Human Paraoxonase 1 is the Enzyme Responsible for Pilocarpine Hydrolysis

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Abbreviations: BNPP, bis-(nonylphenyl)-phenylphosphate; CaCl₂, calcium chloride; CYP, cytochrome P450; DFP, diisopropylphosphorofluorid; EDTA, ethylenediamin-N, N, N', N'-tetraacetic acid; 5-HETE, (±) 5-Hydroxy-6E, 8Z, 11Z, 14Z-eicosatetraenoic acid; 5-HETEL, (±) 5-hydroxy-6E, 8Z, 11Z, 14Z-eicosatetraenoic acid, 1, 5-lactone; HLM, human liver microsomes; HPLC, high-performance liquid chromatography; NaF, sodium fluoride; PMSF, phenylmethylsulfonyl fluoride; PON, paraoxonase.
Abstract

Pilocarpine has widely been used in ophthalmic preparations for the treatment of glaucoma and in oral preparations for the treatment of radiation-induced xerostomia and Sjögren syndrome. The major metabolic pathways of pilocarpine in human are hydrolysis and hydroxylation. Recently, it was found that CYP2A6 is responsible for the 3-hydroxylation, but the enzymes responsible for the hydrolysis have not been characterized. In this study, we attempted to identify esterases responsible for pilocarpine hydrolysis. Pilocarpine hydrolase activities in human liver microsomes and plasma were stimulated by the addition of CaCl₂, suggesting that the calcium-dependent esterase, paraoxonase (PON), was responsible for pilocarpine hydrolysis. To confirm this hypothesis, the pilocarpine hydrolase activity was measured using the recombinant human PONs (PON1, PON2, and PON3) established in this study and the result was that only PON1 showed the pilocarpine hydrolase activity. The effect of PON1 polymorphism (Q192R) on the pilocarpine hydrolase activity was analyzed using the recombinant human PON1 192Q and 192R, and human plasma from 50 volunteers. The results showed that the recombinant PON1 192R revealed significantly higher catalytic efficiency than PON1 192Q. In human plasma, the activity of R/R genotype (117.0 ± 25.2 pmol/min/µl: n = 23) was significantly higher than those of Q/R and Q/Q genotypes (97.3 ± 21.0 pmol/min/µl: n = 20 and 90.4 ± 26.2 pmol/min/µl: n = 7, respectively). It is suggested that this polymorphism affects the pilocarpine hydrolase activity. In this study, we found that human PON1 is the major enzyme for the catalytic efficiency of pilocarpine hydrolysis.
Introduction

Pilocarpine is a muscarinic cholinergic agonist and is widely used as eyedrops for the treatment of glaucoma (Hoyng et al., 2000). In addition, orally administered pilocarpine increases the salivary flow rate by stimulating salivary glands. Therefore, it is also used for the treatment of radiation-induced xerostomia and Sjögren syndrome, which is accompanied by oral discomfort and pain, difficulty in speaking, chewing, swallowing, and sleeping, leading to a decline in the quality of life. It has been shown that pilocarpine is metabolized to its open lactone-ring form, pilocarpic acid (Ellis et al., 1972; Aromdee et al., 1996), and 3-hydroxypilocarpine (Endo et al., 2007) (Fig. 1), which are pharmacologically inactive and extremely weak compared with pilocarpine, respectively. These metabolites are detected in human blood and excreted into the urine at a level approximately equal to that of the parent pilocarpine after oral administration (Aromdee et al., 1999; Van de Merbel et al., 1998; Endo et al., 2008). Recently, Endo et al. (2007) found that CYP2A6 is responsible for the 3-hydroxylation of pilocarpine in human liver. However, the enzymes responsible for the pilocarpine hydrolysis, which is the lactone-ring opening reaction, remain to be determined.

Esterases contribute to the hydrolysis of 10% of clinical therapeutic drugs including ester, amide, and thioester bonds (Williams et al., 2004). In particular, carboxylesterase (CES) is the most thoroughly characterized esterase, which is responsible for the hydrolysis of various prodrugs such as irinotecan and oseltamivir (Li et al., 2005). Butyrylcholinesterase (BChE) and arylacetamide deacetylase (AADAC) are also known to catalyze drug hydrolysis (Morton et al., 1999; Watanabe et al., 2009; Watanabe et al., 2010). Because they possess a serine residue of the conserved –Gly-X-Ser-X-Gly- motif at the active site, they are called serine esterases, which are generally inhibited by organophosphate esters. Esterases that do not belong to the serine
esterase group are also involved in the hydrolysis of xenobiotics. PON is one of such esterases. The PON family consists of three isoforms, PON1, PON2, and PON3, and they require calcium to exert their activities and stabilities (Kuo and La Du, 1998). It has been reported that PON1 and PON3 are synthesized primarily in the liver and secreted into the plasma, whereas PON2 is ubiquitously expressed in human tissues except plasma (Ng et al., 2001). PON1 is responsible for the hydrolysis of not only organophosphates such as paraoxon, sarin, and soman (Aldridge, 1953; Davies et al., 1996), but also a number of lactone- or cyclic carbonates-containing drugs (Billecke et al., 2000). There is little information available on PON2. PON3 has been also reported to be responsible for the hydrolysis of lactone-containing prodrugs such as lovastatin and simvastatin (Draganov and La Du, 2004; Draganov et al., 2005). Because it was reported that the pilocarpine hydrolysis in serum was strongly inhibited by EDTA, a chelating agent (Lavallee and Rosenkrantz, 1965), PON was considered to be one of the candidate enzymes responsible for the pilocarpine hydrolysis. In the present study, we tried to identify the enzymes responsible for the pilocarpine hydrolysis in human, and particularly investigated whether PON can hydrolyze pilocarpine.
Materials and Methods

