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Lactonase and lactonizing activities of human serum paraoxonase (PON1) and rabbit serum PON3

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Abstract

Human paraoxonase (PON1) was previously shown to hydrolyze over 30 different lactones (cyclic esters). In the present study purified human PON1 was found to catalyze the reverse reaction (lactonization) of a broad range of hydroxy acids. Hydroxy acid lactonization or lactone hydrolysis is catalyzed until equilibrium between the open and closed forms is reached. Lactonization by PON1 was calcium-dependent, had a pH optimum of 5.5–6 and could be stimulated with dilauroylphosphatidylcholine. Rabbit serum PON3 and a serine esterase in mouse plasma, presumably a carboxylesterase, also catalyzed hydroxy acid lactonization. Two endogenous oxidized unsaturated fatty acids, (±)4-hydroxy-5*E*,7*Z*,10*Z*,13*Z*,16*Z*,19*Z*-docosahexaenoic acid (4-HDoHE) and (±)5-hydroxy-6*E*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid (5-HETE) lactone, were very efficiently lactonized and hydrolyzed, respectively, by PON1. Human and mouse plasma samples also catalyzed 4-HDoHE lactonization and 5-HETE lactone hydrolysis. Studies with the PON1 inhibitor EDTA and the serine esterase inhibitor phenylmethylsulfonylfluoride suggest that about 80–95% of both activities can be attributed to PON1 in the human samples. In the mouse sample, PON1 accounted for about 30% of the 4-HDoHE lactonizing activity and 72% of the 5-HETE lactonase activity. Our results demonstrate that PON1 can lactonize the hydroxy acid form of its lactone substrates and that reversible hydrolysis of lactones may be a property of lactonases that is not generally considered. Also, the high activity of PON1 towards 4-HDoHE and 5-HETE lactone suggests that oxidized eicosanoids and docosanoids may be important physiological substrates for PON1.

Keywords: Paraoxonase; Lactones; Lactonase; Lactonization; 5-Hydroxyeicosatetraenoic acid; 4-Hydroxydocosahexaenoic acid

1. Introduction

Paraoxonase (PON1; EC 3.1.8.1) is a calcium-dependent esterase that is synthesized primarily in the liver and is secreted into the serum where it is associated with high density lipoproteins. PON1 was initially characterized based on its ability to hydrolyze aromatic carboxylic acid esters and toxic organophosphate compounds [1]. Recently, PON1 was claimed to hydrolyze platelet activating factor and oxidized arachidonate derivatives at the *sn*-2 position of phosphatidylcholine [2,3]. Studies have also shown that this enzyme has antioxidative properties, protecting low density lipoproteins from oxidation *in vitro* [4,5]. Due to its antioxidative properties, many believe that PON1 may play an important role in diseases where oxidative damage has been implicated, such as atherosclerosis [6], however, the mechanism(s) by which PON1 protects against oxidative damage is uncertain. Human PON1 has two polymorphic sites, Leu/Met at position 55 and Arg/Glu at position 192 [7,8]. The latter polymorphism affects its catalytic efficiency for various organophosphate substrates and its ability to protect LDL against oxidation *in vitro* [5,9]. Mammals also have two other paraoxonases, PON2 and PON3, and together the PON proteins make up a structurally unique family [10].

Recently, PON1 was shown to hydrolyze over 30 lactones and cyclic carbonate esters including drugs and endogenous compounds [11–13]. Purified rPON3 was also found to hydrolyze lactones, however, it has very low arylesterase activity and no paraoxonase activity [14]. Over 30 years ago,

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Abbreviations: DLPC, L-α-dilauroylphosphatidylcholine; DHP, 5,6-dihydro-2*H*-pyran-2-one; 4-HDoHE, (±)4-hydroxy-5*E*,7*Z*,10*Z*,13*Z*,16*Z*,19*Z*-docosahexaenoic acid; 5-HETE, (±)5-hydroxy-6*E*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid; KO, PON1 knock out mouse; LV, lovastatin; LVA, lovastatin acid; PMSF, phenylmethylsulfonylfluoride; PON1, paraoxonase 1; rPON3, rabbit serum PON3; r_t, retention time; SV, simvastatin; SVA, simvastatin acid.

