Paraoxonase 1 as a Major Bioactivating Hydrolase for Olmesartan Medoxomil in Human Blood Circulation: Molecular Identification and Contribution to Plasma Metabolism

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Running Title:

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Text Pages: 31
Tables: 4
Figures: 5
References: 32

Abstract: 237 words
Introduction: 407 words
Discussion: 1375 words

Abbreviations:  PON1, paraoxonase/arylesterase 1; OM, olmesartan medoxomil; CMBL, carboxymethylenebutenolidase homolog; HPLC, high performance liquid chromatography; PVDF, polyvinylidene difluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; IgG,
immunoglobulin G; KPB, potassium phosphate buffer; $K_m$, Michaelis constant; $V_{max}$, maximum velocity; VLDL, very-low-density lipoprotein; LDL, low-density lipoprotein; HDL, high-density lipoprotein, LPDS, lipoprotein deficient serum; HPBP, human phosphate binding protein.
Abstract

Olmesartan medoxomil (OM) is a prodrug type angiotensin II type 1 receptor antagonist. The OM-hydrolyzing enzyme responsible for the prodrug bioactivation, was purified from human plasma by successive column chromatography and was molecularly identified by N-Terminal amino acid sequencing, resulting in the identical sequence of 20 amino acids to that of human paraoxonase 1 (PON1). Two recombinant allozymes of human PON1 (PON1_{192QQ} and PON1_{192RR}) were constructed and clearly demonstrated to hydrolyze OM, where hydrolysis by the latter allozyme was slightly faster than by the former. Also, we evaluated the contribution of PON1 to the OM bioactivation in human plasma. Enzyme kinetic studies demonstrated that OM is more effectively hydrolyzed by the recombinant PON1 proteins than by purified albumin. The OM-hydrolyzing activities of the recombinant PON1 proteins as well as diluted plasma were greatly reduced in the absence of calcium ions. Immunoprecipitation with anti-PON1 IgG completely abolished the OM-hydrolyzing activity in human plasma, while it was partially inhibited with anti-albumin IgG. The distribution pattern of the OM-hydrolyzing activity in human serum lipoprotein fractions and lipoprotein deficient serum was examined, and showed that most of the OM-hydrolyzing activity was located in the HDL fraction with which PON1 is closely associated. In conclusion, we identified for the first time PON1 as the OM bioactivating hydrolase in human plasma on a molecular basis and demonstrated that PON1, but not albumin, plays a major role in the OM bioactivation in human plasma.
Introduction

Several prodrug strategies have been developed to enable drugs to exhibit optimal pharmacokinetics and pharmacological actions by overcoming a number of barriers to drug-like properties. In particular, an esterification strategy has been historically used to increase transcellular absorption of poorly permeable drugs administrated orally. Esterases, which are involved in the prodrug bioactivation process, are widely distributed in the blood, liver, intestine and many other biological fluids and tissues (Testa and 023, 2003). In most cases, intestinal esterases serve as the major enzymes in bioactivation of prodrugs during the first pass through the gut after absorption. However, in some cases, prodrug molecules escape the activation process by intestinal esterases, enter the blood circulation as the prodrug, and are then activated by serum (plasma) and liver esterases. Several esterases in human plasma have been investigated as a key enzyme responsible for prodrug bioactivation (Ettmayer et al., 2004; Testa, 2004; Li et al., 2005; Satoh and Hosokawa, 2006): paraoxonase/arylesterase 1 (PON1), cholinesterase, and albumin. For example, human PON1, which is localized predominantly in plasma and associated with high-density lipoprotein, was reported to be a major bioactivating enzyme of the antibacterial agent prulifloxacin (Tougou et al., 1998).

Olmesartan medoxomil (OM) is a prodrug-type angiotensin receptor blocker which is prescribed worldwide as monotherapy and in combination with a thiazide diuretic and/or a calcium channel blocker (Scott and McCormack, 2008; Chrysant, 2008; Rump and Sellin, 2010; Ram, 2011). As shown in Fig. 1, OM is one of the exemplary cases of bioavailability improvement by derivatization into
(5-methyl-2-oxo-1,3-dioxol-4-yl) methyl ester (medoxomil-ester) prodrug (Scott and McCormack, 2008). It has been reported that multiple enzymes are capable of OM bioactivation in humans: plasma albumin (Ma et al., 2005) as well as an intestinal and liver hydrolase carboxymethylenebutenolidase homolog (CMBL) (Ishizuka et al., 2010).

The identification and characterization of responsible enzymes for prodrug bioactivation are important since the properties of them become key determinants for the pharmacokinetics of the pharmacologically active metabolites, and thus pharmacodynamics of the drug entities, such as the onset of drug action and potency of therapeutic efficacy.

In this study, we purified the OM bioactivating hydrolase from human plasma, molecularly identified it as PON1, and directly demonstrated the involvement of PON1 using its recombinant proteins. Furthermore, we compared the enzyme characteristics between the two plasma esterases reported to hydrolyze OM, namely PON1 and albumin, and estimated their contribution to the overall OM bioactivation in human blood circulation.
Materials and Methods

Materials.