Chemicals and Reagents. Pilocarpine hydrochloride, phenyl acetate, diisopropylphosphorofluoridate (DFP), physostigmine sulfate (eserine), and phenylmethylsulfonyl fluoride (PMSF) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Bis-(nonylphenyl)-phenylphosphate (BNPP), and sodium fluoride (NaF) were purchased from Sigma-Aldrich (St. Louis, MO). Pilocarpic acid was kindly provided from Kissei Pharmaceutical (Nagano, Japan). (±) 5-Hydroxy-6E, 8Z, 11Z, 14Z-eicosatetraenoic acid (5-HETE) and (±) 5-hydroxy-6E, 8Z, 11Z, 14Z-eicosatetraenoic acid, 1, 5-lactone (5-HETEL) were purchased from Cayman Chemical (Ann Arbor, MI). Endoglycosidase H (Endo H) was purchased from New England Biolabs (Ipswich, MA). Primers were commercially synthesized at Hokkaido System Sciences (Sapporo, Japan). All the other chemicals used in this study were of analytical or the highest quality commercially available.

Liver Microsomes and Plasma. Pooled human liver microsomes (HLM) were purchased from BD Gentest (Woburn, MA). Human blood was obtained from 50 healthy volunteers who had not been taking medications (age, 21 - 53 years; male, n = 31; female, n = 19). The use of human plasma was approved by the Ethics Committees of Kanazawa University (Kanazawa, Japan). The blood was allowed to stand for 30 minutes and centrifuged at 2,000 g for 15 minutes for clear separation of the plasma. All the assays were performed immediately after separation of the plasma.

Expression of human PONs in Sf9 Cells. The cDNAs of human PON1, PON2, and PON3 were prepared by a reverse transcription-polymerase chain reaction (PCR) technique using total RNA from human liver (Stratagene, La Jolla, CA) with sense and
antisense primers (Table 1). The PCR product was first subcloned into pTARGET Mammalian Expression Vector (Promega, Madison, WI). The nucleotide sequences were determined using a Thermo Sequenase Cy5.5 Dye Terminator Cycle Sequencing kit (GE Healthcare, Buckinghamshire, UK) with a Long-Read Tower DNA sequencer (GE Healthcare). In this study, the nucleotide sequences of PON1, PON2, and PON3 are referred to by accession no. NM 000446.5, NM 000305.2, and NM 000940.2, respectively.

To construct the PON variant with arginine at the 192 position, single-mutation c.575A>G (the nucleotide numbering refers to the ATG in translation starting with A as 1) was inserted into the PON1 cDNA by a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Primers used for the site-directed mutagenesis were PON1 SDM-S and PON1 SDM-AS (Table 1). The nucleotide sequences of the product were confirmed by DNA sequence analysis.

The expression of human PONs using a Bac-to-Bac Baculovirus Expression System (Invitrogen, Carlsbad, CA) was carried out according to the method described previously (Watanabe et al., 2010) except that Spodoptera frugiperda Sf9 cells were used. Non-recombinant bacmid DNA (mock) was also prepared by the same procedures. The protein concentrations were determined according to Bradford (1976).

**Enzymatic deglycosylation of the recombinant PON1.** To compare the expression level between recombinant PON1 192Q and PON1 192R, the recombinant PON1s were enzymatically deglycosylated with Endo H as follows: the homogenates were adjusted to a 1 mg/ml protein concentration with 1 × glycoprotein denaturing buffer [20 mM Tris-HCl (pH 7.5), 50 mM NaCl, 5 mM Na2EDTA], and were denatured at 95°C for 10 min. The denatured proteins (70 µg) were incubated with 700 U of Endo H in 1 × G5 reaction buffer [50 mM sodium citrate (pH 5.5)] in a final volume of 100 µl at 37°C for
1 hr. To analyze the effect of the deglycosylation of the recombinant PON1s on the enzyme activity, the deglycosylation under a non-denaturing condition was carried out at 37°C for 2 hr with excessive amounts (10 unit/µg protein) of Endo H. The incubation mixture is the same as described above except for 1 × glycoprotein denaturing buffer. The deglycosylation was confirmed by the immunoblot analysis described as follows.

**Immunoblot Analysis.** SDS-polyacrylamide gel electrophoresis and immunoblot analysis were performed according to Laemmli (1970). Enzyme sources were separated on 10% polyacrylamide gels and electrotransferred onto polyvinylidene difluoride membrane, Immobilon-PVDF (Millipore Corporation, Billerica, MA). The membranes were probed with polyclonal goat anti-human PON1 (Santa Cruz Biotechnology, Santa Cruz, CA), monoclonal mouse anti-human PON2 (Abnova, Taipei, Taiwan), and polyclonal goat anti-human PON3 antibodies (Santa Cruz Biotechnology), and the corresponding fluorescent dye-conjugated second antibody and an Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE) were used for the detection. The relative expression level was quantified using ImageQuant TL Image Analysis software (GE Healthcare).