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a calcium-dependent lactonase was partially purified from human serum and characterized [15,16]. Based on the overlapping enzymatic activities and biochemical properties of this lactonase, we believe that it was PON1. Their partially purified lactonase was also shown to catalyze the reverse reaction (lactonization) of the open form of aliphatic lactones. However, lactonizing activity of purified PON1 remained to be demonstrated.

Lactones are internal esters that exist in equilibrium between their closed (lactone) and open (hydroxy acid) forms in an aqueous environment. The lactone/hydroxy acid ratio at equilibrium is pH-dependent, with the closed form being favored at lower pH values, and can be greatly influenced by structural features of the lactone such as the ring size, substituents on the ring and the presence of double bonds within the ring [17–20]. Many drugs and endogenous compounds are lactones or hydroxy acids and an enzyme capable of catalyzing the interchange between the open and closed forms *in vivo* could have pronounced effects upon their biological activity and/or distribution. For example, some of the cholesterol lowering drugs, statins, are administered in the closed form but must be hydrolyzed to the pharmacologically active hydroxy acid [21,22]. Some statins are almost three orders of magnitude more lipophilic in their closed forms and this large difference in physical properties between the open and closed forms can dramatically alter their ability to diffuse across membranes and their metabolism by cytochrome P450s [23,24].

In the present study, we investigate the ability of PON1 to lactonize a broad range of hydroxy acids, including two endogenous unsaturated hydroxy fatty acids that may represent an important class of substrates for the enzyme. The general requirements for the lactonization activity are also described. Additionally, in an effort to determine if reversible lactone hydrolysis may be a common feature shared by other members of the PON family, the ability of rPON3 to lactonize hydroxy acids was investigated.

2. Materials and methods

2.1. Materials

4-HDoHE, 5-HETE and 5-HETE lactone were purchased from Cayman Chemical. Lovastatin and simvastatin were provided by Merck and Co. All other lactones were from Sigma–Aldrich. All other chemicals were reagent grade or better from commercial sources.

2.2. Plasma samples

Plasma was separated from heparinized human and Balb/c mouse blood and stored at 4°. The Balb/c KO mouse strain was originally kindly provided by Dr. Diana M. Shih (UCLA). Wild type Balb/c mice, used as controls, were from Harlan-Sprague Dawley, Inc.

2.3. Purification of PON1 and rPON3

Human PON1 type Q_{192} and R_{192} were purified from outdated citrated plasma (University of Michigan blood bank) and rPON3 was purified from rabbit serum as previously described [14,25]. The PON proteins were stored at 4° in storage buffer (25 mM Tris pH 8, 1 mM $CaCl_2$, 20% glycerol). rPON3 storage buffer also contained 0.1% tergitol (2,6,8-trimethyl-4-nonyloxypolyethylen-oxyethanol).

2.4. Preparation of hydroxy acids

Hydroxy acids, other than 4-HDoHE and 5-HETE, were prepared from the lactones by hydrolysis in a large molar excess of NaOH. The acids were freshly made before each assay and analyzed to ensure complete hydrolysis.

2.5. Lactonization and hydrolysis assays

Standard incubations of 0.1 mL contained 45 μL of buffer (NaAcetate for pH 4, Mes/KOH for pH values of 5.5–6.5, Hepes for pH 7 and Tris–HCl for pH values above 7) and $CaCl_2$ at final concentrations of 100 and 10 mM, respectively. For lactonization reactions, 25 μL of the hydroxy acid/NaOH solution was added to the buffer following the addition of 25 μL of HCl at a concentration equal to that of the NaOH in the hydroxy acid/NaOH solution. For 5-HETE, a 1 μL aliquot in methanol was added directly to the reaction buffer. For lactone hydrolysis reactions, 50 μL of the lactone in water was added to the buffer. Reactions were initiated by adding 5 μL of either PON1, rPON3, mouse plasma or buffer solution for controls and incubated at 25°. Calcium dependence reactions were performed in a final volume of 0.2 mL with buffer containing varying concentrations of $CaCl_2$ and initiated by adding 2.5 μL of PON1 or PON storage buffer.

