) formation was lower than when the hydrolyzed metabolites were incubated with HLM. In addition, Met-Hb formation when prilocaine and *o*-toluidine were incubated with HLM was higher than that when lidocaine and 2,6-xylidine were incubated with HLM. Incubation with diisopropyl fluorophosphate and bis-(4-nitrophenyl) phosphate, which are general inhibitors of CES, significantly decreased Met-Hb formation when prilocaine and lidocaine were incubated with HLM. An anti-CYP3A4 antibody further decreased the residual formation of Met-Hb. Met-Hb formation after the incubation of *o*-toluidine and 2,6-xylidine with HLM was only markedly decreased by incubation with an anti-CYP2E1 antibody. *o*-Toluidine and 2,6-xylidine were further metabolized by CYP2E1 to 4- and 6-hydroxy-*o*-toluidine and 4-hydroxy-2,6-xylidine, respectively, and these metabolites were shown to more efficiently induce Met-Hb formation than the parent compounds. Collectively, we found that the metabolites produced by human CES-, CYP2E1-, and CYP3A4-mediated metabolism were involved in prilocaine- and lidocaine-induced methemoglobinemia.

Introduction

Prilocaine and lidocaine are classified as amide-type local anesthetics, which prevent and relieve pain by interrupting nerve excitation and conduction via direct interaction with voltage-gated Na^+ channels to block the Na^+ current (Lipkind and Fozzard, 2005). In general, prilocaine and lidocaine are safely used in patients, although methemoglobinemia is occasionally induced (Rehman, 2001; Maimo and Redick, 2004). Methemoglobinemia is defined as a methemoglobin (Met-Hb) level >1.0% in the blood. Met-Hb is an abnormal form of hemoglobin in which iron is oxidized from the ferrous (Fe^{2+}) to the ferric state (Fe^{3+}). Because Met-Hb cannot bind and transport oxygen, increased levels of Met-Hb are associated with clinically severe symptoms (Moore et al., 2004). Met-Hb concentrations are normally maintained at roughly 1% of total hemoglobin by the action of Met-Hb reductase (Guay, 2009). Cyanosis usually occurs when Met-Hb concentrations increase above 10% and is followed by anxiety, fatigue, and tachycardia when Met-Hb levels reach 20%–50% of total hemoglobin levels. When Met-Hb levels reach 50%–70% of total hemoglobin levels, coma, and death may occur (Rodriguez et al., 1994).

When prilocaine was used for epidural analgesia and peripheral nerve block, some patients were reported to develop methemoglobinemia (Climie et al., 1967; Vasters et al., 2006). For example, when 288 mg of prilocaine was used in a 19-year-old white woman, her Met-Hb levels were observed to be 37.8% of total hemoglobin levels approximately 4 hours after injection (Kreutz and Kinni, 1983). Fetuses and infants under 6 months of age seem to be more susceptible. Lidocaine also causes methemoglobinemia, although only rarely. In fact, the number of articles that were published from 1949 through 2007 concerning lidocaine-related methemoglobinemia (12 episodes) is lower than the number of articles concerning prilocaine-related methemoglobinemia (68 episodes) (Guay, 2009). In humans, prilocaine and lidocaine are hydrolytically metabolized to the aromatic amines *o*-toluidine and 2,6-xylidine, respectively. These metabolites have been reported to cause increased levels of Met-Hb after intravenous administration to cats or rats (Onji and Tyuma, 1965; Lindstrom et al., 1969). On the basis of the results of these studies, prilocaine and lidocaine hydrolysis pathways have been suggested to play an important role in methemoglobinemia, but the metabolic enzymes that are involved in methemoglobinemia remain to be experimentally characterized. Moreover, it is unclear whether differences in methemoglobinemia frequency after prilocaine and lidocaine treatment are attributable to differences in enzymatic metabolism or differences in the potency of Met-Hb formation by their metabolites.

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ABBREVIATIONS: AADAC, arylacetamide deacetylase; BNPP, bis-(4-nitrophenyl) phosphate; CES, carboxylesterase; DFP, diisopropyl fluorophosphate; HLM, human liver microsomes; HPLC, high-performance liquid chromatography; Met-Hb, methemoglobin; NADPH-GS, NADPH-generating system; NPR, NADPH-P450 reductase; P450, cytochrome P450.

Esterases, which are expressed in human liver, plasma, and other tissues, contribute to the hydrolysis of approximately 10% of clinically therapeutic drugs, including ester, amide, and thioester bonds (Fukami and Yokoi, 2012). In particular, human carboxylesterases (CES), especially the CES1A and CES2 enzymes, are the major serine esterases that are responsible for the hydrolysis of various drugs and xenobiotics (Imai et al., 2006). Recently, we demonstrated that human arylacetamide deacetylase (AADAC) is involved in the metabolism of drugs such as flutamide, phenacetin, and rifamycins (Watanabe et al., 2009, 2010; Nakajima et al., 2011). We have more recently demonstrated that the hydrolysis of phenacetin by AADAC, which produced *p*-phenetidine, an aromatic amine metabolite, is predominantly involved in the phenacetin-induced formation of Met-Hb (Kobayashi et al., 2012). Thus, it is conceivable that CES1A, CES2, and AADAC are involved in the hydrolysis of prilocaine and lidocaine.

Ganesan et al. (2010) reported that dapsone-hydroxylamine, which is an *N*-hydroxylated metabolite of dapsone, was suspected to be a cause of dapsone-induced methemoglobinemia. The formation of dapsone-hydroxylamine is catalyzed by cytochromes P450 (P450) CYP2C19, CYP2E1, and CYP3A4. We recently reported that metabolic activation by CYP1A2 and CYP2E1 and hydrolysis of AADAC play a predominant role in phenacetin-induced methemoglobinemia (Kobayashi et al., 2012). Thus, it is conceivable that P450(s) are also involved in prilocaine- and lidocaine-induced methemoglobinemia.

On the basis of the aforementioned background studies, in the present study, we investigated the human enzymes responsible for the metabolism of prilocaine and lidocaine to clarify the mechanisms of prilocaine- and lidocaine-induced methemoglobinemia. In addition, the efficiencies of enzymatic metabolism and Met-Hb formation were compared between prilocaine and lidocaine.

Materials and Methods

Chemicals and Reagents. Lidocaine hydrochloride, 2,6-xylidine, *o*-toluidine, 4-hydroxyl-*o*-toluidine, and diisopropyl fluorophosphate (DFP) were purchased from Wako Pure Chemical Industries (Osaka, Japan). 6-Hydroxyl-*o*-toluidine and 4-hydroxyl-2,6-xylidine were purchased from Tokyo Chemical Industry (Tokyo, Japan). Prilocaine hydrochloride and bis-(4-nitrophenyl)-phosphate (BNPP) were obtained from Sigma-Aldrich (St. Louis, MO). Human liver microsomes (HLM; pooled, $n = 50$); recombinant human CYP1A2, CYP2A6, CYP2C8, CYP2D6 [with NADPH-P450 reductase (NPR)], CYP2B6, CYP2C9, CYP2C19, CYP2E1, and CYP3A4 (with NPR and cytochrome b_5) enzymes expressed in baculovirus-infected insect cells; monoclonal mouse anti-human CYP1A2 antibody; anti-human CYP2E1 antibody; and anti-human CYP3A4 antibody were purchased from BD Gentest (Woburn, MA). Other chemicals were of the highest commercially available grade.

Mouse and Human Red Blood Cells. Animals were maintained in accordance with the National Institutes of Health Guide for Animal Welfare of Japan, and the protocols were approved by the Institutional Animal Care and Use Committee of Kanazawa University, Japan. The use of human red blood cells was approved by the Ethics Committees of Kanazawa University (Kanazawa, Japan). Mouse blood (pooled samples from 5 C57BL/6J mice: 6-week-old male, 20–25 g) that had been obtained from SLC Japan (Hamamatsu, Japan) and human blood samples (from five healthy Japanese volunteers: 22–30-year-old males) were obtained according to our previous report (Kobayashi et al., 2012). All assays were performed immediately after the separation of the red blood cells.

Prilocaine and Lidocaine Hydrolase Activities. Prilocaine and lidocaine hydrolase activities were determined as follows: a typical incubation mixture (final volume of 0.2 ml) contained 100 mM potassium phosphate buffer (pH 7.4) and various enzyme sources (HLM or Sf21 cell homogenates expressing esterases, 0.4 mg/ml). Sf21 cell homogenates expressing CES1A, CES2, or AADAC were prepared as previously described (Fukami et al., 2010; Watanabe et al., 2010). In a preliminary study, we confirmed that the formation rates of *o*-toluidine and 2,6-xylidine from prilocaine and lidocaine, respectively, were linear with respect to protein concentration (< 2.0 mg/ml) and incubation time (<120 minutes). Prilocaine and lidocaine were dissolved in distilled water. The reactions were initiated by the addition of prilocaine and lidocaine (0.2–10 mM for HLM or 0.1–4 mM for Sf21 cell homogenates expressing esterases) after a 2-minute preincubation at 37°C. After a 30-minute incubation, the reactions were terminated by the addition of 10 μl of ice-cold 60% perchloric acid. After removal of the protein by centrifugation at 9500*g* for 5 minutes, a 60-μl portion of the supernatant was subjected to high-performance liquid chromatography (HPLC). The HPLC analysis was performed using an L-7100 pump (Hitachi, Tokyo, Japan), an L-7200 autosampler (Hitachi), an L-7405 UV detector (Hitachi), and a D-2500 Chromato-Integrator (Hitachi) equipped with a Wakopak eco-ODS column (5-μm particle size, 4.6 mm i.d.$\times$150 mm; Wako Pure Chemical Industries). The eluent was monitored at 210 nm with a noise-base clean Uni-3 (Union, Gunma, Japan), which can reduce the noise by integrating the output and increase the signal by 3-fold by differentiating the output and by 5-fold by further amplification with an internal amplifier, resulting in a maximum 15-fold amplification of the signal. The mobile phase was 35% methanol containing 0.2% phosphoric acid and 2.2 mM sodium 1-octanesulfonate. The flow rate was 1.0 ml/min. The column temperature was 35°C. The quantification of *o*-toluidine and 2,6-xylidine was performed by comparing the HPLC peak height with that of an authentic standard. The limit of quantification in the reaction mixture for *o*-toluidine and 2,6-xylidine was 100 nM, with a coefficient of variation $<5.6\%$. The activity at each concentration was determined as the mean value in triplicate. For kinetic analyses of the prilocaine and lidocaine hydrolase activities, the parameters were estimated from the fitted curves with use of a computer program (KaleidaGraph; Synergy Software, Reading, PA) that was designed for use in nonlinear regression analyses.