**Phenyl Acetate Hydrolase Activity.** Phenyl acetate has been often used as a specific substrate for PON1 (Eckerson et al., 1983; Smolen et al., 1991). The phenyl acetate hydrolase activity was determined as follows: a typical incubation mixture (final volume of 1.0 ml) contained 20 mM Tris-HCl (pH 8.0), 1 mM CaCl₂, and enzyme source (Sf9 cell homogenates expressing PONs: 0.02 mg/ml, human plasma: 2.5 µl). The reaction was determined spectrophotometrically by adding phenyl acetate (1 mM) and following formation of phenol (ε = 1310 M⁻¹ cm⁻¹) at 270 nm at 30°C for 2 min, with data gathered at 5-second intervals. Phenyl acetate was dissolved in ethanol, and the
final concentration of ethanol in the incubation mixture was 1.0%.

5-HETEL Hydrolase Activity. 5-HETEL has been known to be hydrolyzed by all PONs (Teiber et al., 2003). The 5-HETEL hydrolase activity was determined as follows: a typical incubation mixture (final volume of 0.2 ml) contained 50 mM Tris-HCl (pH 7.4), 1 mM CaCl₂ and enzyme sources (Sf9 cell homogenates expressing PONs: 75 ng/ml). The reaction was initiated by the addition of 3.3 µM 5-HETEL after 2-min preincubation at 37°C. After the 2-min incubation at 37°C, the reaction was terminated by the addition of 200 µl of ice-cold 100% acetonitrile. After removal of the protein by centrifugation at 9,000 g for 1 min, an 80-µl portion of the supernatant was subjected to HPLC. The HPLC analysis was performed using an L-2130 pump (Hitachi, Tokyo, Japan), an L-2200 autosampler (Hitachi), an L-2400 UV detector (Hitachi), and a D-2500 chromato-integrator (Hitachi) equipped with a Capcell Pak C₁₈ UG120 (5-µm particle size, 4.6 mm i.d. × 250 mm; Shiseido, Tokyo, Japan). The eluent was monitored at 236 nm with a noise-base clean Uni-3 (Union, Gunma, Japan), which can reduce the noise by integrating the output and increase the signal 3-fold by differentiating the output, and 5-fold by further amplification with an internal amplifier, resulting in a maximum 15-fold amplification of the signal. The mobile phase was 85% acetonitrile containing 0.2% acetic acid. The flow rate was 1.0 ml/min. The column temperature was 35°C. The quantification of 5-HETE was performed by comparing the HPLC peak height with that of an authentic standard. The activity was determined as the mean value in triplicate.

Pilocarpine Hydrolase Activity. The pilocarpine hydrolase activity was determined as follows: a typical incubation mixture (final volume of 0.2 ml) contained 100 mM Tris-HCl (pH 7.4) and various enzyme sources (HLM and Sf9 cell homogenates
expressing human PONs, 0.5 mg/ml; human plasma, 2.5 µl). When the effects of CaCl₂ and EDTA on pilocarpine hydrolase activity were analyzed, they were used at the concentration of 1 mM. The reaction was initiated by the addition of 0.2 – 20 mM pilocarpine after 2-min preincubation at 37°C. After the 60-min incubation at 37°C, the reaction was terminated by the addition of 100 µl of ice-cold 100% methanol. After removal of the protein by centrifugation at 9,500 g for 5 min, a 20 µl portion of the supernatant was subjected to HPLC. The HPLC apparatus was the same as described above except that a YMC C₁₈ column (5-µm particle size, 4.6 mm i.d. × 150 mm; YMC, Tokyo, Japan) was used. The eluent was monitored at 217 nm with a noise-base clean Uni-3. The mobile phase was 1% methanol containing 50 mM potassium dihydrogenphosphate, pH 3.9. The flow rate was 1.0 ml/min. The column temperature was 35°C. The quantification of pilocarpic acid was performed by comparing the HPLC peak height with that of an authentic standard. The activity in each concentration was determined as the mean value in triplicate. For kinetic analyses of pilocarpine hydrolase activity, the parameters were estimated from the fitted curves using a computer program (KaleidaGraph, Synergy Software, Reading, PA) designed for nonlinear regression analysis.

To clarify the major involvement of PON1 in the pilocarpine hydrolysis, the inhibition analyses of pilocarpine hydrolysis in plasma were performed by using representative esterase inhibitors. Organophosphates such as BNPP and DFP are known as general CES inhibitors (Heymann and Krisch, 1967). Eserine and NaF are cholinesterase inhibitors (Iwatsubo et al., 1965; Preuss and Svensson, 1996). PMSF is a serine hydrolase inhibitor (Johnson and Moore, 2000). The concentrations of inhibitors were 1 mM. PMSF was dissolved in DMSO such that the final concentration of DMSO in the incubation mixture was 1.5%. Other inhibitors were dissolved in distilled water. The experimental procedure and condition were the same as described above except that
3 mM pilocarpine was added. It was confirmed that 1.5% DMSO did not inhibit the pilocarpine hydrolase activity, and the control activity was determined in the presence of 1.5% DMSO.