2.6. Initial rate and inhibition assays with 5-HETE lactone and 4-HDoHE

Reactions were performed in a total volume of 0.1 mL containing 50 mM Tris–HCl buffer, pH 7.4 with 1 mM $CaCl_2$ (5-HETE lactone) or 100 mM Mes/KOH pH 6 with 10 mM $CaCl_2$ (4-HDoHE) and substrate at a concentration of 5 μM. For initial rate determinations, reactions contained 0.05 μg/mL PON1 or plasma (1:4000 dilution) for 5-HETE lactonase activities and 0.5 μg/mL PON1 or plasma (1:80 dilution) for 4-HDoHE lactonization activities. Reactions were initiated by adding 1 μL of the substrate (0.5 mM in methanol) and incubated at room temperature for up to 2.5 min. Initial rates were estimated under conditions in which less than 10% of the substrate was metabolized.

PMSF inhibition was performed by adding 2 μL of the inhibitor (200 mM in methanol), or methanol for controls, to plasma that had been diluted 1:4 in buffer (50 mM Tris–HCl, 1 mM $CaCl_2$, pH 7.4), so that the final concentration of PMSF was 2 mM. Samples were then incubated at room temperature for 2 hr. Inhibition by EDTA was performed by diluting the samples 1:1 in buffer (50 mM Tris–HCl, 40 mM EDTA, pH 7.4) and incubating for 16 hr at room temperature. Buffer for the controls contained 1 mM $CaCl_2$ instead of EDTA. Aliquots (5 μL) of the inhibited and control samples were assayed as described above. No spontaneous 5-HETE lactone hydrolysis or 4-HDoHE lactonization was detected.

2.7. GC analysis of lactones

γ-Butyrolactone, γ-valerolactone, γ-heptalactone, δ-valerolactone, δ-hexalactone and DHP were analyzed by GC. Incubations containing γ-valerolactone, γ-hydroxyvaleric acid or γ-hydroxyheptanoic acid were stopped by adding 0.1 mL of a Tris–HCl solution (1 M, pH 7) followed by 10 μL of an acetonitrile solution containing γ-hexalactone (0.5 μg/mL) or γ-octalactone (0.1 μg/mL) as an internal standard. The samples were immediately extracted with 0.1 mL of ethyl acetate by vortexing for 1 min and briefly centrifuged. A 2 μL aliquot of the ethyl acetate phase was injected on to a Varian 3600 gas chromatograph equipped with a flame ionization detector and a Rtx-1 dimethyl polysiloxane capillary column (15 m length, 0.53 mm i.d., 5.0 μm df; Restek) using He as the carrier gas. The injector was run in the splitless mode and the detector temperature was 300°. The conditions for analysis of the lactone/internal standard pairs were as follows: γ-valerolactone/γ-hexalactone (retention time (r_t) = 5.0 and 7.5 min): injector temperature, 70°; initial column temperature, 50°; column hold time, 6 min; final column temperature, 225°; ramp rate, 20°/min; γ-heptalactone/γ-octalactone (r_t = 6.5 and 8.1 min): injector temperature, 125°; initial column temperature, 70°; column hold time, 5 min; final column temperature, 200°; ramp rate, 15°/min.

All other reactions processed for GC analysis were terminated by adding 75 μL of ethyl acetate, immediately following the addition of the internal standard (2.5 μL of 2 μg/mL γ-valerolactone, or γ-octalactone for analysis of δ-hexalactone). After extraction and centrifugation, 1 μL of the ethyl acetate phase was injected into the gas chromatograph. Analysis of δ-hexalactone and γ-octalactone (r_t = 5.6 and 6.4 min) was performed at an injector temperature of 150° with an initial column temperature of 60° that was held for 3 min and then ramped to 200° at 25°/min. For the rest of the lactones, analysis was performed on an Rtx-1 30 m column (Restek) with an initial column temperature of 90° which was held for 0.5 min and then ramped to 225° at 15°/min. r_ts were as follows: γ-butyrolactone, 8.5; γ-thiobutyrolactone, 10.4; δ-valerolactone, 10.7; DHP, 10.5 and γ-valerolactone, 9.1 min. Identification of the lactones was determined by their co-elution with authentic compounds. Standard curves for each lactone were generated based on the peak height of standards at varying lactone concentrations. Standards were processed in the same manner as the samples except that PON was omitted.