Met-Hb Formation. A Met-Hb formation assay was conducted according to the methods outlined in our previous study (Kobayashi et al., 2012), with a slight modification. A typical incubation mixture (final volume of 0.2 ml) contained 5% of the mouse red blood cell fraction except in (7), where human red blood cell fraction was used, 100 mM potassium phosphate buffer (pH 7.4), an NADPH-generating system (NADPH-GS: 0.5 mM $NADP^+$, 5 mM glucose 6-phosphate, 5 mM $MgCl_2$, and 1 U/ml glucose-6-phosphate dehydrogenase), and various enzyme sources.

1. To investigate the time-dependence of Met-Hb formation, HLM (1.0 mg/ml) were used as enzyme sources. The reactions were initiated by the addition of prilocaine, lidocaine, *o*-toluidine, or 2,6-xylidine (1 mM) after a 2-minute preincubation at 37°C. After the 0–120-minute incubation at 37°C, the reaction was terminated by placing the samples on ice.
2. To investigate the concentration-dependence of Met-Hb formation, HLM (1.0 mg/ml) were used as enzyme sources. The reactions were initiated by the addition of prilocaine, lidocaine, *o*-toluidine, or 2,6-xylidine (0.01, 0.1, 1, or 10 mM) after a 2-minute preincubation at 37°C. After the 60-minute incubation at 37°C, the reaction was terminated by placing the samples on ice.
3. To determine the P450 enzymes that were involved in prilocaine-, lidocaine-, *o*-toluidine-, and 2,6-xylidine–induced Met-Hb formation, recombinant human P450 enzymes (25 pmol /ml) were used as enzyme sources. The reactions were initiated by the addition of prilocaine (10 mM), lidocaine (10 mM), *o*-toluidine (1 mM), or 2,6-xylidine (1 mM) after a 2-minute preincubation at 37°C. After the 120-minute incubations (prilocaine and lidocaine) and the 60-minute incubations (*o*-toluidine and 2,6-xylidine) at 37°C, the reactions were terminated by placing the samples on ice.
4. To investigate the involvement of various esterase(s) in the HLM, inhibition analyses of prilocaine- and lidocaine-induced Met-Hb formation were performed using the general CES inhibitors DFP and BNPP (Watanabe et al., 2009). HLM (1.0 mg/ml) were used as enzyme sources, and the concentrations of inhibitors were 100 μM. DFP and BNPP were dissolved in distilled water. The reaction conditions were the same as those described in (3).
5. To investigate the involvement of P450 enzymes in the HLM in Met-Hb formation, inhibition analyses of the formation of Met-Hb by prilocaine

(10 mM), lidocaine (10 mM), *o*-toluidine (1 mM), or 2,6-xylidine (1 mM) were performed using anti-P450 antibodies. HLM (0.5 mg/ml) were used as enzyme sources. Ten microliters of antibody-mixtures [4 μl of anti-CYP2E1 antibody mixed with 6 μl of 25 mM Tris-buffer (pH 7.5) or 10 μl of anti-CYP1A2 or anti-CYP3A4 antibody] was incubated on ice for 30 minutes with enzyme sources, after which typical incubation mixtures (final volume of 0.2 ml) that included the antibody mixtures were prepared. To investigate the involvement of P450 enzymes in prilocaine- and lidocaine-induced Met-Hb formation in the absence of a hydrolysis reaction, DFP (100 μM) was added into the incubation mixture. The reaction conditions were the same as those described in (3).

6. To investigate whether the hydroxylated metabolites of *o*-toluidine and 2,6-xylidine induced Met-Hb formation, *o*-toluidine, 4-hydroxy-*o*-toluidine, 6-hydroxy-*o*-toluidine, 2,6-xylidine, and 4-hydroxy-2,6-xylidine (1 mM) were incubated with HLM (1.0 mg/ml) and mouse red blood cells both in the presence and absence of an NADPH-GS. After a 60-minute incubation at 37°C, the reactions were terminated by placing the samples on ice.
7. To investigate the sensitivity of human red blood cells to prilocaine- and lidocaine-induced Met-Hb formation, a Met-Hb formation assay was conducted using human red blood cells instead of mouse red blood cells. HLM (1.0 mg/ml) were used as enzyme sources, and DFP (100 μM) was added into the incubation mixture. The reaction was initiated by the addition of 10 mM prilocaine and lidocaine after a 2-minute preincubation at 37°C. The reaction conditions were the same as those described in (3).

Prilocaine and lidocaine were dissolved in distilled water. *o*-Toluidine and 2,6-xylidine were dissolved in acetonitrile, and the final concentration of acetonitrile in the incubation mixture was 1%. It has been reported that 1% acetonitrile does not inhibit the activities of CYP1A2, CYP2E1, and CYP3A4 (Chauret et al., 1998).

The Met-Hb levels in red blood cells were determined as the percentage of total hemoglobin according to the methods outlined in our previous study (Kobayashi et al., 2012)

Normalizing Met-Hb Formation by P450 Levels in HLM. Met-Hb formation was normalized by the levels of each P450 enzyme in HLM and was calculated using the following equation, where the $P450_{HLM}$ activity and $P450_{expression}$ activity values are the marker activities of each P450 in HLM and recombinant P450 expression systems, respectively:

$$\text{Met-Hb formation normalized by P450 levels in HLM (\%)} = \text{Met-Hb formation by recombinant human P450 expression systems (\%)} \times P450_{HLM}\ \text{activity}/P450_{expression}$$

The reaction conditions for Met-Hb formation by recombinant human P450 expression systems were determined as follows: prilocaine, lidocaine (10 mM), and their hydrolyzed metabolites (1 mM) were incubated with each P450 expression system (25 pmol P450/ml), an NADPH-GS, and mouse red blood cells. The incubation time was either 120 minutes (prilocaine and lidocaine) or 60 minutes (*o*-toluidine and 2,6-xylidine).

The reaction conditions for Met-Hb formation in HLM were determined as described in the above paragraph, except HLM (1.0 mg/ml) were used in place of the P450 expression systems. Met-Hb formation was detected as described in (3) above.

Methoxyresorufin *O*-demethylase, chlorzoxazone 6-hydroxylase, and midazolam 1′-hydroxylase activities were measured as markers for the activities of CYP1A2, CYP2E1, and CYP3A4, respectively, by HPLC, according to methods described in our previous reports (Nakajima et al., 2002; Fukami et al., 2007, 2008).

***o*-Toluidine and 2,6-Xylidine Hydroxylase Activities.** The activities of *o*-toluidine and 2,6-xylidine hydroxylase were determined as follows: a typical incubation mixture (final volume of 0.2 ml) contained *o*-toluidine or 2,6-xylidine (4–200 μM), 100 mM potassium phosphate buffer (pH 7.4), an NADPH-GS, and HLM (0.4 mg/ml). In a preliminary study, we confirmed that the formation rates of 4-hydroxyl-*o*-toluidine and 6-hydroxyl-*o*-toluidine from *o*-toluidine, as well as 4-hydroxyl-2,6-xylidine from 2,6-xylidine, in HLM were linear with respect to protein concentration (< 1.0 mg/ml) and incubation time (<60 minutes). *o*-Toluidine and 2,6-xylidine were dissolved in acetonitrile, and the final concentration of acetonitrile in the incubation mixture was 1%.

Inhibition analyses of *o*-toluidine or 2,6-xylidine hydroxylation were performed using anti-P450 antibodies. HLM (0.4 mg/ml) were used as enzyme sources. Eight microliters of antibody-mixtures [3.2 μl of anti-CYP2E1 antibody mixed with 4.8 μl of 25 mM Tris-buffer (pH 7.5) or 8 μl of anti-CYP1A2 or anti-CYP3A4 antibody] was incubated with enzyme sources on ice for 30 minutes. Then, typical incubation mixtures (final volume of 0.2 ml) were prepared by the addition of *o*-toluidine (50 μM) or 2,6-xylidine (30 μM).