**Genotyping of PON1 Q192R.** Written informed consent was obtained from 50 Japanese volunteers, and this study was approved by the Ethics Committees of Kanazawa University. Blood samples were collected from a cubital vein, and genomic DNA was extracted from peripheral lymphocytes using a Puregene DNA isolation kit (Gentra Systems, Minneapolis, Minn). A PCR-restriction fragment length polymorphism (RFLP) method was developed for the genotyping of the PON1 Q192R. The primers were PON1ex6S and PON1int6AS (Table 1). The reaction mixture contained genomic DNA (100 ng), 1 × PCR buffer [67 mM Tris-HCl (pH 8.8), 16.6 mM (NH₄)₂SO₄, 0.45% Triton X-100, 0.02% gelatin], 1.5 mM MgCl₂, 0.25 mM dNTPs, 0.4 µM each primer, and 1 U of Taq DNA polymerase in a final volume of 25 µl. After an initial denaturation at 94°C for 2 min, the amplification was performed by denaturation at 94°C for 25 s, annealing at 56°C for 25 s, and extension at 72°C for 30 s for 35 cycles. The PCR product (211 bp) was digested with Mbo I at 37°C for 6 hr. The digestion patterns were determined by electrophoresis in a 4% agarose gel. The PON1 192Q allele yields 179- and 32-bp fragment and the PON1 192R allele yields 151-, 32-, and 29-bp fragments.

**Statistical Analysis.** Comparison of two groups was made with unpaired, two-tailed Student’s t test. Comparison of several groups was made with nonparametric ANOVA test. A value of \( P < 0.05 \) was considered statistically significant.
Results

Kinetic Analyses of Pilocarpine Hydrolase Activities in HLM and Human Plasma.

The pilocarpine hydrolase activities in HLM and human plasma were measured (Fig. 2, Table 2). The pilocarpine hydrolase activity in HLM was detected in the presence of 1 mM CaCl₂ with the Km and Vmax values of 7.7 ± 1.0 mM and 1.0 ± 0.0 nmol/min/mg, respectively, resulting in a CLint value of 0.12 ± 0.01 µl/min/mg (Table 2). However, the activity was not detected when 1 mM CaCl₂ was not added. In human plasma (pooled from 5 subjects randomly selected), the pilocarpine hydrolase activity was detected with the Km and Vmax values of 2.7 ± 0.6 mM and 61.7 ± 4.1 pmol/min/µl, respectively, resulting in a CLint value of 0.02 ± 0.00 µl/min/µl (Table 2). However, the activity was completely inhibited by 1 mM EDTA (data not shown). The activity in human plasma was significantly stimulated by the addition of 1 mM CaCl₂, with the result that the Vmax value was significantly increased to 134.5 ± 10.2 pmol/min/µl, although the Km value was not changed (3.3 ± 0.8 mM). Therefore, the CLint value was also significantly increased to 0.04 ± 0.01 µl/min/µl (Table 2). Because the units used for pilocarpine hydrolase activity in HLM and human plasma were different, their activities could not be simply compared. These results suggested that pilocarpine hydrolysis in HLM and human plasma is catalyzed by calcium-dependent esterase.

Identification of the Human Esterase Responsible for Pilocarpine Hydrolysis.

Among esterases responsible for drug metabolism, PON is well-known to be activated and stable in the presence of calcium ion (Kuo and La Du, 1998). Therefore, it was conceivable that PON is responsible for the pilocarpine hydrolysis. In this study, baculovirus expression systems for human PONs (PON1, PON2, and PON3) were constructed. The protein expression was confirmed with immunoblotting (Fig. 3A). The
phenyl acetate hydrolase activity was measured using the constructed recombinant PONs, but the activity at the concentration of 1 mM was detected only by recombinant PON1 (4.43 ± 0.28 nmol/min/mg). The 5-HETEL hydrolase activity at the concentration of 3.3 µM was detected by the recombinant PON1, PON2, and PON3 (1.2 ± 0.3 µmol/min/mg, 0.5 ± 0.0 µmol/min/mg, and 1.7 ± 0.2 µmol/min/mg, respectively). It has been reported that phenyl acetate can be used as a specific substrate for PON1 (Eckerson et al., 1983; Smolen et al., 1991). In addition, Teiber et al. (2003) reported that 5-HETEL is the common substrate of the PONs, but its hydrolase activity of PON1 or PON3 is higher than that of PON2. Our results were consistent with these previous studies and indicate that the recombinant PONs have the specific functions of each isoform.

Using the recombinant PONs, the pilocarpine hydrolase activity at a concentration of 5 mM was measured. PON1 showed potent pilocarpine hydrolase activity (13.4 ± 2.2 nmol/min/mg) (Fig. 3B). PON2 and PON3 showed extremely low activity (0.73 ± 0.41 nmol/min/mg and 0.56 ± 0.09 nmol/min/mg, respectively), the values of which were similar to those of mock Sf9 cells (0.44 ± 0.18 nmol/min/mg). These results indicated that PON1 is the responsible enzyme for pilocarpine hydrolysis.