2.8. HPLC analysis of lactones

An aliquot of the incubations containing the aromatic, 5-HETE or 4-HDoHE lactone/acids was added to an equal volume of acetonitrile. Samples were then vortexed and centrifuged at about 15,000 *g* for 1 min. A 20 μL aliquot of the supernatant was injected onto a Beckman System Gold 126 HPLC equipped with a Beckman model 128 diode array detector, Rheodyne model 7125 injector with a 20 μL loop and a Discovery 25 cm × 4.6 mm C-18 column (Supelco). The flow rate was 1 mL/min. Aromatic lactone/acids were eluted isocratically with a 35:65:0.2 ratio of acetonitrile–water–acetic acid. 5-HETE and 4-HDoHE lactone/acids were eluted isocratically with a 90:10:0.2 ratio of acetonitrile–water–acetic acid. Statins were analyzed as previously described [14].

The wavelength settings for the detection of the compounds were as follows: coumarin/acid (265 nm), dihydrocoumarin/acid and 2-coumaranone/acid (220 nm) and all others (236 nm). r_ts were as follows: coumarin/acid = 7.6/4.7 min, dihydrocoumarin/acid = 11.4/5.7 min, 2-coumaranone/acid = 10.4/4.7 min, 4-HDoHE lactone/acid = 6.9/4.9 min and 5-HETE lactone/acid = 7.3/5.0 min.

Quantitation of the extent of hydrolysis or lactonization was based on the lactone/hydroxy acid peak height ratio after the response factors for each lactone and acid were determined. 4-HDoHE lactone was not commercially available and produced enzymatically by PON1 from its open form. The identity of 4-HDoHE lactone was confirmed by converting it back to 4-HDoHE by increasing the pH of the enzymatic reaction.

2.9. Analysis of mevalonic lactone

Mevalonic lactone was analyzed colorimetrically by the Hestrin method as previously described [15]. Briefly, 0.25 mL of a 1:1 mixture of hydroxyl amine (2 M):NaOH (3.5 M) followed by 0.5 mL of a 1:1 mixture of ferric chloride (10% in 4 M HCl):95% ethanol was added to a 0.1 mL reaction. The samples were briefly vortexed and after 5 min their absorbance was read at 520 nm. The concentration of the lactone was calculated using the experimentally determined extinction coefficient of 28 M^{-1} cm^{-1}.

2.10. Stimulation of PON1 lactonization activity by L-α-dilauroylphosphatidylcholine (DLPC)

DLPC (2 mg) was added to 1.0 mL of buffer (25 mM Tris–HCl, 1 mM $CaCl_2$, 20% glycerol, pH 8) and sonicated for about 1 min. One volume of PON1 (0.125 μg/mL), or

PON storage buffer as the control, was mixed with an equal volume the DLPC suspension, or buffer without DLPC, and incubated at 37° for 30 min. *o*-Coumaric acid lactonization reactions were incubated for 10 min as described above.

2.11. Arylesterase and paraoxonase activity

Arylesterase and paraoxonase activities were determined as previously described [26,27].

2.12. Data analysis

Experimental data was analyzed with the Graph Pad Prism statistical program (Graph Pad Software, Inc., version 3.00). Half-lives (to equilibrium) were estimated by fitting the data to a one phase exponential association or decay curve. The calcium dissociation constant was calculated by fitting the data from the calcium dependence assays to a sigmoidal dose–response curve. Data are presented as the average of two separated assays with error bars representing the range of the values (unless otherwise indicated).