The reactions were initiated by the addition of an NADPH-GS after a 2-minute preincubation at 37°C. After a 30-minute incubation, the reactions were terminated by the addition of 10 μl of ice-cold 60% perchloric acid. After removal of the protein by centrifugation at 9500*g* for 5 minutes, a 50-μl portion of the supernatant was subjected to HPLC. The HPLC equipment was the same as that described above. The mobile phase for *o*-toluidine hydroxylation was 8.5% methanol/8% acetonitrile containing 0.2% phosphoric acid and 2.2 mM sodium 1-octanesulfonate, and the mobile phase for 2,6-xylidine hydroxylation was 10% acetonitrile containing 0.11% phosphoric acid and 2.2 mM sodium 1-octanesulfonate. The quantification of the hydroxylated metabolites of *o*-toluidine and 2,6-xylidine was performed by comparing the HPLC peak height with that of an authentic standard. The activity at each concentration was determined as the mean value in triplicate. For kinetic analyses of the *o*-toluidine and 2,6-xylidine hydroxylase activities, the parameters were estimated as described above.

Statistical Methods. Statistical analyses between two and multiple groups were performed using an unpaired, two-tailed Student's *t* test and analysis of variance, followed by Tukey's post-hoc test. $P < 0.05$ was considered to be statistically significant.

Results

Prilocaine and Lidocaine Hydrolase Activities in HLM. Because hydrolysis of prilocaine and lidocaine was suspected to play an important role in methemoglobinemia, we investigated whether microsomes of the human liver, which is the main organ for drug metabolism and expresses various esterases, could hydrolyze prilocaine and lidocaine (Fig. 1, A and B). Data for these hydrolase activities in HLM followed Michaelis-Menten kinetics. The K_m and V_{max} values for the hydrolysis of prilocaine in HLM were 1.15 ± 0.01 mM and 2.46 ± 0.04 nmol/min/mg protein, respectively, resulting in a CL_{int} value of 2.14 ± 0.04 μl/min/mg protein. The K_m and V_{max} values for the hydrolysis of lidocaine in HLM were 0.96 ± 0.06 mM and 0.62 ± 0.01 nmol/min/mg protein, respectively, resulting in a CL_{int} value of 0.66 ± 0.03 μl/min/mg protein (Table 1). Thus, the CL_{int} value for the hydrolysis of prilocaine was shown to be 3.2 higher than that of lidocaine. These results indicated that HLM have a higher metabolic efficiency for prilocaine hydrolysis than for lidocaine hydrolysis.

Prilocaine and Lidocaine Hydrolase Activities by Recombinant Human CES1A and CES2. To confirm that human CES1A, CES2, and AADAC, which are typical serine esterases that are involved in the hydrolysis of numerous drugs, can hydrolyze prilocaine and lidocaine, prilocaine and lidocaine hydrolase activities were measured using recombinant human CES1A, CES2, and AADAC expressed in Sf21 cells (Fig. 1, C and D). Data for these hydrolase activities followed Michaelis-Menten kinetics. The K_m and V_{max} values for prilocaine hydrolase activity by CES1A were 0.31 ± 0.01 mM and 0.40 ± 0.01 nmol/min/mg protein, respectively, resulting in a CL_{int} value of 1.29 ± 0.04 μl/min/mg protein (Table 1). CES2 displayed the prilocaine hydrolase activity with K_m, V_{max}, and CL_{int} values of 0.39 ± 0.01 mM, 0.10 ± 0.00 nmol/min/mg protein, and 0.26 ± 0.00 μl/min/mg protein, respectively. The K_m, V_{max}, and CL_{int} values for lidocaine hydrolysis by CES1A were 0.35 ± 0.06 mM, 0.14 ± 0.00 nmol/min/mg protein, and 0.40 ± 0.02 μl/min/mg

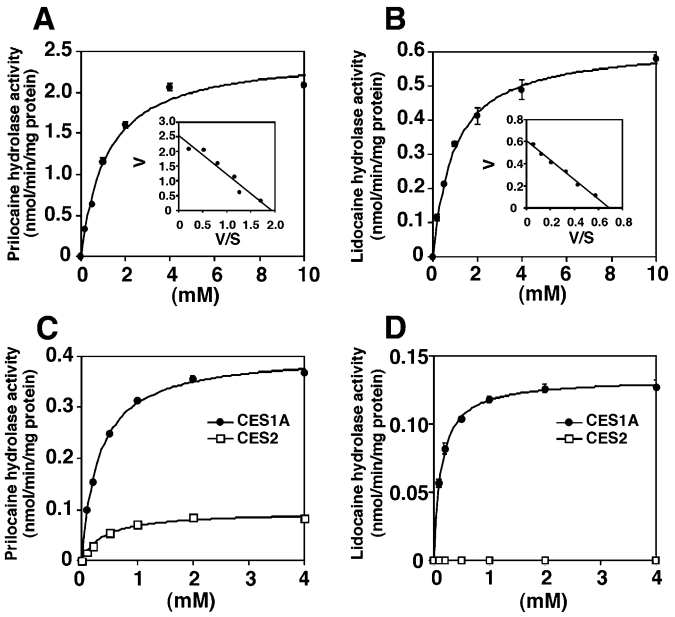

Fig. 1. Kinetic analyses of prilocaine and lidocaine hydrolase activities in HLM and recombinant human CES1A and CES2 expressed in Sf21 cells. HLM (0.4 mg/ml) (A and B) or CES1A and CES2 (0.2 mg/ml) (C and D) were incubated with prilocaine (A and C) and lidocaine (B and D) for 30 minutes. Hydrolase activities for prilocaine and lidocaine were measured by quantitative analyses of *o*-toluidine and 2,6-xylidine, respectively, using HPLC. Each data point represents the mean ± S.D. of triplicate determinations.

protein, respectively, whereas CES2 did not display any lidocaine hydrolase activity (Table 1). AADAC did not display hydrolase activities for either prilocaine or lidocaine. These results indicate that the metabolic efficiency of prilocaine hydrolysis by CES1A was higher than that of lidocaine hydrolysis. CES2 participated in prilocaine hydrolysis but not lidocaine hydrolysis.

Formation of Met-Hb by Prilocaine or Lidocaine and Their Metabolites. To investigate Met-Hb formation in vitro, prilocaine, lidocaine, or their hydrolyzed metabolites, *o*-toluidine and 2,6-xylidine, were incubated with HLM, an NADPH-GS, and mouse red blood cells for 0–120 minutes. Met-Hb formation was linear with respect to incubation time (1 mM prilocaine or lidocaine <120 minutes, 1 mM *o*-toluidine or 2,6-xylidine <60 minutes) (Fig. 2A).

To compare the induction potency of Met-Hb formation among prilocaine, lidocaine, *o*-toluidine, and 2,6-xylidine, the compounds were incubated at various concentrations (Fig. 2B). Met-Hb formation increased in a concentration-dependent manner. *o*-Toluidine and 2,6-xylidine more efficiently induced Met-Hb formation, compared

TABLE 1

Kinetic parameters of the hydrolase activities of prilocaine and lidocaine

Data are the mean ± S.D. of triplicate determinations.

Drug	Enzyme Source	K_m	V_{max}	CL_{int}
		mM	*nmol/min/mg protein*	*μl/min/mg protein*
Prilocaine	HLM	1.15 ± 0.01	2.46 ± 0.04	2.14 ± 0.04
	CES1A	0.31 ± 0.01	0.40 ± 0.01	1.29 ± 0.04
	CES2	0.39 ± 0.01	0.10 ± 0.00	0.26 ± 0.00
	AADAC	ND	ND	—
Lidocaine	HLM	0.96 ± 0.06	0.62 ± 0.01	0.66 ± 0.03
	CES1A	0.35 ± 0.06	0.14 ± 0.00	0.40 ± 0.02
	CES2	ND	ND	—
	AADAC	ND	ND	—

ND, not detected.