Olmesartan medoxomil (OM), olmesartan, and RNH-6272, a structural analog of olmesartan and used as the internal standard for olmesartan determination, were synthesized in Daiichi Sankyo (Tokyo, Japan). Phenylacetate and paraoxon were purchased from Sigma-Aldrich Japan (Tokyo, Japan). Benzoyl choline was purchased from Nakalai Tesque (Kyoto, Japan). Human plasma or serum was prepared from the blood collected from healthy subjects under a protocol approved by the Institutional Human Ethical Committee of Daiichi Sankyo. The animal plasma was prepared from the blood which was collected in-house from Wister-Imamichi rats, ddY mice, Japanese white rabbits, cynomolgus monkeys, and beagle dogs in accordance with the guidelines of the Institutional Animal Care and Use Committee of Daiichi Sankyo. Purified human serum albumin and anti-human albumin rabbit polyclonal IgG were purchased from Sigma-Aldrich.

Hydrolase Activity Measurement.

OM hydrolyzing activity was determined as follows: Animal and human plasma for the species difference examination and each eluent fraction obtained by successive column chromatography for purification of our target hydrolase were appropriately diluted (final 50- to 400-fold and 10- to 100-fold dilution, respectively) with 10 mM potassium phosphate buffer. The diluted protein solutions were incubated with OM as a substrate (final solvent concentration: 2% acetonitrile) at 37°C for 5 to 10 min. After reaction termination by adding ice-cold acetonitrile, the concentration of the active metabolite olmesartan was determined by an HPLC system (SLC-10A system, Shimadzu, Kyoto, Japan). Olmesartan was separated from the OM peak with a
reversed-phase C\textsubscript{18} column (YMC-Pack ODS-A A-312, C-18, 5 µm, 6.0 ID×150 mm, YMC, Kyoto, Japan) and a mobile phase of 40% acetonitrile containing 2% PIC-A (Waters, Milford, MA) at a flow rate of 1.0 ml/min, and was detected with UV detection at 254 nm. The lower limit of quantitation (LLOQ) was set at 0.2 µM. In the other \textit{in vitro} experiments, the OM-hydrolyzing activities of diluted human plasma (final 500-fold dilution), recombinant PON1 proteins (final 0.01 mg protein/ml), and purified human serum albumin (final 5 mg protein/ml) were measured in 100 mM Tris-HCl buffer (pH 7.5) containing 1 mM CaCl\textsubscript{2} at 37°C with OM as a substrate (final solvent concentration: 2.5% acetonitrile). After incubation at 37°C for a designated reaction time, the reaction was terminated by adding ice-cold acetonitrile containing RNH-6272 as an internal standard for the determination of the metabolite concentration and 0.25% trifluoroacetic acid for preventing the non-enzymatic degradation of OM. After filtration and addition of 50% methanol containing 1% formic acid, the concentration of the active metabolite olmesartan was determined by an LC-MS/MS system consisting of Prominence LC-20A (Shimadzu) and API 3200 (AB Sciex, Foster City, CA). Olmesartan was separated with a reversed-phase C\textsubscript{18} column (Atlantis T3, S-5 µm, 2.1 mm ID×150 mm, Waters) and a mobile phase of 64% methanol containing 0.2% formic acid at a flow rate of 0.2 ml/min, and was determined by monitoring the ion transition of \textit{m/z} 447 to \textit{m/z} 207 with multiple reaction monitoring in the positive electrospray ionization mode. The LLOQ was set at 20 nM. The enzymatic activity was expressed as a metabolite formation rate (\(\nu;\) nmol/min/mg protein) based on the production of olmesartan for the reaction by the recombinant protein which was subtracted from that in the buffer control as non-enzymatic hydrolysis.
Phenyl acetate hydrolyzing activity was determined as follows: Over the protein purification process from human serum, 20 to 40 µl of each fraction was incubated with 0.2 ml of 10 mM phenyl acetate solution in 50 mM Tris-HCl buffer (pH 8) at 37°C for 10 to 20 min. After reaction termination by adding 1 ml of 0.04% 4-aminoantipyrine in acetonitrile and then color development by adding 2 ml of 0.08% potassium ferricyanide with 50 mM Tris-HCl buffer (pH 8) in acetonitrile, the absorbance at 510 nm was measured. After column purification, the activity of the purified protein was measured as reported previously (Gan et al., 1991) with slight modification. Twenty µl of the purified esterase solution was incubated with 1.5 ml of 1 mM phenyl acetate solution in 50 mM Tris-HCl buffer (pH 8) containing 1 mM CaCl₂ at room temperature. The metabolite phenol formation was determined by monitoring absorbance change at 270 nm. The activity was expressed with a unit of ΔOD₂₇₀/min/mg protein.

Paraoxon hydrolyzing activity was measured as reported previously (Gan et al., 1991) with slight modification. Twenty µl of the appropriately diluted purified protein solution was incubated with 0.8 ml of 1 mM paraoxon solution in 50 mM Tris-HCl buffer (pH 8) containing 1 mM CaCl₂ and 1 M NaCl at room temperature. The metabolite p-nitrophenol formation was determined by monitoring absorbance change at 412 nm. The activity was expressed with a unit of ΔOD₄₁₂/min/mg protein.

Benzoyl choline hydrolyzing activity was measured as follows: One hundred and twenty µl of the appropriately diluted purified protein solution was incubated at 37°C for 10 min with benzoyl choline (final 1 mM). After reaction termination by adding ice-cold acetonitrile, the concentration of the metabolite benzoic acid was determined by an HPLC system (SLC-10A system, Shimadzu). The metabolite was separated
from the benzoyl choline peak with a reversed-phase C<sub>18</sub> column (YMC-Pack ODS-A A-312, C-18, 5 µm, 6.0 ID×150 mm, YMC) and a mobile phase of 55% acetonitrile containing phosphoric acid for pH adjustment (pH 3.0) at a flow rate of 1.0 ml/min.  This was detected with UV detection at 225 nm.  The activity was expressed with a unit of nmol/min/mg protein.