3. Results

3.1. General requirements for lactonization by PON1

The rates of lactonization of γ-hydroxyvaleric acid and hydrolysis of γ-valerolactone by human PON1 at pH 6 and 6.5 are shown in Fig. 1. In the presence of PON1, the time necessary for the lactone and hydroxy acid to reach equilibrium was greatly decreased, and no spontaneous hydrolysis or lactonization was detectable even after 5 hr. The ratio of the lactone to acid at equilibrium was dependent upon the pH, as shown, but not upon the substrate concentration (data not shown). The percent lactone at equilibrium was 34.5 and 14.2 at pH 6 and 6.5, respectively, while at pH 7.5 only about 1% was in the lactone form (data not shown). Under the assay conditions, the apparent initial rates were also dependent upon the pH (Fig. 1). At pH 6.5, the estimated hydrolytic rate was 11.1 μmol/min/mg and the lactonization rate was 3.4 μmol/min/mg while at pH 6 the respective rates were 5.8 and 4.4 μmol/min/mg. For comparison, the initial rates for the lactonization of γ-hydroxyheptanoic acid and *o*-coumaric acid were determined at pH 6 and were 15.0 and 43.2 μmol/min/mg, respectively.

The pH activity curve for the lactonization of γ-hydroxyheptanoic acid is shown in Fig. 2A. The optimal pH for lactonization activity with γ-hydroxyheptanoic acid (Fig. 2A) and *o*-coumaric acid (data not shown) was about 5.75. At pH 7.5, PON1 could still appreciably catalyze the lactonization of γ-hydroxyheptanoic acid.

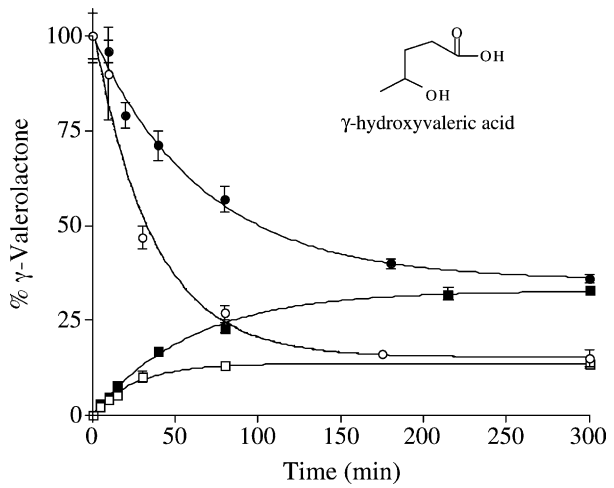


Fig. 1. Time-dependent hydrolysis of γ-valerolactone and lactonization of γ-hydroxyvaleric acid by PON1. γ-Valerolactone (circles) or γ-hydroxyvaleric acid (squares), at a concentration of 1 mM, was incubated in the presence of 1.25 μg/mL of PON1 at pH 6 (filled symbols) or pH 6.5 (open symbols) as described in Section 2. γ-Valerolactone was analyzed by GC as described in Section 2.