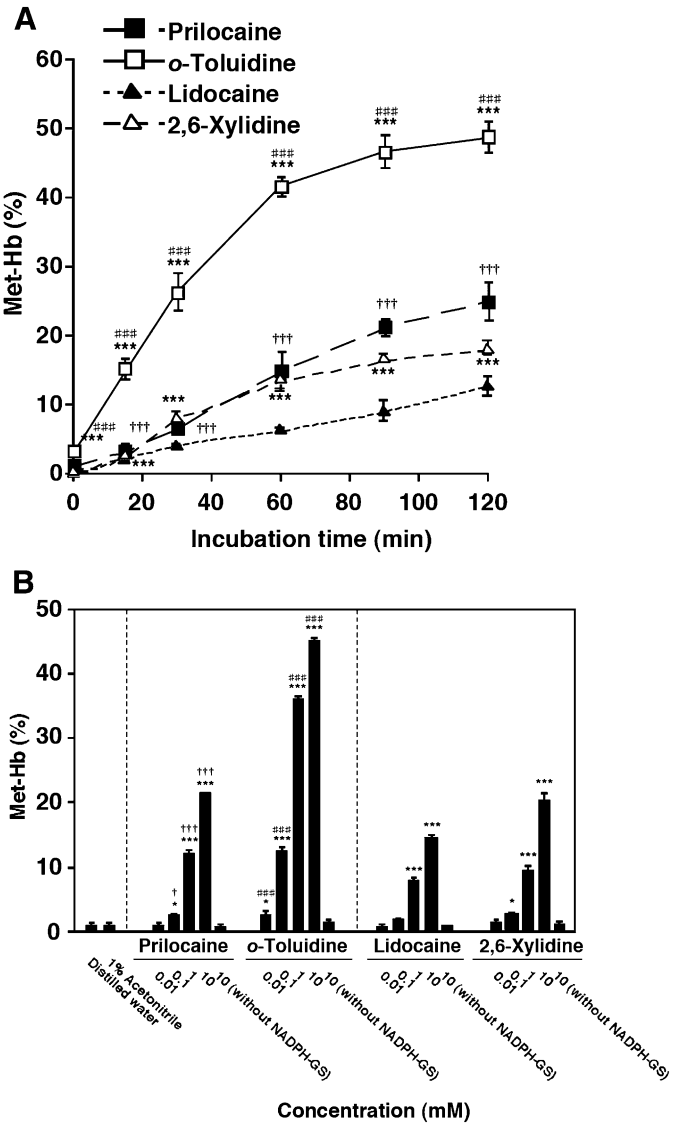

Fig. 2. (A) Time-dependent prilocaine-, lidocaine-, *o*-toluidine–, and 2,6-xylidine–induced Met-Hb formation. Prilocaine, lidocaine, and their hydrolyzed metabolites (1 mM) were incubated for 0–120 minutes with HLM (1.0 mg/ml), an NADPH-GS, and mouse red blood cells. Each data point represents the mean ± S.D. of triplicate determinations. Differences in Met-Hb formation, compared with the corresponding parent compounds at the same incubation time, were considered to be significant at $^{***}P < 0.001$. Differences in prilocaine- and lidocaine-induced Met-Hb formation at the same incubation time were considered to be significant at $^{\dagger\dagger\dagger}P < 0.001$. Differences in *o*-toluidine– and 2,6-xylidine–induced Met-Hb formation at the same incubation time were considered to be significant at $^{\#\#\#}P < 0.001$. (B) Concentration-dependent prilocaine-, lidocaine-, *o*-toluidine–, and 2,6-xylidine–induced Met-Hb formation. Prilocaine, lidocaine, and their hydrolyzed metabolites (0.01–10 mM) were incubated with HLM (1.0 mg/ml), an NADPH-GS, and mouse red blood cells for 60 minutes. Each column represents the mean ± S.D. of triplicate determinations. Differences in Met-Hb formation, compared with the corresponding vehicle-treated controls, were considered to be significant at $^{*}P < 0.05$; $^{***}P < 0.001$. Differences in prilocaine- and lidocaine-induced Met-Hb formation at the same concentration were considered to be significant at $^{\dagger}P < 0.05$; $^{\dagger\dagger\dagger}P < 0.001$. Differences in *o*-toluidine– and 2,6-xylidine–induced Met-Hb formation at the same concentration were considered to be significant at $^{\#\#\#}P < 0.001$.

with their corresponding parent drugs. This result suggested that the hydrolyzed metabolites enhanced prilocaine- or lidocaine-induced Met-Hb formation. Prilocaine-induced Met-Hb formation was significantly higher than lidocaine-induced formation at each concentration that was greater than 0.1 mM. In the same manner, *o*-toluidine–induced Met-Hb formation was significantly higher than 2,6-xylidine–induced

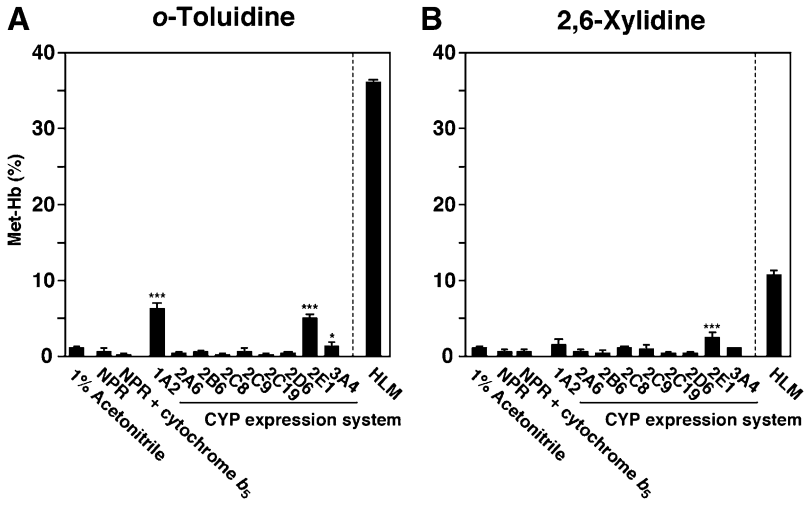

Fig. 3. The effects of P450 (CYP) enzymes on *o*-toluidine– and 2,6-xylidine–induced Met-Hb formation. Each individual recombinant human P450 expression system (25 pmol P450/ml) or HLM (1.0 mg/ml) was incubated with 1 mM *o*-toluidine (A) or 2,6-xylidine (B), an NADPH-GS, and mouse red blood cells for 60 minutes. Each column represents the mean ± S.D. of triplicate determinations. Differences in Met-Hb formation, compared with NPR or NPR + cytochrome b_5 (control supersomes expressing no P450s), were considered to be significant at $^*P < 0.05$; $^{***}P < 0.001$.

formation at each concentration that was greater than 0.1 mM. The increased Met-Hb formation that was induced by prilocaine, lidocaine, and their hydrolyzed metabolites was not observed in the absence of an NADPH-GS (Fig. 2B). These results suggest that the potency of prilocaine to induce Met-Hb formation was approximately 1.5-fold higher than that of lidocaine at the same concentration and that the NADPH-dependent enzymes that are expressed in HLM are essential for Met-Hb formation.

Met-Hb Formation after the Metabolic Activation of *o*-Toluidine and 2,6-Xylidine by Human P450(s). As outlined above, NADPH-dependent enzyme(s) are essential for prilocaine- and lidocaine-induced Met-Hb formation. Therefore, the involvement of representative NADPH-dependent enzymes (the P450 enzymes) in *o*-toluidine and 2,6-xylidine metabolism was investigated (Fig. 3). Either *o*-toluidine or 2,6-xylidine (1 mM) was incubated with recombinant human P450s, an NADPH-GS, and mouse red blood cells. *o*-Toluidine–induced Met-Hb formation was significantly increased by CYP1A2 (6.2% ± 0.7%), CYP2E1 (5.0% ± 0.4%), and CYP3A4 (1.3% ± 0.5%), and 2,6-xylidine–induced Met-Hb formation was increased by CYP2E1 (2.3% ± 0.8%).

Prilocaine- and Lidocaine-Induced Met-Hb Formation Inhibition Analyses in the Presence of Esterase Inhibitors. To investigate the involvement of human CES in prilocaine- and lidocaine-induced methemoglobinemia, inhibition analyses were performed with DFP and BNPP, which are potent CES inhibitors (Watanabe et al., 2009) (Fig. 4). The hydrolase activities of prilocaine and lidocaine (1 mM) in HLM (1.0 mg/ml) were preliminarily confirmed by HPLC to be completely inhibited by 10 μM DFP and 10 μM BNPP (unpublished data). When prilocaine and lidocaine (10 mM) were incubated with HLM, an NADPH-GS, and mouse red blood cells in the presence of DFP or BNPP (100 μM), both prilocaine- and lidocaine-induced Met-Hb formation were decreased (prilocaine, percentage of control: 31.6% in the presence of DFP and 34.0% in the presence of BNPP; lidocaine, percentage of control: 56.6% in the presence of DFP and 56.6% in the presence of BNPP) (Fig. 4). DFP and BNPP did not alter Met-Hb formation (unpublished data). Thus, CES were determined to be involved in Met-Hb formation.

Met-Hb Formation after the Metabolic Activation of Prilocaine and Lidocaine by Human P450(s). As shown in Fig. 4, DFP and BNPP could not completely inhibit prilocaine- and lidocaine-induced Met-Hb formation, suggesting that the metabolic activation of prilocaine and lidocaine by P450 enzyme(s) may mediate Met-Hb formation in the absence of hydrolysis. When prilocaine and lidocaine (10 mM) were incubated with representative recombinant human P450s, an NADPH-GS, and mouse red blood cells, Met-Hb formation increased in the presence of CYP3A4 (1.7% ± 0.2% for prilocaine and 1.1% ± 0.4% for lidocaine) (Fig. 5). These results suggest that prilocaine- and lidocaine-induced Met-Hb formation in the absence of hydrolysis is catalyzed by metabolic activation by human CYP3A4.

Normalizing Met-Hb Formation by P450 Levels in HLM. Because each P450 enzyme is expressed at different levels in HLM, we were unable to simply compare the contribution of each P450 enzyme to prilocaine-, lidocaine-, *o*-toluidine–, or 2,6-xylidine–induced Met-Hb formation in HLM with use of human P450 expression systems. Therefore, to estimate the contributions of P450 enzymes in HLM, Met-Hb formation in the presence of recombinant P450 expression systems was normalized by the levels of each P450 enzyme in HLM (Fig. 6). For *o*-toluidine– and 2,6-xylidine–induced Met-Hb formation, CYP2E1 had the highest contribution in HLM,

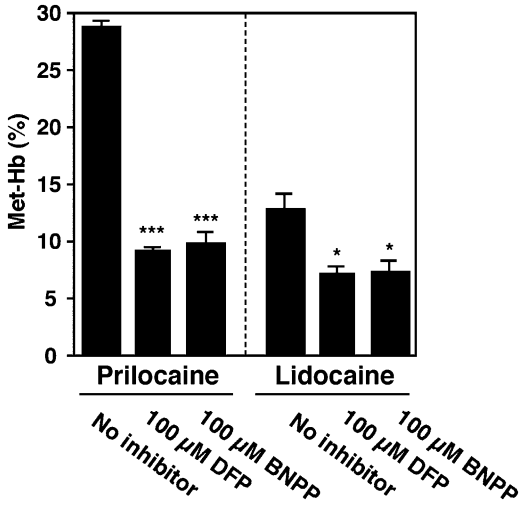

Fig. 4. The effects of DFP or BNPP on prilocaine- and lidocaine-induced Met-Hb formations. HLM (1.0 mg/ml) were incubated with prilocaine or lidocaine (10 mM), an NADPH-GS, and mouse red blood cells for 120 minutes. The concentration of DFP or BNPP was 100 μM. Each column represents the mean ± S.D. of triplicate determinations. Prilocaine- and lidocaine-induced Met-Hb formation in the absence of inhibitors was 28.8% ± 0.5% and 12.9% ± 1.3%, respectively. Differences compared with the controls lacking inhibitor were considered to be significant at $^*P < 0.05$; $^{***}P < 0.001$.