**Purification of OM Hydrolase from Human Plasma.**

Since there were findings of species difference in the plasma OM-hydrolyzing activity (Fig. 2) and chemical inhibition property previously reported (Ishizuka et al., 2010) that resembled the enzymatic characters of human PON1, all column purification steps were performed according to the plasma PON1 purification method previously reported (Gan et al., 1991) with slight modification.  The peak fraction was determined by phenylacetate hydrolyzing activity with absorbance at 510 nm, since OM-hydrolyzing activity and phenylacetate hydrolyzing activity were confirmed to behave quite similarly in the preliminary column chromatography.  Over the purification process, each fraction was analyzed by SDS-PAGE according to Laemmli’s method (Laemmli, 1970) using 8% or 10% SDS-polyacrylamide gel (Bio-Rad, Hercules, CA).  Human plasma was added to two volumes of column buffer A (50 mM Tris-HCl buffer (pH 8) containing 1 mM CaCl<sub>2</sub>, 5 µM EDTA, and 5% glycerol) containing 3M NaCl.  The mixture was allowed to stand still for about 1 hr.  After centrifugation, the supernatant was loaded onto a Blue Sepharose column (5×7 cm, GE Healthcare Japan, Tokyo, Japan).  This was equilibrated beforehand with column buffer A containing 3M NaCl, first washed with column buffer A containing 3M NaCl followed by the same without NaCl, and then eluted with column buffer A.
containing 0.1% sodium deoxycholate. The active fractions were dialyzed against column buffer A, concentrated, and loaded onto an ion exchange column (DEAE Sephacel column, 2.5×8 cm, GE Healthcare Japan) equilibrated beforehand with 25 mM Tris-HCl buffer (pH 8) containing 1 mM CaCl₂, 5 µM EDTA, and 5% glycerol (referred to as column buffer B), washed with column buffer B containing 1% Emulgen 911 (Kao Corp., Tokyo, Japan) and 5% dimethylacetamide followed by that containing 0.1% Emulgen, and then eluted with a linear gradient of 0 to 350 mM NaCl in column buffer B. The active fractions were again loaded onto the DEAE Sephacel column which was washed firstly with column buffer B containing 1% Emulgen and 5% dimethylacetamide, secondly with that containing 0.1% Emulgen, followed with that containing 100 mM NaCl, and then eluted with a linear gradient of 100 to 350 mM NaCl in column buffer B. The resultant active fractions were dialyzed against the column buffer B containing 0.1% Emulgen, concentrated, and stored frozen at -80°C until use as the final purified esterase, afterward determined as human arylesterase/paraoxonase 1 (PON1). A portion of the active fractions from each column purification step was loaded onto SDS-polyacrylamide gel (8% gel, Bio-Rad) according to Laemmli’s method (Laemmli, 1970), and the gel was stained with Coomassie brilliant blue. The protein concentration was determined using DC Protein Assay (Bio-Rad) with bovine serum albumin as a standard.

**N-Terminal Amino Acid Sequencing.**

The N-terminal sequence of the target protein was determined from the purified protein electrophoretically transferred onto a PVDF membrane (Immun-Blot PVDF membrane, Bio-Rad) after SDS-PAGE. Amino acids were sequenced by automated Edman degradation using a gas-phase protein sequencer, model PPSQ-10 (Shimadzu),
according to the manufacturer's procedure.

**Immunoblotting and Immunoprecipitation Analysis.**

Specific antiserum against the purified protein, which was later identified as human PON1, was raised in female Japanese white rabbits. The animals received three boosts with equal volume of the purified protein as an antigen in complete Freund's adjuvant with a two-week interval. The antiserum was bled from the animals and the IgG was purified from the antiserum by 50% saturated ammonium sulfate precipitation followed by DEAE-Sephacel column chromatography. The IgG was further purified by Econo-Pac Serum IgG Purification Column Kit (Bio-Rad) to remove components with the OM-hydrolyzing activity.

The plasma proteins in each purification step that were separated by SDS-PAGE and then blotted on a PVDF membrane were detected with the purified anti-PON1 IgG described above, followed by ECL Donkey anti-rabbit IgG HRP-linked (GE Healthcare, Little Chalfont, UK) as primary and secondary antibodies, respectively. These immunoblots were visualized by chemiluminescence with an ECL Detecting Reagent (GE Healthcare).

Inhibitory effects of the purified IgG against PON1 and albumin on the OM-hydrolyzing activity in human plasma were investigated to estimate the contribution of each protein. Diluted human plasma at an appropriate dilution ratio was incubated overnight at 4°C with respective IgG fractions at various IgG fraction/plasma volume ratios. After separating the antigen-antibody complex by centrifugation, the supernatant was used as an enzyme source for the OM-hydrolyzing activity measurement.