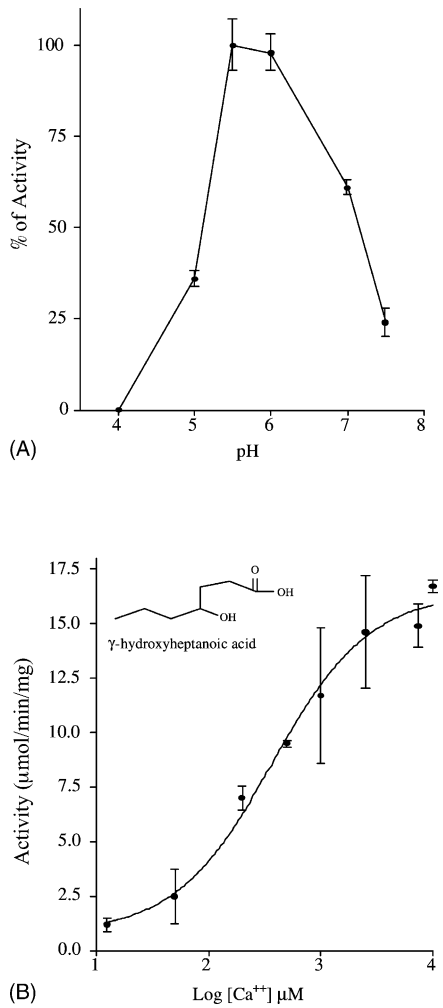


Fig. 2. pH (A) and Ca^{++} (B) dependence for the lactonization of γ-hydroxyheptanoic acid by PON1. γ-Hydroxyheptanoic acid (1 mM) was incubated for 10 min in the presence of 1.25 μg/mL of PON1 (A) or 0.3 μg/mL of PON1 at pH 6 (B) as described in Section 2. γ-Heptalactone was analyzed by GC as described in Section 2.

PON1 contains two calcium binding sites, a high affinity site required for stabilization and a lower affinity site required for its hydrolytic activity [28]. The calcium dependence for the lactonization of γ-hydroxyheptanoic acid by PON1 is shown in Fig. 2B. The apparent K_d for the low affinity binding site at pH 6 was calculated to be 370 μM; a concentration of 10 mM $CaCl_2$ is nearly saturating for maximum activity.

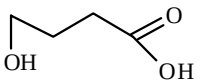
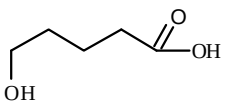
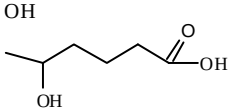
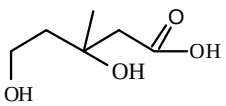
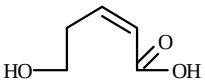
Pre-incubation of purified human serum PON1 with DLPC has been shown to stimulate its hydrolytic activity towards paraoxon, aromatic esters and lactones [11,29]. The aromatic hydroxy acid *o*-coumaric acid was found to be a good substrate for PON1 (see below). Therefore, it was used to determine if DLPC could also stimulate PON1's lactonization activity under conditions similar to those used in previous investigations, i.e. slightly alkaline pH. Pre-incubation of PON1 for 30 min with DLPC resulted in a 71 ± 5% increase in the rate of *o*-coumaric acid lactonization. This degree of stimulation was similar to that observed for the hydrolysis of phenyl acetate and some lactones [11,29].

3.2. Substrate specificity for lactonization by PON1

PON1 has a broad substrate specificity with respect to lactone hydrolysis. Based on our findings that the hydrolysis of γ-valerolactone and γ-heptalactone by PON1 is reversible, we hypothesized that the enzyme can lactonize the corresponding hydroxy acid of any lactone substrate as long as the initial concentration of the acid is greater than its equilibrium concentration. To test this hypothesis, the ability of PON1 to lactonize a diverse group of hydroxy acids, including a thio-acid, was examined. The PON1-mediated lactonization of γ-hydroxybutyric acid and four non-aromatic δ-hydroxy acids was tested and the results are given in Table 1. The equilibrium ratios of the lactone/hydroxy acid pairs were estimated by incubating the lactone or hydroxy acid in the presence of PON1 at pH 5.5 until equilibrium was attained and exemplify how the ring size and structure of the lactone can influence this ratio. The lactone and acid forms of the compounds shown in Table 1 were all substrates for PON1 since no appreciable spontaneous hydrolysis or lactonization was observed in the absence of the enzyme (data not shown). An estimate of the hydroxy acid lactonization activities was determined by incubating the acids for 1 hr in the presence of PON1 at pH 5.5. The low concentration of the δ-valerolactone present at equilibrium precluded activity measurements with its hydroxy acid. Under these conditions no mevalonic lactone was detected. However, the detection limit using the Hestrin method was rather high, approximately 0.25 mM. Of the other three hydroxy acids, δ-hydroxyhexanoic acid was the best substrate. It reached its equilibrium concentration within 1 hr. Despite substantially greater equilibrium concentrations of the lactones with γ-hydroxybutyric acid and DHP, the extent of conversion to the lactone was less with both of these acids than with δ-hydroxyhexanoic acid after the 1-hr incubation.