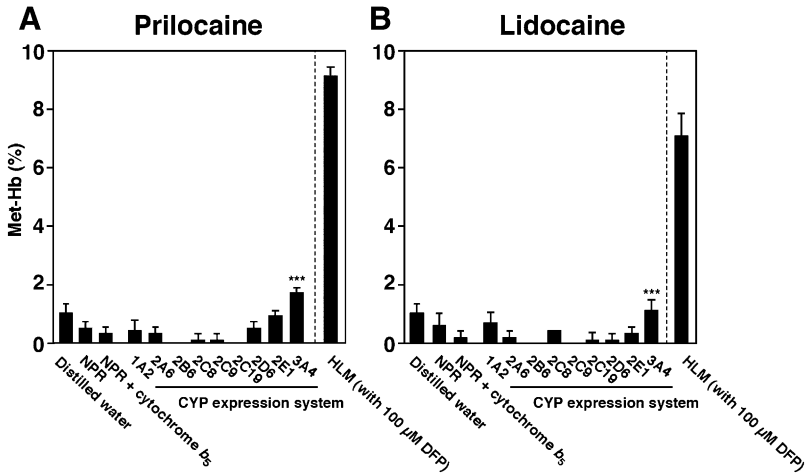

Fig. 5. The effects of P450 (CYP) enzymes on prilocaine- and lidocaine-induced Met-Hb formation without hydrolysis pathway. Each individual recombinant human P450 expression system (25 pmol P450/ml) or HLM (1.0 mg/ml) was incubated with 10 mM prilocaine (A) or lidocaine (B), an NADPH-GS, and mouse red blood cells for 120 minutes. Each column represents the mean ± S.D. of triplicate determinations. Differences in Met-Hb formation, compared with NPR or NPR + cytochrome b_5 (control supersomes expressing no P450s), were considered to be significant at $*P < 0.05$; $***P < 0.001$.

whereas CYP1A2 and CYP3A4 had relatively low contributions (Fig. 6, A and B). For prilocaine- and lidocaine-induced Met-Hb formation, CYP3A4 had the highest contribution in HLM (Fig. 6, C and D). Although flavin-containing monooxygenase is also known to be an NADPH-dependent enzyme, Met-Hb formation that was induced by prilocaine, lidocaine, and their hydrolyzed metabolites in HLM was not inhibited by 1 mM methimazole, which is a competitive flavin-containing monooxygenase inhibitor (unpublished data) (Rawden et al., 2000). Thus, CYP2E1 was determined to contribute highly to *o*-toluidine– and 2,6-xylidine–induced Met-Hb formation, whereas CYP3A4 was determined to contribute highly to prilocaine- and lidocaine-induced Met-Hb formation.

Met-Hb Formation Inhibition Analyses after the Incubation of Prilocaine, Lidocaine, and Their Hydrolyzed Metabolites with Anti-P450 Antibodies. To further investigate the contributions of CYP1A2, CYP2E1, and CYP3A4 to the *o*-toluidine– and 2,6-xylidine–induced Met-Hb formation in HLM, inhibition analyses were performed using anti-P450 antibodies (Fig. 7A). *o*-Toluidine– and 2,6-xylidine–induced Met-Hb formation were markedly decreased by incubation with an anti-CYP2E1 antibody (from 19.5% ± 0.6% to 7.1% ± 0.4% for

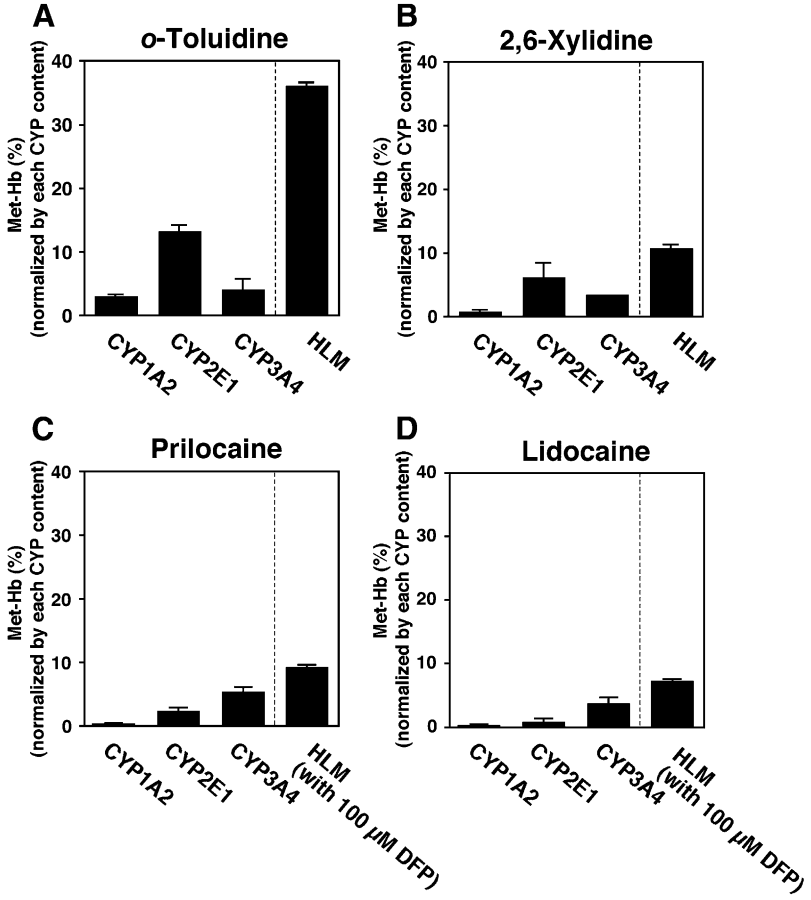

Fig. 6. Met-Hb formation normalized by the levels of each P450 (CYP) in HLM. Met-Hb formation was normalized by the levels of each P450 and calculated according to the equation described in *Materials and Methods*. Prilocaine, lidocaine (10 mM) (A and C), and their hydrolyzed metabolites (1 mM) (B and D) were incubated with each P450 expression system (25 pmol P450/ml), an NADPH-GS, and mouse red blood cells. The incubation time was 120 minutes (prilocaine and lidocaine) or 60 minutes (*o*-toluidine and 2,6-xylidine). Each column represents the mean ± S.D. of triplicate determinations.

o-toluidine and from 6.2% ± 0.3% to 2.6% ± 0.2% for 2,6-xylidine) and slightly decreased by incubation with an anti-CYP3A4 antibody (to 16.9% ± 0.5% for *o*-toluidine and to 4.3% ± 0.3% for 2,6-xylidine). Incubation with an anti-CYP1A2 antibody also slightly decreased 2,6-xylidine–induced Met-Hb formation (to 5.0% ± 0.5%).

To investigate the contribution of CYP3A4 to prilocaine- and lidocaine-induced Met-Hb formation in the absence of hydrolysis, inhibition analyses were performed using anti-P450 antibodies (Fig. 7B). When 100 μM DFP was used to inhibit CES enzyme activity, incubation with an anti-CYP3A4 antibody substantially decreased prilocaine- and lidocaine-induced Met-Hb formation (from 5.7% ± 0.4% to 0.6% ± 0.3% for prilocaine and from 3.4% ± 0.4% to 0.7% ± 0.2% for lidocaine).