**Inhibition Analyses of Pilocarpine Hydrolase Activity in Human Plasma.**

Pilocarpine hydrolase activity was detected in human plasma without the addition of CaCl₂ to the incubation mixture. To confirm whether the major enzyme responsible for pilocarpine hydrolysis in plasma is PON1, inhibition analyses were performed by using representative esterase inhibitors. The pilocarpine hydrolase activity in human plasma was inhibited by EDTA, but not by other esterase inhibitors (DFP, BNPP, eserine, NaF, and PMSF) (Fig. 4). This result suggested that PON1 is the major enzyme responsible for pilocarpine hydrolysis in human plasma.
Effects of Genetic Polymorphisms of PON1 on Pilocarpine Hydrolase Activity. The PON1 gene has a common polymorphism (c.575A>G) within the coding region resulting in an amino acid substitution of Q192R (Humbert et al., 1993). In the present study, to investigate whether the PON1 polymorphism (Q192R) affects the pilocarpine hydrolase activity, recombinant PON1 192R was also constructed. Because PON1 has three potential N-linked glycosylation sites (Gan et al., 1991), several bands were detected with immunoblot analysis for PON1 expressed in Sf9 cell homogenates (Fig. 5A). To compare the expression levels between PON1 192Q and PON1 192R, immunoblot analysis was performed using Sf9 homogenates treated with Endo H (Fig. 5B), resulting that the expression level of the recombinant PON1 192R was nearly equal to that of the recombinant PON1 192Q (the relative band intensities of PON1 192Q and PON1 192R were 1.00:1.09, which was calculated with the initial slope of the plots of band intensities). It has been reported that the glycosylation of PON1 is not important for its activity (Draganov et al., 2005), and this polymorphism (Q192R) does not affect the phenyl acetate hydrolase activity. This study also confirmed that the phenyl acetate hydrolase activities of PON1 192Q and PON1 192R after deglycosylation revealed similar activity (5.50 ± 0.16 mmol/min/mg and 4.82 ± 0.37 mmol/min/mg, respectively).

As shown in Fig. 5C, the Km and Vmax values of pilocarpine hydrolase activity by the recombinant human PON1 192Q were 14.1 ± 3.1 mM and 52.1 ± 3.6 nmol/min/mg, respectively, resulting in a CLint value of 3.8 ± 0.6 µl/min/mg (Table 3). In contrast, the Km value of the recombinant PON1 192R was 8.3 ± 1.9 mM, which was significantly lower than that of PON1 192Q, although the Vmax value of PON1 192R (45.0 ± 2.8 nmol/min/mg) was similar to that of PON1 192Q. Therefore, the CLint value of PON1 192R (5.6 ± 0.9 µl/min/mg) was significantly (P < 0.05) higher than that
of PON1 192Q (3.8 ± 0.6 µl/min/mg). These results suggested that the genetic polymorphism of PON1 Q192R could affect the pilocarpine hydrolase activity.

**Relationship between PON1 Genotypes and Pilocarpine Hydrolase Activities in Plasma Samples from 50 Japanese Volunteers.** To analyze the effect of the genetic polymorphism of PON1 Q192R on the pilocarpine hydrolase activity in human, the relationship between the PON1 genotypes and the pilocarpine hydrolase activity was investigated using plasma samples from 50 Japanese subjects. In 50 Japanese, 20 were heterozygotes and 23 subjects were homozygotes of PON1 192R. The PON 192R genotype frequencies were in accordance with the Hardy-Weinberg equation. The allele frequencies of PON1 192Q and PON1 192R were 34 and 66%, respectively. This result is in accordance with a previous study (Ueno et al., 2003).

The pilocarpine hydrolase activities in plasma at a concentration of 3 mM, a value that is similar to the Km value (Table 2), were compared among the 50 Japanese. The activity in the 50 Japanese plasma samples was 105.4 ± 25.7 pmol/min/µl. The pilocarpine hydrolase activity in plasma from the subjects with the R/R genotype (117.0 ± 25.2 pmol/min/µl: n = 23) was significantly \(P < 0.05\) higher than those with the Q/R and Q/Q genotypes (97.3 ± 21.0 pmol/min/µl: n = 20 and 90.4 ± 26.2 pmol/min/µl: n = 7, respectively) (Fig. 6A).