**Expression of Human PON1 in Mammalian Cell Line.**
The open reading frame of the full-length human PON1 (1-355aa) and that of Q192R mutant were subcloned into a vector plasmid pcDNA6-myc-his (Invitrogen, Carlsbad, CA) providing a C-terminal myc-polyhistidine-epitope tag, confirmed by DNA sequencing, and expressed in mammalian FreeStyleTM 293-F Cells (Invitrogen). The transfected cells were cultured for 7 days in FreeStyle™ 293 Expression Medium (Invitrogen) and the conditioned media from the cells overexpressing human PON1<sub>192QQ</sub> and PON1<sub>192RR</sub>, respectively, were filtered with a polyethersulfone membrane filter (0.45 µm, Thermo Fisher Scientific, Rockford, IL). After dialysis against 20 mM Tris-HCl buffer (pH 7.5), the overexpressed histidine-tagged proteins were purified with a two-step purification process: anion chromatography with HiTrap Q-XL followed by Ni-affinity chromatography with HisTrap FF (both by GE Healthcare). The eluates were collected, desalted with PD-10 (GE Healthcare), and then the resultant proteins were stored frozen at -80°C until use. The protein concentration was determined using Micro BCA Pierce Protein Assay (Thermo Fisher Scientific) with bovine serum albumin as a standard.

**Kinetic analysis.**

The enzyme kinetics for OM hydrolysis by human plasma, recombinant PON1 proteins, and purified human serum albumin were evaluated at substrate concentrations [S] ranging from 3.125 to 400 μM. For reactions by the purified serum albumin, 100 mM potassium phosphate buffer (KPB, pH 7.4) was used instead of 100 mM Tris-HCl buffer (pH 7.5) containing 1 mM CaCl<sub>2</sub>. Kinetic parameters, namely Michaelis constant (K<sub>m</sub>) and maximum velocity (V<sub>max</sub>), were estimated from the data of the substrate concentrations [S] and initial velocity (v) using WinNonlin Professional (version 5.2.1, Pharsight, Sunnyvale, CA) by a non-linear least-square regression.
analysis fitted to the Michaelis-Menten equation, \( v = \frac{V_{\text{max}} [S]}{K_m + [S]} \).

**Distribution of OM-hydrolyzing activity to Serum Lipoprotein Fractions.**

The lipoprotein fractions, namely very-low-density lipoprotein (VLDL) including chylomicron, low-density lipoprotein (LDL), and high-density lipoprotein (HDL), were separated from human serum by sequential ultracentrifugation in continuous density gradients (Havel et al., 1955) (CS150GXL with S120AT2 rotor, Hitachi Koki, Tokyo, Japan), desalted (PD-10 Desalting Column, GE Healthcare), and concentrated by centrifugation (Amicon Ultra, molecular weight cut off: 10K, Millipore, Billerica, MA). The lipoprotein deficient serum (LPDS) fraction after the lipoprotein separation, that is supposed to include serum albumin, was also desalted with PD-10 and used for the activity measurement. The OM-hydrolyzing activity was measured at a substrate concentration of 10 \( \mu \text{M} \) as described above. Incubations with the LPDS fraction in 100 mM KPB (pH 7.4) were additionally carried out, since purified serum albumin showed higher OM-hydrolyzing activity in KPB than in Tris-HCl buffer containing CaCl\(_2\).

**Other methods.**

The protein concentration was determined using Bradford Protein Assay (Bio-Rad) with bovine serum albumin as a standard if not otherwise mentioned.
Results

Characteristics of Plasma OM Hydrolase.

In plasma fractions from six different species including humans, OM was substantially hydrolyzed and converted into the active metabolite, olmesartan. The OM-hydrolyzing activities in the human and animal plasma are comparatively shown in Fig. 2. The rabbit plasma demonstrated the highest activity followed by the dog and human plasma. The hydrolysis in rat plasma was much slower than that in human plasma.

Purification of OM Hydrolase from Human Plasma.

The OM hydrolase was purified from human plasma by successive column chromatography. As shown in Table 1, the purification resulted in a 386-fold increase in specific activity of OM hydrolysis in accordance with those of phenyl acetate hydrolase and paraoxon hydrolase activities, which were monitored as markers of PON1 activity, while the marker activity for choline esterase, the benzoyl choline hydrolyzing activity, was completely removed during the purification process. The fractions containing the OM hydrolase were separated by SDS-PAGE and Coomassie-stained, showing a highly purified enzyme preparation with a dominant protein band exhibiting an apparent molecular mass of 48.5 kDa (Fig. 3A, lane 5). After transfer onto the PVDF membrane, the band was excised from the membrane and subjected to the following amino acid sequencing.

N-Terminal Amino Acid Sequencing.

The automated Edman degradation procedure provided the N-terminal sequence of the first 20 amino acids of the column-purified OM hydrolase from human plasma. The following sequence was obtained: Ala - Lys - Leu - Ile - Ala - Leu - Thr - Leu -
Leu - Gly - Met - Gly - Leu - Ala - Leu - Phe - Arg - Asn - His - Gln ---. A BLAST search against human protein database (NCBI's RefSeq database) demonstrated that PON1, which has been postulated as our target protein in plasma, is the only human protein showing the perfect match to the determined 20 amino-acid sequence.