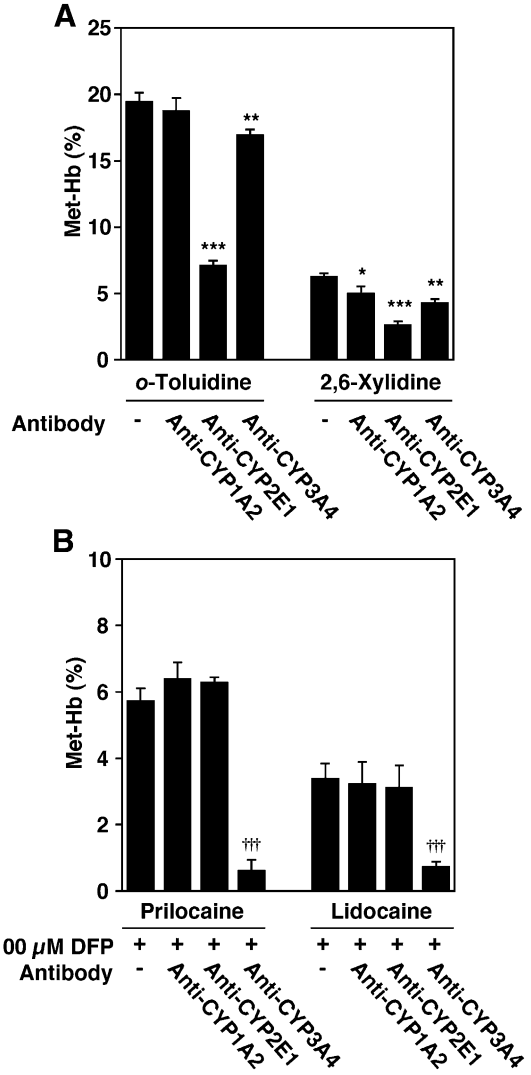

Fig. 7. Inhibitory effects of anti-human CYP1A2, CYP2E1, and CYP3A4 antibodies on *o*-toluidine–, 2,6-xylidine–, prilocaine-, and lidocaine-induced Met-Hb formations in HLM. HLM (0.5 mg/ml) were incubated with 1 mM *o*-toluidine, 1 mM 2,6-xylidine (A), 10 mM prilocaine, or 10 mM lidocaine (in the presence of 100 μM DFP) (B), an NADPH-GS, mouse red blood cells, and each P450 antibody. Met-Hb formation in the absence of antibody was 19.5% ± 0.6% (*o*-toluidine) and 6.2% ± 0.3% (2,6-xylidine), and Met-Hb formation in the absence of antibody but in the presence of DFP was 5.7% ± 0.4% (prilocaine) and 3.4% ± 0.4% (lidocaine). The incubation time was 120 minutes (prilocaine and lidocaine) and 60 minutes (*o*-toluidine and 2,6-xylidine). Each column represents the mean ± S.D. of triplicate determinations. Differences compared to control with no antibody were considered significant at $^{*}P < 0.05$; $^{**}P < 0.005$; and $^{***}P$ or $\dagger\dagger\dagger P < 0.001$.

Met-Hb Formation in the Presence of the Hydroxylated Metabolites of *o*-Toluidine and 2,6-Xylidine. 4-Hydroxy-*o*-toluidine, 6-hydroxy-*o*-toluidine, or 4-hydroxy-2,6-xylidine has been detected in human urine after the administration of prilocaine or lidocaine (Hjelm et al., 1972; Keenaghan and Boyes, 1972). To investigate whether these metabolites could induce Met-Hb formation, in vitro analyses were performed. 4-Hydroxy-*o*-toluidine–,6-hydroxy-*o*-toluidine–, and 4-hydroxy-2,6-xylidine–induced Met-Hb formation were markedly increased, in both the presence and the absence of an NADPH-GS (Fig. 8). A higher degree of Met-Hb formation was detected in the presence of these metabolites than in the presence of *o*-toluidine and 2,6-xylidine. Thus, it was suggested that these hydroxylated metabolites of *o*-toluidine and 2,6-xylidine were one of the causative factors underlying prilocaine- and lidocaine-induced methemoglobinemia.

Formation of the Hydroxylated Metabolites of *o*-Toluidine and 2,6-Xylidine in HLM. Because the hydroxylated metabolites of *o*-toluidine and 2,6-xylidine could induce Met-Hb formation, we investigated whether HLM could catalyze the hydroxylation of *o*-toluidine and 2,6-xylidine (Fig. 9, A and B). Data for these hydroxylase activities in HLM followed Michaelis-Menten kinetics. The K_m and V_{max} values for 4-hydroxylation of *o*-toluidine in HLM were 50.2 ± 2.8 μM and 0.61 ± 0.03 nmol/min/mg protein, respectively, resulting in a CL_{int} value of 12.2 ± 0.2 μl/min/mg protein. The K_m and V_{max} values for 6-hydrolxylation of *o*-toluidine in HLM were 70.3 ± 6.2 μM and 0.05 ± 0.00 nmol/min/mg protein, respectively, resulting in a CL_{int} value of 0.7 ± 0.1 μl/min/mg protein (Table 2). Thus, the CL_{int} value for 4-hydroxylation of *o*-toluidine in HLM was

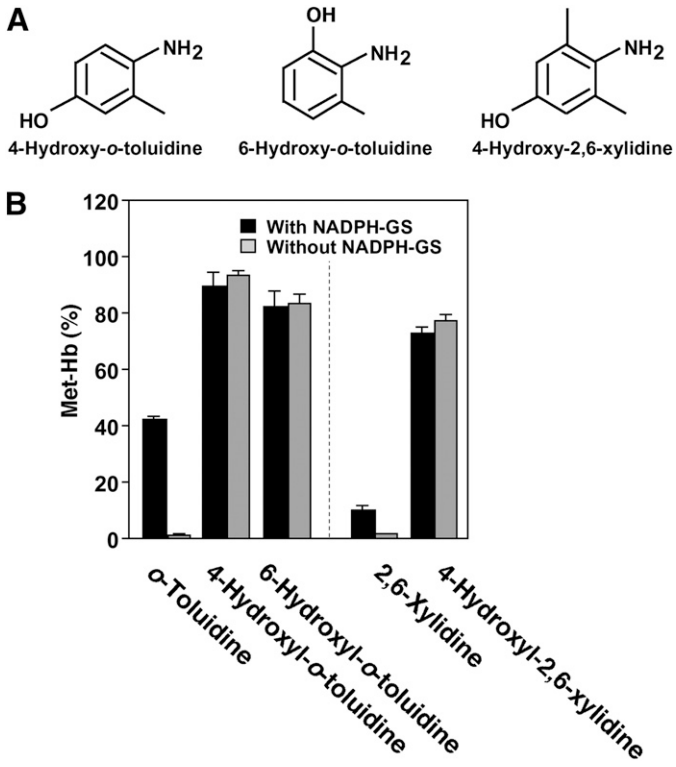

Fig. 8. (A) Chemical structures of 4-hydroxy-*o*-toluidine, 6-hydroxy-*o*-toluidine, and 6-hydroxy-2,6-xylidine. (B) Met-Hb formation induced by the hydroxylated metabolites of *o*-toluidine and 2,6-xylidine. *o*-Toluidine, 4-hydroxy-*o*-toluidine, 6-hydroxy-*o*-toluidine, 2,6-xylidine, or 4-hydroxy-2,6-xylidine (1 mM) was incubated with HLM (1.0 mg/ml) and mouse red blood cells in the presence or absence of an NADPH-GS for 60 minutes. Each column represents the mean ± S.D. of triplicate determinations.

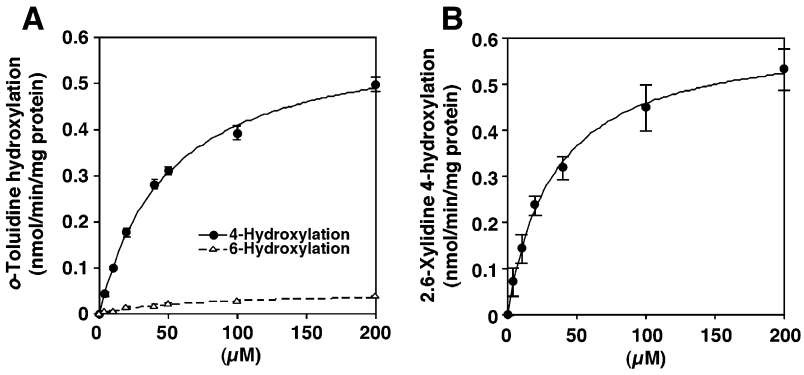

Fig. 9. Kinetic analyses of *o*-toluidine and 2,6-xylidine hydroxylase activities in HLM. HLM (0.4 mg/ml) was incubated with *o*-toluidine (A) and 2,6-xylidine (B) for 30 minutes. *o*-Toluidine and 2,6-xylidine hydroxylase activities were measured by quantitative analyses of 4- or 6-hydroxyl-*o*-toluidine and 4-hydroxy-2,6-xylidine, respectively, using HPLC. Each data point represents the mean ± S.D. of triplicate determinations.

shown to be 18-fold higher than that for 6-hydroxylation. The K_m and V_{max} values for 4-hydroxylation of 2,6-xylidine in HLM were 34.1 ± 5.1 μM and 0.61 ± 0.04 nmol/min/mg protein, respectively, resulting in a CL_{int} value of 18.3 ± 3.4 μl/min/mg protein. These results indicate that *o*-toluidine and 2,6-xylidine were efficiently metabolized to their hydroxylated forms in HLM.

Inhibition Analyses of *o*-Toluidine and 2,6-Xylidine Hydroxylase Activities in the Presence of Anti-P450 Antibodies. To further investigate the contributions of CYP1A2, CYP2E1, and CYP3A4 to *o*-toluidine and 2,6-xylidine hydroxylation in HLM, inhibition analyses were performed using anti-P450 antibodies (Fig. 10). *o*-Toluidine 4- and 6-hydroxylase activities were markedly decreased (percentage of control: 35.4% ± 1.6% for *o*-toluidine 4-hydroxylation and 47.1% ± 2.3% for *o*-toluidine 6-hydroxylation) by incubation with an anti-CYP2E1 antibody. 2,6-Xylidine 4-hydroxylase activity was also markedly decreased by incubation with an anti-CYP2E1 (percentage of control: 25.6% ± 0.9%) and slightly decreased by incubation with an anti-CYP3A4 antibody (percentage of control: 92.9% ± 0.3%). Thus, it was suggested that CYP2E1 could mainly catalyze *o*-toluidine and 2,6-xylidine hydroxylations, leading to Met-Hb formation.