It was considered that the expression levels of PON1 might affect the difference of the pilocarpine hydrolase activity between PON1 192Q and 192R. It has been reported that the PON1 Q192R polymorphism does not affect the phenyl acetate hydrolase activity (Smolen et al., 1991). Therefore, the effect of PON1 Q192R on the pilocarpine hydrolysis in human plasma was evaluated with the activity corrected with the phenyl acetate hydrolase activity (Fig. 6B). The corrected activity in plasma from subjects with the R/R genotype (3.24 ± 0.62: n = 23) was significantly higher \(P <
0.001) than those with the Q/R and Q/Q genotypes (2.42 ± 0.44: n = 20 and 2.17 ± 0.29: n = 7, respectively). Thus, it was demonstrated that the polymorphism of Q192R affects the pilocarpine hydrolase activity in human plasma. This result was consistent with the finding that recombinant PON1 192R showed a significantly higher CLint value of pilocarpine hydrolysis than the recombinant PON1 192Q.
Discussion

Pilocarpine has been widely used orally to treat xerostomia and Sjögren syndrome. It has been shown that pilocarpine is metabolized by esterase to pilocarpic acid (Ellis et al., 1972; Aromdee et al., 1996) and by CYP2A6 to 3-hydroxypilocarpine (Endo et al., 2007) (Fig. 1), and that these metabolites are pharmacologically inactive and extremely weak compared with pilocarpine, respectively. These metabolites are detected in human blood and excreted into the urine at a level approximately equal that of the parent pilocarpine after oral administration (Aromdee et al., 1999; Van de Merbel et al., 1998; Endo et al., 2008). Despite the fact that the metabolism of pilocarpine is important for its efficacy, the enzymes responsible for the pilocarpine hydrolysis have not been identified. The previous report showed that pilocarpine can be hydrolyzed in HLM, human serum, and ocular tissues (Ellis et al., 1972). However, we found that the pilocarpine hydrolase activity in HLM was not detected without the addition of CaCl₂. From this result, it was suggested that major esterases expressed in HLM, such as CES, BChE, and AADAC, did not contribute to the pilocarpine hydrolysis. Unlike in HLM, the pilocarpine hydrolase activity was detected in human plasma without the addition of CaCl₂ to the incubation mixture. The activity in human plasma was completely inhibited by EDTA, a chelating agent, but not by other general esterase inhibitors such as DFP, BNPP, eserine, NaF, and PMSF. DFP, BNPP, and PMSF are serine esterase inhibitors. Therefore, serine esterases expressed in human plasma would not be involved in the pilocarpine hydrolysis. It has been reported that serum albumin has the esterase-like activity (Li et al., 2005), but we confirmed that the pilocarpine hydrolysis was not catalyzed by human purified albumin (fatty acid free) (Sigma-Aldrich) (data not shown). The calcium concentration in plasma is normally approximately 1 mM (Kuo and La Du, 1998). In contrast, that in the endoplasmic reticulum is normally approximately 0.1 to 1
µM (Kuo and La Du, 1998). The difference in the pilocarpine hydrolase activity between HLM and human plasma without the addition of CaCl₂ would be due to differences in their endogenous calcium concentration. From these results, we have judged that the esterase responsible for pilocarpine hydrolysis in human is a calcium-dependent esterase, PON. To our knowledge, PON is the only esterase that requires calcium to exert its activity and is expressed in HLM and human plasma.

To identify the PON isoform responsible for the pilocarpine hydrolysis, recombinant human PONs were established using a baculovirus expressing system. The human PON gene family consists of three members, PON1, PON2, and PON3. These isoforms share approximately 65% identity at the amino acid level. Among them, only recombinant PON1 showed pilocarpine hydrolase activity (Fig. 3B). In human, PON1 is synthesized primarily in the liver and secreted into the blood, where it is associated with high density lipoprotein (HDL) particles (Mackness et al., 1989; Hassett et al., 1991). PON1 is responsible for the hydrolysis of not only numerous organophosphates including nerve agents such as sarin and soman (Davies et al., 1996), and pesticides such as paraoxon and diazoxon (La Du, 1992), but also a number of lactone- or cyclic carbonates-containing drugs such as spironolactone and prulifloxacin (Billecke et al., 2000). Thus, it was conceivable that pilocarpine was hydrolyzed by PON1 expressed in HLM and human plasma.

PON1 has a common polymorphism, Q192R. It has been reported that the Q192R polymorphism affects the hydrolase activities of various substrates. For example, it has been demonstrated using purified PON enzymes that paraoxon is more efficiently hydrolyzed by PON1 192R, but soman and sarin are more efficiently hydrolyzed by PON1 192Q (Billecke et al., 2000). In this study, the CLint value of the pilocarpine hydrolase activity by recombinant PON1 192R was significantly higher than that of recombinant PON1 192Q (Fig. 5). In addition, the activity in plasma from subjects with
the 192R/R genotype was significantly higher than that in plasma from subjects with the 192Q/R and 192Q/Q genotypes (Fig. 6A). The pilocarpine hydrolase activities in human plasma were corrected with the phenyl acetate hydrolase activities, which are not affected by the polymorphism of PON1 Q192R (Smolen et al., 1991) (Fig. 6B). In this analysis, the similar result in Fig. 6A has been obtained. Thus, we found that the polymorphism of PON1 Q192R may be a causal factor for the high pilocarpine hydrolase activity. It has been known that the allele frequency of PON1 Q192R is different among ethnic groups as follows: The allele frequency of 192R was higher than that of 192Q in African-Americans and Asians (Japanese) (the allele frequency of 192R: 63% and 60%, respectively), whereas the opposite was reported in Caucasians (26%) (Scacchi et al., 2003; Ueno et al., 2003; Chen et al., 2003). Therefore, the pilocarpine concentrations in Japanese and African-Americans are considered to be lower than that in Caucasians after oral dosing of pilocarpine. However, it has been reported that the Cmax and AUC values of pilocarpine between Japanese and Caucasians were not significantly different (Wasnich et al., 2003). Our previous study demonstrated that CYP2A6 enzyme activity in Japanese was significantly lower than that in Caucasians by calculating the nicotine metabolic ratio (Nakajima et al., 2006). Thus, ethnic differences in CYP2A6 enzyme activity may negate differences in the pilocarpine pharmacokinetics between Japanese and Caucasians. In this study, the effect of PON1 Q192R polymorphism on the pilocarpine hydrolase activity was clarified on in vitro analyses. To elucidate the effect of this polymorphism, an in vivo study would be required.