**OM Hydrolysis by recombinant PON1 Proteins.**

To confirm our protein identification results of N-terminal amino acid sequencing, we overexpressed recombinant human PON1 in FreeStyle™ 293-F cells, a mammalian cell line derived from Human embryonic kidney 293 cells, and the OM-hydrolyzing activity by the recombinant protein was measured. Since it is a well-investigated polymorphic site of human PON1 accounting for marked qualitative differences (Harel et al., 2004; Ginsberg et al., 2009), two types of allele homozygotes at residue 192, PON1<sub>192QQ</sub> and PON1<sub>192RR</sub>, were generated. To assure the produced recombinant proteins, we examined the tryptic fragments by mass spectrometry and achieved 78% and 95% amino acid sequence coverage of PON1<sub>192QQ</sub> and PON1<sub>192RR</sub>, respectively, with covering the Q192R mutated sequence. Both recombinant PON1 allozymes rapidly hydrolyzed OM and converted into the active metabolite, olmesartan, and showed calcium-ion dependency of the enzymatic activity (Table 2).

**Immunoblotting of Plasma OM Hydrolase.**

Specific immunoreactivity of the IgG fraction against the PON1 protein was confirmed by western blotting (Fig. 3B). This anti-PON1 IgG immunostained the targeted protein in active fractions of each column purification step as a single band with the same migration point, whose intensity increased in accordance with the purification step proceeding.
Metal Ion Requirement

In Table 2, the OM-hydrolyzing activities by several plasma esterases in the incubation buffer containing Ca$^{2+}$ ions are compared with those in which Ca$^{2+}$ ions were replaced by either Zn$^{2+}$ or Mg$^{2+}$ ions. The enzymatic activities of the diluted plasma and recombinant PON1 proteins were greatly reduced in the absence of Ca$^{2+}$ ions. In contrast, the purified serum albumin equally hydrolyzed OM in all incubations regardless of the metal ions contained.

Kinetic Analysis.

The OM hydrolysis in human plasma, the recombinant proteins PON1$_{192QQ}$ and PON1$_{192RR}$, and purified serum albumin exhibited single-enzyme Michaelis-Menten kinetics as shown in Fig. 4. The parameters of enzyme kinetics were summarized in Table 3. Higher estimated $K_m$ values for the recombinant proteins PON1$_{192QQ}$ and PON1$_{192RR}$ (157 and 102 µM, respectively) than that for plasma ($K_m$: 6.71 µM) indicate a meaningful lower affinity of the substrate OM with the recombinant PON1s than the natural protein in plasma. The comparison of $V_{max}/K_m$ representing enzymatic efficiency between the two recombinant PON1 proteins show that PON1$_{192RR}$ is more active with OM as a substrate than PON1$_{192QQ}$. The $V_{max}/K_m$ value for serum albumin was considerably low compared to those of recombinant PON1 proteins.

Immunoprecipitation of Plasma OM Hydrolase.

The contribution of PON1 and albumin to OM bioactivation in human plasma was estimated by immunoprecipitation using specific antibodies against the respective proteins. As shown in Fig. 5A, the IgG against human PON1 showed a concentration-dependent inhibition of the OM-hydrolyzing activity in human plasma.
and completely abolished the activity at four-fold IgG fraction/plasma ratio. In contrast, the IgG against human serum albumin displayed only incomplete inhibition of the plasma OM-hydrolyzing activity even at the highest IgG fraction/plasma ratio (Fig. 5B), despite showing complete inhibition of the activity in purified albumin at the same volume of IgG fraction added (data not shown). The maximum inhibition magnitude, which was observed at the IgG fraction/plasma ratios above two-fold, was less than 30% of the control activity.

Distribution of the OM-hydrolyzing activity to the Human Serum Lipoprotein Fractions

Distribution of the OM-hydrolyzing activity in lipoprotein fractions (VLDL including chylomicron, LDL, HDL) and LPDS separated from human serum was summarized in Table 4. Most of the activity was located in the HDL fraction (more than 93%) while fairly low enzymatic activity was observed in LPDS which is supposed to include serum albumin.
Discussion

Although several reports state that some human plasma esterases are capable for the bioactivation of prodrug OM (Laeis et al., 2001; Ma et al., 2005), we found no reports showing evidence of the molecular identification of enzymes that were responsible for the hydrolytic reaction. In this report, we directly identified the OM hydrolase purified from human serum to be human PON1 by N-terminal peptide sequencing, and demonstrated the significant OM-hydrolyzing activities of recombinant PON1 proteins. In our previous work (Ishizuka et al., 2010), the OM-hydrolyzing activity in human plasma was strongly inhibited by p-chloromercuribenzoic acid (a free thiol modifier) and EDTA (a divalent cation chelator), which are both PON1 inhibitors, but not sensitive to diisopropyl fluorophosphate (an organophosphate), which inhibits cholinesterases and carboxylesterases. The cross-species difference in the plasma OM-hydrolyzing activity, abundant in rabbits and limited in rats as shown in Fig. 2, also suggested the involvement of PON1 (Costa et al., 1990; Kuo and La Du, 1995) in OM bioactivation in plasma rather than other plasma esterases. Therefore, we purified this OM-hydrolyzing activity according to the method historically used for PON1 purification (Gan et al., 1991). The N-terminal sequence (20 amino acids) of the extruded 48.5 kD protein was, as we expected, identical to the N-terminal region spanning Ala² to Gln²¹ of human paraoxonase/arylesterase 1 (PON1; EC 3.1.8.1./EC 3.1.1.2).

In an attempt to confirm the OM-hydrolyzing activity of human PON1, we constructed recombinant human PON1 proteins, PON1₁₉₂QQ and PON1₁₉₂RR, in a mammalian cell line. We compared their enzyme characteristics with diluted human
plasma and purified serum albumin that has been reported as another OM-hydrolyzing enzyme in human plasma. Consistent with the well-known feature of paraoxonases as a calcium-dependent metalloenzyme, both recombinant PON1 allozymes as well as diluted plasma showed calcium-ion dependent OM-hydrolyzing activities, while the activity of the purified serum albumin was hardly affected by the metal-ion replacements (Table 2).