Prilocaine- and Lidocaine-Induced Met-Hb Formation in Human Red Blood Cells. In the aforementioned Met-Hb formation analyses, mouse red blood cells were used. To assess species-specific differences in Met-Hb formation between mice and humans, an analysis using human red blood cells that were obtained from 5 individuals was conducted as follows: prilocaine or lidocaine (10 mM) was incubated with HLM, an NADPH-GS, and individual human red blood cells that had been obtained from the five healthy donors. The levels of Met-Hb formation in the absence of prilocaine or lidocaine were 0.6–1.5% (mean, 1.1%). High Met-Hb formation was observed after incubation with both prilocaine and lidocaine [prilocaine: mean, 34.8% (26.8–41.5%); lidocaine: mean, 9.9% (9.0–11.3%)], but the formation of Met-Hb was significantly decreased by incubation with 100 μM DFP [prilocaine: mean, 7.6% (6.8–8.6%); lidocaine: mean, 6.4% (5.1–7.4%)] (Fig. 11). This result was similar to that obtained using mouse red blood cells (Fig. 4). These results indicate the comparable Met-Hb formation sensitivity between human and mouse red blood cells.

TABLE 2

Kinetic parameters of the hydroxylase activities of *o*-toluidine and 2,6-xylidine

HLM: 0.4 mg/ml. Data are the mean ± S.D. of triplicate determinations.

Hydroxylase Reaction	K_m	V_{max}	CL_{int}
	μM	*nmol/min/mg protein*	*μl/min/mg protein*
o-Toluidine			
4-Hydroxylation	50.2 ± 2.8	0.61 ± 0.03	12.2 ± 0.2
6-Hydroxylation	70.3 ± 6.2	0.05 ± 0.00	0.7 ± 0.1
2,6-Xylidine			
4-Hydroxylation	34.1 ± 5.1	0.61 ± 0.04	18.3 ± 3.4

Discussion

Prilocaine and lidocaine are typical amide-type local anesthetics that carry the risk of a serious adverse reaction known as methemoglobinemia (Maimo and Redick, 2004). Prilocaine and lidocaine are hydrolytically metabolized to the aromatic amines *o*-toluidine and 2,6-xylidine, respectively. Although these metabolites were suspected to be causes of prilocaine- and lidocaine-induced methemoglobinemia (Neuhaeuser et al., 2008), the enzymes responsible for Met-Hb formation remained to be characterized. In the present study, we found that metabolic activation by human CES, CYP2E1, and CYP3A4 was involved in prilocaine- and lidocaine-induced methemoglobinemia.

We demonstrated that prilocaine was hydrolyzed by CES1A and CES2, whereas lidocaine was specifically hydrolyzed by CES1A (Fig. 1), and that hydrolysis reactions that were catalyzed by CES enzymes enhanced prilocaine- and lidocaine-induced Met-Hb formation (Figs. 2 and 4). Because prilocaine and lidocaine hydrolase activities were detected in HLM, but not in human plasma where cholinesterases and paraoxonases are expressed (unpublished data), these enzymes could be excluded from the candidate enzymes catalyzing their hydrolysis. Recombinant human AADAC showed no activity (Fig. 1; Table 1). Moreover, prilocaine and lidocaine hydrolase activities at 1 mM were completely inhibited by 100 μM DFP and BNPP (unpublished data), which are potent CES inhibitors (Watanabe et al., 2009). Collectively, these results suggest that CES enzymes are major enzymes that are responsible for the hydrolysis of prilocaine and lidocaine.

Met-Hb formation after the incubation of the parent compounds (prilocaine and lidocaine) with HLM was lower than that after incubation of their hydrolyzed metabolites (*o*-toluidine and 2,6-xylidine) (Fig. 2). Prilocaine- and lidocaine-induced Met-Hb formation was significantly decreased by DFP and BNPP (Fig. 4), indicating that CES enzymes may be involved in the prilocaine- and lidocaine-induced Met-Hb formation. When metabolic efficiency was analyzed, prilocaine was shown to be more efficiently hydrolyzed in HLM than was lidocaine (Fig. 1, A and B). Furthermore, prilocaine- and *o*-toluidine–induced Met-Hb formation was higher than that induced by incubating lidocaine and 2,6-xylidine (Fig. 2, A and B). These results support the previous report (Guay, 2009), which indicates that the number of prilocaine-related methemoglobinemia episodes is higher than that of lidocaine-related methemoglobinemia.

We considered the possibility that hydrolysis may not be the sole cause of prilocaine- and lidocaine-induced methemoglobinemia, because Met-Hb formation was not completely inhibited by DFP and BNPP.

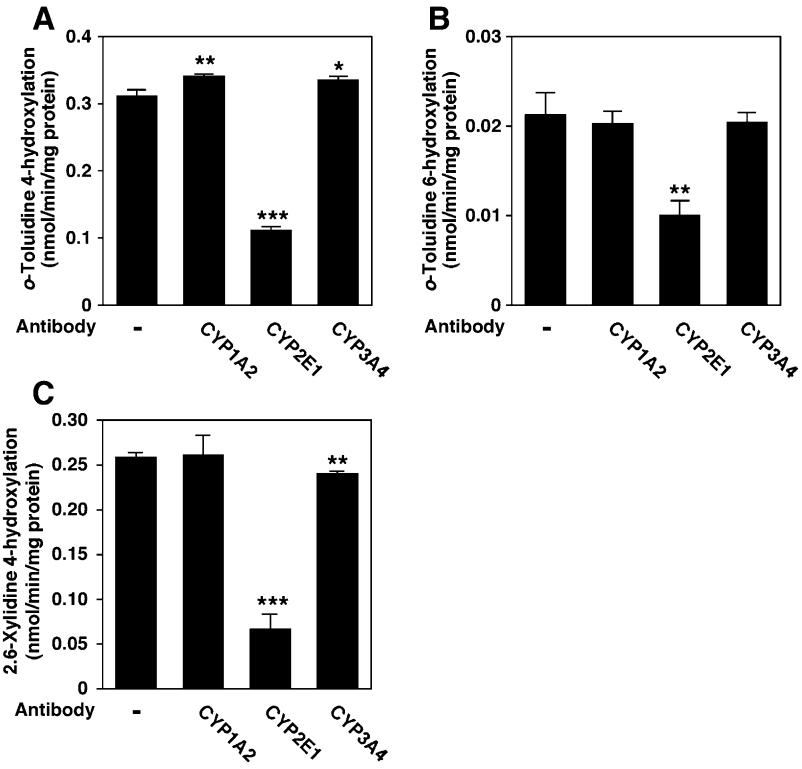

Fig. 10. Inhibitory effects of anti-human CYP1A2, CYP2E1, and CYP3A4 antibodies on *o*-toluidine- or 2,6-xylidine hydroxylation in HLM. HLM (0.4 mg/ml) was incubated with 50 μM *o*-toluidine or 30 μM 2,6-xylidine, an NADPH-GS, and each P450 antibody for 30 minutes. The control activities for *o*-toluidine-4-hydroxylation (A), *o*-toluidine-6-hydroxylation (B), and 2,6-xylidine-4-hydroxylation (C) were 0.31 ± 0.01, 0.02 ± 0.00, and 0.26 ± 0.00 nmol/min/mg protein, respectively. Each column represents the mean ± S.D. of triplicate determinations. Differences compared to control with no antibody were considered significant at $*P < 0.05$; $**P < 0.005$; and $***P < 0.001$.

Supporting this assumption, we observed that metabolism by P450 enzymes was also required for prilocaine- and lidocaine-induced Met-Hb formation in the presence and absence of the hydrolysis reaction (Figs. 2, 3, and 5–7). Human P450 enzymes, which are major NADPH-dependent enzymes, account for ~75% of the metabolism of clinical drugs (Guengerich, 2008). In the absence of an NADPH-GS, Met-Hb formation after incubation with prilocaine, lidocaine, and their hydrolyzed metabolites was not increased (Fig. 2B). In fact, we demonstrated that Met-Hb formation was increased by CYP3A4 (prilocaine and lidocaine) by using recombinant P450 enzymes in the absence of the hydrolysis reaction (Fig. 5). The formation of Met-Hb after incubation with the hydrolyzed metabolites was indeed increased by CYP1A2, CYP2E1, and CYP3A4 (*o*-toluidine) or CYP2E1 (2,6-xylidine) (Fig. 3). When we accounted for the levels of each P450 enzyme in HLM, CYP2E1 appeared to contribute highly to the formations of Met-Hb after incubation with the hydrolyzed metabolites (Fig. 6). These data were consistent with those obtained in the analyses that were conducted in the presence of anti-P450 antibodies (Fig. 7A). Met-Hb formation in the presence of HLM was similar to the sum of Met-Hb formation by each of the individual P450 expression systems (Fig. 6), indicating that these P450 enzymes may be responsible for Met-Hb formation in the presence of prilocaine, lidocaine, *o*-toluidine, or 2,6-xylidine in the presence and absence of the hydrolysis reaction. Collectively, our data indicate that two metabolic pathways, the hydrolysis pathway, which is catalyzed by CES enzymes and CYP2E1, and the nonhydrolysis pathway, which is catalyzed by CYP3A4, may be involved in prilocaine- and lidocaine-induced methemoglobinemia (Fig. 12).