PON1 has another common polymorphism, a leucine (L) to methionine (M) change at position 55 (L55M). To investigate whether the PON1 polymorphism of L55M affects the pilocarpine hydrolase activity, we performed genotyping analysis for this polymorphism in 50 subjects. The genotyping method and results are described in
the Supplemental Data. The pilocarpine hydrolase activity in plasma from subjects with the 55L/M genotype (84.9 ± 14.2 pmol/min/μl: n = 7) was lower than that from subjects with the 55L/L genotype (108.7 ± 25.3 pmol/min/μl: n = 43), although the difference was not statistically significant. In this study, all 7 subjects with PON1 55M were judged as homozygotes or heterozygotes of PON1 192Q. In addition, there were no homozygotes of PON1 55M among the subjects in this study. From these data, this study could not clarify whether or not the polymorphism of PON1 L55M affects the pilocarpine hydrolase activity in human plasma.

In conclusion, we first found that human PON1 is responsible for the pilocarpine hydrolysis, and that PON1 polymorphism (Q192R) would affect the pilocarpine hydrolase activity. The present study could provide useful information about the metabolism of pilocarpine.
Acknowledgments

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AUTHORSHIP CONTRIBUTION

Participated in research design: Hioki, Fukami, Nakajima and Yokoi.

Conducted experiment: Hioki and Fukami.

Contributed new reagent or analytic tools: Hioki and Fukami.

Performed data analysis: Hioki.

Wrote or contributed to the writing of the manuscript: Hioki, Fukami and Yokoi.
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and distinct substrate specificities *J Lipid Res* **46**: 1239-1247.


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nicotine metabolism and CYP2A6 polymorphic alleles in four ethnic populations.


Footnotes

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Figure legends

Fig. 1. Metabolic pathways of pilocarpine.

Fig. 2. Kinetic analyses of pilocarpine hydrolase activities in (A) HLM and (B) human plasma with/without 1 mM CaCl₂. The kinetic parameters were estimated from the fitted curve using the computer program Kaleida Graph designed for nonlinear regression analysis. Each data point represents the mean ± SD of triplicate determinations.

Fig. 3. The PON protein expression and pilocarpine hydrolase activity in Sf9 cell membranes expressing recombinant PON enzymes. (A) Immunoblot analysis of recombinant human PON1, PON2 and PON3 expressed in Sf9 cells. Total cell homogenates from Sf9 cells (30 µg) were separated by electrophoresis using 10% SDS-polyacrylamide gel. (B) Pilocarpine hydrolase activities by mock, recombinant human PON1, PON2 and PON3. The homogenates of Sf9 cells expressing these enzymes were incubated with 5 mM pilocarpine. Each column represents the mean ± SD of triplicate determinations.

Fig. 4. Inhibitory effects of representative esterase inhibitors on pilocarpine hydrolysis in human plasma. Pilocarpine hydrolase activities in plasma were determined at a substrate concentration of 3 mM. The control activity was 22.4 pmol/min/µL plasma. Each column represents the mean ± SD of triplicate determinations.

Fig. 5. The effects of PON1 polymorphisms (Q192R) on the pilocarpine hydrolase activity. (A, B) Enzymatic deglycosylation of the recombinant human PON1 enzymes expressed in Sf9 cells. Total cell homogenates from Sf9 cells (A) with no treatment (20 µg) and (B) after deglycosylation with Endo H (10 unit/µg) treatment (3-25 µg) were separated by electrophoresis using 10% SDS-polyacrylamide gel. (C) Kinetic analyses
of pilocarpine hydrolase activities by recombinant human PON1 192Q and PON1 192R. The kinetic parameters were estimated from the fitted curve using the computer program Kaleida Graph designed for nonlinear regression analysis. Each data point represents the mean ± SD of triplicate determinations.

Fig. 6. The effects of PON1 polymorphisms (Q192R) on the pilocarpine hydrolase activity in human plasma. (A) The pilocarpine hydrolase activities and (B) the activities corrected with the phenyl acetate hydrolase activity in human plasma with different PON1 genotypes. Human plasma samples from 50 Japanese subjects were used. The 7, 20 and 23 subjects were judged as PON1 192Q/Q, 192Q/R and 192R/R genotypes, respectively. Pilocarpine hydrolase activities in plasma were determined at a substrate concentration of 3 mM. *P < 0.05 compared with PON1 192Q/Q genotype. **P < 0.05 and ***P < 0.005 compared with PON1 192Q/R genotype.
### TABLE 1