OM was hydrolyzed slightly faster by the recombinant PON\textsubscript{192RR} than PON\textsubscript{192QQ} (Table 2). Genetic variability in human PON1 activity has been of interest and widely studied over the years. PON1 has two common coding region polymorphisms, M55L and Q192R, and more attention has been paid to the latter because it accounts for marked qualitative differences in the affinity for and catalytic activity with a number of substrates between the two allozymes (Mackness et al., 1998a; Mackness et al., 1998b; Costa et al., 2003). Some ester substrates, such as phenyl acetate, are hydrolyzed by the PON1 192Q and R allozymes at approximately equivalent rates, whereas most organophosphates are hydrolyzed at different rates by them. Prulifloxacin, a prodrug-type antibacterial agent having a medoxomil moiety like OM, was reported to be hydrolyzed by mainly PON1 and form its active metabolite (Tougou et al., 1998). The authors showed that the prulifloxacin-hydrolyzing activity positively correlated with the paraoxon-hydrolyzing activity which is catalyzed faster by PON\textsubscript{192R}, suggesting PON\textsubscript{192R} allozyme is more active than PON\textsubscript{192Q} allozyme on the prodrug activation in the same manner as OM bioactivation. Interestingly, the interindividual variation in the prulifloxacin-hydrolyzing activity was reported to be only two fold whereas that in the paraoxon-hydrolyzing activity was nine fold (Tougou et al., 1998). Taken together with the result regarding OM, the effects of the PON1 Q192R
polymorphism on the bioactivation of the prodrugs with medoxomil moiety is not considered to be significant. As another example, the latest research revealing the PON1 involvement in the hydrolysis of pilocarpine, used as a treatment for xerostomia, demonstrated the higher activity of R/R genotype than those of Q/R and Q/Q genotypes using 50 individual human plasma samples (Hioki et al., 2011). The analogy of this polymorphic phenomenon in the pilocarpine lactone-ring opening reaction to those of the medoxomil prodrugs described above seems to be attributed to the structural similarity in the hydrolyzed groups of these drugs.

Next, we evaluated the contribution of the two plasma hydrolases, PON1 and albumin, to the OM bioactivation in human plasma. By comparing the kinetic parameters between these two proteins (Table 3), significantly lower $K_m$ and higher $V_{\text{max}}$, resulting in higher $V_{\text{max}}/K_m$, in recombinant PON1s demonstrated larger capacity of PON1 for OM hydrolysis than albumin. However, since albumin is the most abundantly existing protein in plasma, it is still possible that albumin plays a certain role in OM bioactivation in human plasma. Indeed, a several hundred-fold difference in PON1 (~0.2 mg protein/ml; Connelly et al., 2008; Garin et al., 1997) and albumin (~40 mg protein/ml) concentrations in human plasma numerically offset the difference in the in vitro catalytic efficiency in these purified proteins. We therefore directly examined the contribution of PON1 and albumin using human plasma by immunoprecipitation assays with specific IgG fractions against these two proteins. As a result, the anti-PON1 IgG completely abolished the OM-hydrolyzing activity in human plasma, while only 30% inhibition magnitude was observed at maximum by anti-albumin IgG (Fig. 5), apparently suggesting predominant contribution of PON1 rather than albumin. Similarly to OM, Tougou et al. reported that human serum
albumin also had prulifloxacin-hydrolyzing activity, but the contribution of albumin to the total activity in human serum was <5% and concluded that PON1 is mainly responsible for the hydrolysis of prulifloxacin (Tougou et al., 1998).

Furthermore, the distribution pattern of the OM-hydrolyzing activity in serum lipoprotein fractions and LPDS, where most of the activity was located in the HDL fraction (Table 4), brings us proof showing the practically exclusive involvement of PON1 in plasma OM hydrolysis. PON1 is an enzyme secreted into the blood where it resides on HDL particles. Recent works have shown that both the activity and stability of PON1 are highly dependent on the HDL components (La Du et al., 1993; Sorenson et al., 1999). Interestingly, Gaidukov and Tawfik demonstrated that recombinant PON1’s stability and lactonase activity was dramatically stimulated by treatment with apolipoprotein A-I (apoA-I)-containing HDL, besides the paraoxonase and arylesterase activities were stimulated with HDL particles regardless of the apolipoprotein content (Gaidukov and Tawfik, 2005). In addition, association of PON1 with human phosphate binding protein (HPBP), one of the HDL components having a similar molecular weight to that of PON1, is highlighted to be essential for preserving active conformation(s) of the enzyme (Rochu et al., 2007; Renault et al., 2010). Lacking these HDL components as a chaperone might explain the considerably lower affinity to OM of our recombinant PON1 proteins than that of natural PON1 in diluted human plasma in the enzyme kinetic analysis (Table 3).