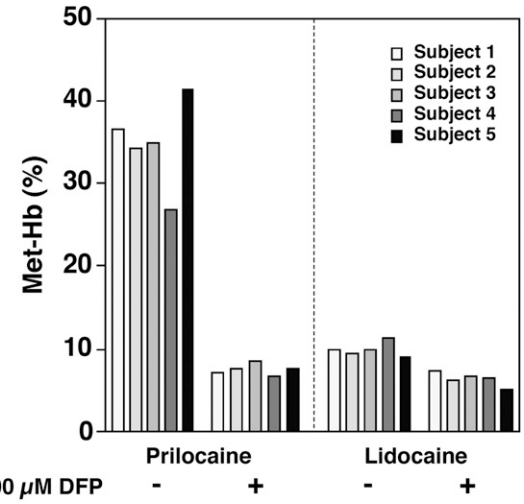

Fig. 11. Prilocaine- and lidocaine-induced Met-Hb formation after incubation with human red blood cells in the absence or presence of 100 μM DFP. HLM (1.0 mg/ml) was incubated for 120 minutes with prilocaine or lidocaine (10 mM), an NADPH-GS, and human red blood cells that had been obtained from five healthy individuals.

By comparing the decreased levels of Met-Hb after incubation with DFP to the residual formation of Met-Hb (Fig. 4), the contributions of the hydrolytic and nonhydrolytic metabolic pathways to prilocaine- and lidocaine-induced Met-Hb formation in HLM can be roughly predicted. The decreases in prilocaine- and lidocaine-induced Met-Hb formation were 2.16- and 0.82-fold greater, respectively, than the residual formation of Met-Hb. Thus, the hydrolysis of prilocaine by CES enzymes highly contributed to prilocaine-induced formation, whereas the hydrolytic and nonhydrolytic pathways of lidocaine seemed to contribute equally to Met-Hb formation.

Methemoglobinemia is reported to be induced by certain types of clinically used drugs, including the analgesic antipyretic phenacetin, the antileprosy drug dapsone, and the antibiotic sulfamethoxazole (Reilly et al., 1999; Ganesan et al., 2010; Kobayashi et al., 2012), which all possess an aromatic amine moiety. *N*-Hydroxylamines, which are *N*-hydroxylated metabolites of aromatic amines, were suspected to be a cause of aromatic amine-induced methemoglobinemia (Spooren and

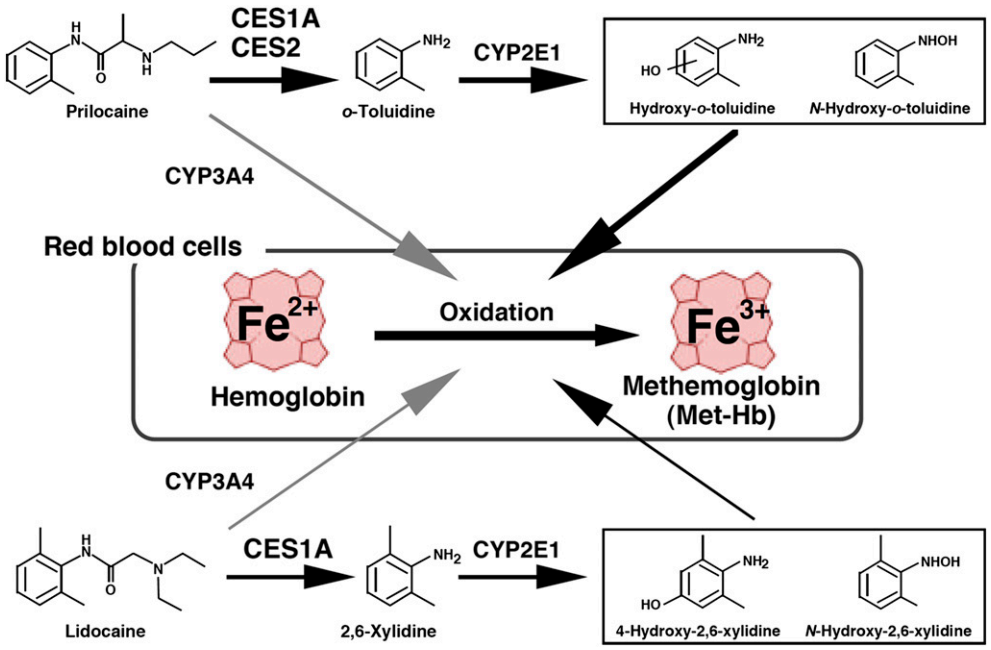

Fig. 12. Mechanisms suggested to underlie prilocaine- and lidocaine-induced Met-Hb formation. Two metabolic pathways are proposed: the hydrolysis pathway, which is mediated by CES and CYP2E1, and the nonhydrolysis pathway, which is mediated by CYP3A4.

Evelo, 2000). For instance, in vitro experimental data indicate that dapsone-hydroxylamine, which is an *N*-hydroxylated metabolite of dapsone that is catalyzed by CYP2C19, CYP2E1, and CYP3A4, may cause dapsone-induced methemoglobinemia (Reilly et al., 1999). Therefore, *N*-hydroxyl-*o*-toluidine and *N*-hydroxyl-2,6-xylidine, which are *N*-hydroxylated metabolites of *o*-toluidine and 2,6-xylidine, respectively, may cause Met-Hb formation after exposure to *o*-toluidine and 2,6-xylidine. Because *N*-hydroxylamines are generally unstable (Fuller, 1978), *N*-hydroxyl-*o*-toluidine and *N*-hydroxyl-2,6-xylidine could not be obtained. 4-Hydroxy-*o*-toluidine and 6-hydroxy-*o*-toluidine or 4-hydroxy-2,6-xylidine (Fig. 8A) have been detected in human urine after the administration of prilocaine or lidocaine, respectively (Hjelm et al., 1972; Keenaghan and Boyes, 1972). We also confirmed that *o*-toluidine and 2,6-xylidine were converted to the hydroxylated metabolites 4- and 6-hydroxy-*o*-toluidine or 4-hydroxy-2,6-xylidine in HLM (Fig. 9) and that hydroxylation of *o*-toluidin and 2,6-xylidine was mainly catalyzed by CYP2E1 (Fig. 10). These hydroxylated metabolites could efficiently induce Met-Hb formation in the absence of an NADPH-GS (Fig. 8B), suggesting that 4- and 6-hydroxy-*o*-toluidine or 4-hydroxy-2,6-xylidine may also cause Met-Hb formation after exposure to *o*-toluidine and 2,6-xylidine, respectively. Although *o*-toluidine more efficiently induced Met-Hb formation than did 2,6-xylidine (Fig. 2), the metabolic efficiency of *o*-toluidine 4-hydroxylation was shown to be lower than that of 2,6-xylidine 4-hydroxylation (Table 2). This discrepancy may be accounted for by the *N*-hydroxylated metabolites. Because we could not compare the induction potencies of Met-Hb formation between the *N*-hydroxylated metabolites and the other hydroxylated metabolites, the metabolite that contributed to Met-Hb formation to the greatest degree could not be determined.

As shown in Figs. 6, C and D, and 7B, CYP3A4 may be involved in prilocaine- and lidocaine-induced Met-Hb formation in the absence of the hydrolysis pathway. Lidocaine is known to be metabolized to monoethylglycinexylidide by CYP3A4, but we confirmed that little Met-Hb was formed after incubation with monoethylglycinexylidide (Supplemental Fig. 1). In addition, lidocaine is metabolized to 3-hydroxylidocaine, but this metabolite was excluded as a potential cause of Met-Hb formation, because it is formed by CYP1A2 and CYP3A4 (Wang et al., 2000). P450 enzymes have not been previously reported to participate in the metabolism of prilocaine. It will be worthwhile to identify the metabolites of prilocaine and lidocaine that cause methemoglobinemia and are catalyzed by CYP3A4 in the near future.

An in vitro assay to determine Met-Hb formation used human red blood cells that had been obtained from five individuals, resulting in comparable sensitivity for Met-Hb formation between human and mouse red blood cells (Figs. 4 and 11). Vasters et al. (2006) reported that interindividual differences in the Met-Hb levels (17.1-fold) varied by more than the variation observed after different dosages of prilocaine (1.4-fold). Neuhaeuser et al. (2008) reported that there were large interindividual variations in Met-Hb levels (8.8-fold) despite the similar dosages of lidocaine that had been administered to patients. In this study, no interindividual variability in Met-Hb formation was observed in individual red blood cells that were treated with prilocaine and lidocaine (Fig. 11). Therefore, we suggest that interindividual variations in Met-Hb formation after treatment with prilocaine and lidocaine were not attributable to hemoglobin in red blood cells per se, but rather to the metabolic potencies of enzymes, CES, and P450s, and Met-Hb reductases.

In conclusion, the present study clarified that two metabolic pathways, the hydrolysis pathway, which means the hydroxylation by CYP2E1 after the hydrolysis by CES(s), and the nonhydrolysis pathway, which is catalyzed by CYP3A4, were involved in prilocaine- and lidocaine-induced methemoglobinemia. Furthermore, the catalytic efficiencies of prilocaine and lidocaine metabolism may be involved in the different incidences of methemoglobinemia that were observed for prilocaine and lidocaine. The results obtained in this study will provide valuable information regarding the importance of CES and P450 enzymes in drug toxicity.

Authorship Contributions

Participated in research design: Higuchi, Fukami, Nakajima, Yokoi.
Conducted experiment: Higuchi, Fukami.
Contributed new reagent or analytic tools: Higuchi, Fukami.
Performed data analysis: Higuchi.
Wrote or contributed to the writing of the manuscript: Higuchi, Fukami, Yokoi.

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Address correspondence to: Dr. Tatsuki Fukami, Drug Metabolism and Toxicology, Faculty of Pharmaceutical Sciences, Kanazawa University, Kakuma-machi, Kanazawa 920-1192, Japan. E-mail: tatsuki@p.kanazawa-u.ac.jp