**Primers used in the present study**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>For construction of expression plasmids</strong></td>
<td></td>
</tr>
<tr>
<td>PON1 S</td>
<td>5’- TGTCTATCCCCGACCATGGC -3’</td>
</tr>
<tr>
<td>PON1 AS</td>
<td>5’- TCGGTCTGTAGAGCTCACA -3’</td>
</tr>
<tr>
<td>PON2 S</td>
<td>5’- ATGGGCGGCTGGTGCTGTGGGCTT -3’</td>
</tr>
<tr>
<td>PON2 AS</td>
<td>5’- GTGAACCTCATTGTACTTTTGCGATGAA -3’</td>
</tr>
<tr>
<td>PON3 S</td>
<td>5’- TGTCTATCCCCGACCATGGC -3’</td>
</tr>
<tr>
<td>PON3 AS</td>
<td>5’- TCGGTCTGTAGAGCTCACA -3’</td>
</tr>
<tr>
<td>PON1 SDM-S</td>
<td>5’- CTTGACCCCTACTTACGATCCTGGGAGATGTATTTG -3’</td>
</tr>
<tr>
<td>PON1 SDM-AS</td>
<td>5’- CAAATACATCTCCAGGATCGTAAGTGGGTCAG -3’</td>
</tr>
<tr>
<td><strong>For genotyping of PON1 Q192R</strong></td>
<td></td>
</tr>
<tr>
<td>PON1ex6S</td>
<td>5’- CTGTGGGACCTGAGCAGATTTT -3’</td>
</tr>
<tr>
<td>PON1int6AS</td>
<td>5’- CCACTACATTTCAGAGAGTTTCA -3’</td>
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</tbody>
</table>

Underlined letters indicate the mutated nucleotides.
**TABLE 2**

*Kinetic parameters of pilocarpine hydrolase activities by HLM and human plasma*

<table>
<thead>
<tr>
<th>Enzyme source</th>
<th>1 mM CaCl₂</th>
<th>Km</th>
<th>Vmax</th>
<th>CLint</th>
</tr>
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<tbody>
<tr>
<td>HLM</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>7.7 ± 1.0</td>
<td>1.0 ± 0.0</td>
<td>0.12 ± 0.01</td>
</tr>
<tr>
<td>Human plasma</td>
<td>-</td>
<td>2.7 ± 0.6</td>
<td>61.7 ± 4.1</td>
<td>0.02 ± 0.00</td>
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<tr>
<td></td>
<td>+</td>
<td>3.3 ± 0.8</td>
<td>134.5 ± 10.2 ***</td>
<td>0.04 ± 0.01 *</td>
</tr>
</tbody>
</table>

Data are mean ± SD (n = 3).

ND: Not detected.

*P < 0.05 and ***P < 0.001 compared with 1 mM CaCl₂ (-).
**TABLE 3**

*Kinetic parameters of pilocarpine hydrolase activities by recombinant human PON1*

<table>
<thead>
<tr>
<th>Enzyme source</th>
<th>Km</th>
<th>Vmax</th>
<th>CLint</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recombinant</td>
<td>mM</td>
<td>nmol/min/mg</td>
<td>μl/min/mg</td>
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<tr>
<td>PON1 192Q</td>
<td>14.1 ± 3.1</td>
<td>52.1 ± 3.6</td>
<td>3.8 ± 0.6</td>
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<tr>
<td>PON1 192R</td>
<td>8.3 ± 1.9*</td>
<td>45.0 ± 2.8</td>
<td>5.6 ± 0.9*</td>
</tr>
</tbody>
</table>

Data are mean ± SD (n = 3).

*P < 0.05 compared with PON1 192Q.

The incubation mixture contained 1 mM CaCl₂.
Fig. 1

Pilocarpine $\xrightarrow{\text{Esterase}}$ Pilocarpic acid

Pilocarpine $\xrightarrow{\text{CYP2A6}}$ 3-Hydroxy pilocarpine
Fig. 3

A

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Mock</th>
<th>PON1</th>
<th>PON2</th>
<th>PON3</th>
</tr>
</thead>
<tbody>
<tr>
<td>PON1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PON2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PON3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B

Pilocarpine hydrolase activity (nmol/min/mg)

- **Mock**
- **PON1**: High activity
- **PON2**: Moderate activity
- **PON3**: Low activity
Fig. 4

Bar graph showing the percentage of control for different compounds and concentrations.

- **BNPP (1 mM)**
- **DFP (0.1 mM)**
- **DFP (1 mM)**
- **EDTA (1 mM)**
- **Eserine (1 mM)**
- **NaF (0.1 mM)**
- **NaF (1 mM)**
- **PMSF (1 mM)**

Each bar represents the percentage of control with error bars indicating variability.
Fig. 6

A

Pilocarpine hydrolase activity (pmol/min/µL)

90.4 ± 26.2  97.3 ± 21.0  117.0 ± 25.2 *

Q/Q  n=7
Q/R  n=20
R/R  n=23

B

Pilocarpine hydrolase activity (pmol/min/µL) / Phenyl acetate hydrolase activity (µmol/min/µL)

2.17 ± 0.29  2.42 ± 0.44  3.24 ± 0.62 *

Q/Q  n=7
Q/R  n=20
R/R  n=23