OM is an orally administered prodrug. After oral administration of the prodrug, the first pass bioactivation may occur in the intestine, followed by the portal blood and liver before it reaches the systemic circulation. Recently we discovered the involvement of an unknown human protein, CMBL, in OM bioactivation in the
intestine and liver (Ishizuka et al., 2010). High metabolic clearance of intestinal CMBL suggests that the intestinal bioactivation firstly and predominantly contributes to the quick onset of drug action after oral administration of OM. For reference, the intestinal first-pass availability in the prodrug form was estimated to be several percent by \(Q_{\text{Gut}}\)-model prediction (Yang et al., 2007) using \textit{in vitro} clearance for intestinal CMBL and a permeability estimate of OM (see Supplemental Method 1). Importantly however, plasma esterase PON1, presumably in the portal blood, may play a supplemental role to complete the bioactivation of prodrug molecules that escape from the hydrolysis by CMBL in the intestine. Although the transit time through portal blood is quite short, the possibility of a significant contribution of plasma PON1 has been indicated according to our previous publication (Kobayashi et al., 2000), that shows OM hydrolysis greatly proceeds in human plasma with a half-life of less than several seconds. This multiple-enzyme contribution in multiple sites is considered to effectuate the minimal risk of significant interindividual variation regardless of the possible inhibition by concomitant drugs or genetic polymorphism in CMBL that may cause a varied production of the pharmacologically active metabolite. In fact, no components other than the active metabolite olmesartan were detected in plasma following oral administration of radiolabeled OM in healthy volunteers (Laeis et al., 2001). After all, the multiple bioactivating enzymes in multiple sites in humans \textit{in vivo} are considered to achieve the rapid and complete drug action of the orally administered prodrug OM.

In conclusion, we reported for the first time the purification and identification of the OM bioactivating hydrolase in human plasma as PON1 in molecular basis. Moreover, we clearly demonstrated that PON1 plays a major role in the OM
bioactivation in human plasma by comparison of enzyme characteristics between PON1 and albumin.
Acknowledgements

*We gratefully acknowledge Kazumi Abiko and Junko Kawaguchi for the N-terminal amino acid sequencing. We would also like to express our appreciation to Dr. Shinji Yamaguchi and Dr. Kazuishi Kubota for their expert advices on the lipoprotein fractionation and LC-MS/MS peptide mapping, respectively, Dr. Masakatsu Kotsuma for assistance with the in vitro experiments and writing the manuscript, as well as Miho Kazui and Eiko Suzuki for many helpful discussions.

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Performed data analysis: Ishizuka, Fujimori, and Sakurai.

Wrote or contributed to the writing of the manuscript: Ishizuka, Yoshigae, Nakahara, Kurihara, Ikeda, and Izumi.
References


Footnotes

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Figure Legends

Figure 1  Bioactivation of olmesartan medoxomil. Hydrolysis liberates its active metabolite olmesartan and generates a diketone. Another possible product RNH-8097 was not detected in the *in vitro* reaction mixture.

Figure 2  Cross-species difference in the activity of OM hydrolysis in human and animal plasma. Data represent the results of single determinations of pooled plasma of three individuals.

Figure 3  Purification of PON1 from human plasma. The OM hydrolase was purified from human plasma by successive column chromatography. The SDS-PAGE gel stained with Coomassie brilliant blue (A) and immunoblot membrane stained with anti-PON1 IgG (B) of the pooled active fractions of each purification step are shown. Lane 1, molecular mass marker; lane 2, human plasma; lane 3, eluate from Blue Sepharose column; lane 4 and 5, the first- and second-step eluate from DEAE Sephacel column, respectively. Arrowheads indicate 48.5 kDa bands, which in panel A, lane 5 was subjected to the following amino acid sequencing.

Figure 4  Enzymatic kinetics for OM hydrolysis by human plasma esterases. Enzyme kinetics of recombinant proteins PON1<sub>192QQ</sub> (A) and PON1<sub>192RR</sub> (B), diluted human plasma (C), and purified serum albumin (D) was investigated. Data represent the mean values of duplicate determinations. The *solid line* is the best fit by non-linear least-squares regression to the Michaelis-Menten equation. The respective
Eadie-Hofstee plot is presented in the inset.

Figure 5  Immunoprecipitation analysis of the OM-hydrolyzing activity in human plasma. Inhibitory effects of the rabbit IgG raised against two human serum proteins, PON1 and albumin, on the OM-hydrolyzing activity in human plasma are shown in the panels A and B, respectively. Diluted human plasma was incubated overnight at 4°C with the two purified IgG fractions at various IgG fraction/plasma ratios. The solid lines are the best fit by non-linear least-squares regression to the inhibitory effect sigmoid E\text{max} model.
Table 1  Purification of OM bioactivating hydrolase from human plasma and three marker activities of typical plasma esterases

<table>
<thead>
<tr>
<th>Purification step</th>
<th>OM hydrolase</th>
<th>Phenyl acetate hydrolase</th>
<th>Paraoxon hydrolase</th>
<th>Benzyol choline hydrolase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>activity</td>
<td>activity</td>
<td>activity</td>
<td>activity</td>
</tr>
<tr>
<td></td>
<td>n mol/min/mg protein</td>
<td>ΔOD 270/min/mg protein</td>
<td>ΔOD 412/min/mg protein</td>
<td>n mol/min/mg protein</td>
</tr>
<tr>
<td>Plasma</td>
<td>13.0</td>
<td>1.014</td>
<td>0.001</td>
<td>13.4</td>
</tr>
<tr>
<td>Blue Sepharose</td>
<td>312.5</td>
<td>24.92</td>
<td>2.09</td>
<td>0.0</td>
</tr>
<tr>
<td>DEAE-Sephacel (1)</td>
<td>2457.2</td>
<td>189.3</td>
<td>110.4</td>
<td>0.0</td>
</tr>
<tr>
<td>DEAE-Sephacel (2)</td>
<td>5020.4</td>
<td>386.8</td>
<td>288.1</td>
<td>0.0</td>
</tr>
</tbody>
</table>

The activities of phenyl acetate hydrolysis and paraoxon hydrolase, as markers of PON1 activity, and the activity of benzoyl choline hydrolysis, as a marker for choline esterase, were simultaneously monitored over the purification process.
Table 2  Metal ion requirement for OM hydrolysis by various proteins in human plasma

<table>
<thead>
<tr>
<th>Enzyme source</th>
<th>+ Ca²⁺</th>
<th>+ Mg²⁺</th>
<th>+ Zn²⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmol/min/mg protein (% activity)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma</td>
<td>7.91 (100)</td>
<td>0.266 (3.4)</td>
<td>0.0918 (1.2)</td>
</tr>
<tr>
<td>Recombinant PON1192QQ</td>
<td>9.50 (100)</td>
<td>0.650 (6.8)</td>
<td>0.750 (7.9)</td>
</tr>
<tr>
<td>Recombinant PON1192RR</td>
<td>20.6 (100)</td>
<td>2.25 (10.9)</td>
<td>1.75 (8.5)</td>
</tr>
<tr>
<td>Serum Albumin</td>
<td>0.0102 (100)</td>
<td>0.0106 (103.9)</td>
<td>0.0164 (160.8)</td>
</tr>
</tbody>
</table>

Percent (%) was determined as the percent activity compared to values in the presence of Ca²⁺ ions. Substrate concentration was 10 µM.
Table 3  Kinetic parameters for OM-hydrolyzing activities by various proteins in human plasma

<table>
<thead>
<tr>
<th>Enzyme source</th>
<th>$K_m$</th>
<th>$V_{max}$</th>
<th>$V_{max}/K_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\mu$M</td>
<td>nmol/min/mg protein</td>
<td>ml/min/mg protein</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma</td>
<td>6.71</td>
<td>18.6</td>
<td>2.77</td>
</tr>
<tr>
<td>Recombinant PON1(192Q)</td>
<td>157</td>
<td>124</td>
<td>0.790</td>
</tr>
<tr>
<td>Recombinant PON1(192R)</td>
<td>102</td>
<td>140</td>
<td>1.37</td>
</tr>
<tr>
<td>Serum Albumin</td>
<td>354</td>
<td>2.61</td>
<td>0.00737</td>
</tr>
</tbody>
</table>

Data generated in duplicate determinations were fitted to the single enzyme Michaelis-Menten model by nonlinear least-squares regression.
### Table 4  Distribution of the OM-hydrolyzing activity to the lipoprotein fractions and LPDS separated from human serum.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Incubation buffer</th>
<th>Velocity (nmol/min/mg protein)</th>
<th>Protein Content (mg proteine/ml serum)</th>
<th>Distribution of OM-hydrolyzing Activity (nmol/min/ml serum (% distribution))</th>
</tr>
</thead>
<tbody>
<tr>
<td>VLDL, chylomicron</td>
<td>Tris-HCl/Ca²⁺</td>
<td>13.9</td>
<td>0.174</td>
<td>2.42 (0.4)</td>
</tr>
<tr>
<td>LDL</td>
<td>Tris-HCl/Ca²⁺</td>
<td>12.6</td>
<td>0.323</td>
<td>4.07 (0.6)</td>
</tr>
<tr>
<td>HDL</td>
<td>Tris-HCl/Ca²⁺</td>
<td>277</td>
<td>2.15</td>
<td>596 (93.4)</td>
</tr>
<tr>
<td>LPDS a</td>
<td>Tris-HCl/Ca²⁺</td>
<td>0.516</td>
<td>69.0</td>
<td>35.6 (5.6)</td>
</tr>
<tr>
<td>LPDS b</td>
<td>KPB</td>
<td>0.131</td>
<td>69.0</td>
<td>9.04 (N.C.)</td>
</tr>
</tbody>
</table>

Human serum was fractionated into three lipoprotein fractions (VLDL including chylomicron, LDL, and HDL) and the LPDS by sequential ultracentrifugation method. Substrate concentration was 10 µM. The activity in the LPDS was tested in potassium phosphate buffer (KPB) as well as in Tris-HCl buffer containing Ca²⁺ ions (LPDS a and b, respectively) since serum albumin previously showed higher activity for OM hydrolysis in KPB than in Tris-HCl buffer. N.C.: not calculated.
Fig. 1

**Chemical Structures**

- **Olmesartan Medoxomil** [ester prodrug]
- **Olmesartan** [active metabolite]
- **RNH-8097** (not detected)
- **Diacetyl**

**Chemical Reactions**

- Hydrolysis reaction

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Fig. 2

![Graph showing V0 (nmol/min/mg protein) for different species. Rabbit has the highest V0, followed by Dog, Human, Mouse, Monkey, and Rat.](image-url)
Fig. 3B
Fig. 4A

![Graph showing substrate concentration vs. enzyme activity]
Fig. 4B

![Graph showing substrate concentration versus reaction rate](image-url)
Fig. 4C
Fig. 4D
Fig. 5A

![Graph showing the relationship between Anti-PON1 IgG/plasma (fold) and Residual activity (% of control). The graph displays a decrease in residual activity as the fold of Anti-PON1 IgG/plasma increases.]
Fig. 5B