γ-Thiobutyrolactone, structurally equivalent to γ-butyrolactone except that the ester bond contains a sulfur instead of an oxygen atom, was previously shown to be a poor

Table 1
Lactonization of hydroxy acids by purified human PON1 at pH 5.5

Hydroxy acid	Percent lactone at equilibrium starting with[a]		Percent lactone after 1 hr[b]
	Acid	Lactone	
γ-Hydroxybutyric acid 	15.4 ± 1.0	14.6 ± 1.7	1.3 ± 0.1
δ-Hydroxyvaleric acid 	0.8 ± 0.2	0.6 ± 0.1	ND
δ-Hydroxyhexanoic acid 	2.1 ± 0.2	2.2 ± 0.3	2.1 ± 0.1
δ-Mevalonic acid 	15.7 ± 0.2	21.6 ± 0.2	<2.5
δ-DHP acid 	46.2 ± 4.3	48.8 ± 2.8	1.0 ± 0.2

ND: not determined.

[a] The acid or lactone was incubated in the presence of PON1 as described in Section 2 until equilibrium was attained. The values are the average of three to four experiments ± SD.

[b] Incubations were performed at a substrate concentration of 10 mM and a PON1 concentration of 6.25 μg/mL as described in Section 2. The values are the average of three experiments ± SD. No spontaneous lactone formation was observed when PON1 was omitted from the incubations.

substrate for PON1 [11]. γ-Thiobutyric acid was lactonized by PON1 but it was a very poor substrate. At pH 6, no lactone was detected (detection limit 25 μM) after a 4-hr incubation with the thio acid (20 mM) and PON1 (6.25 μg/mL). Only $1.1 \pm 0.1\%$ of γ-thiobutyric acid (20 mM) was converted by PON1 (50 μg/mL) to the lactone after 4 days at pH 5.5 and equilibrium was not attained. No spontaneous lactonization of the thio acid was detected.

2-Coumaranone (a γ-lactone) and dihydrocoumarin (a δ-lactone) are aromatic lactones that are efficiently hydrolyzed by PON1 [11]. We found that at pH 5.5, >99% of both lactones were hydrolyzed by PON1 (data not shown) indicating that the equilibrium almost completely favors the hydroxy acids. The very low ratio of lactone to acid, even at low pH values, precluded lactonization experiments with these aromatic compounds.

Coumarin, an aromatic δ-lactone, is similar in structure to dihydrocoumarin except that it has a *cis* α,β-double bond in the lactone ring. Unlike 2-coumaranone and dihydrocoumarin, coumarin is very stable even at alkaline pH values due to the *cis* α,β-double bond, which holds the two functional groups in closer proximity allowing ring closure to be favored [30]. *o*-Coumaric acid was rapidly lactonized by PON1 at pH 7.5 (Fig. 3), a pH at which the ratio of coumarin to *o*-coumaric acid is roughly 1000 [20]. The half-life (for the approach to coumarin's equilibrium value) was 164 and 23 min in the absence and presence of PON1, respectively (Fig. 3). Aromatic lactones are also good substrates for rPON3 [14]. We found that rPON3 could catalyze *o*-coumaric acid lactonization, although it was not as efficient as PON1 under the conditions tested (Fig. 3). Previously we could not detect hydrolysis of coumarin by PON1 [11]. However, the assays were performed at pH 8, conditions under which hydrolysis is highly unfavorable. Here the ability of PON1 to hydrolyze coumarin was examined at pH 10, conditions under which the equilibrium ratio of coumarin to *o*-coumaric acid is about 1 [20]. At this pH, 2.2% of a 100 μM solution of coumarin was hydrolyzed by PON1 (50 μg/mL) in 30 min. No spontaneous hydrolysis was detected.

3.3. Lactonization of lovastatin and simvastatin

The cholesterol lowering lactone drugs LV and SV are aliphatic δ-lactones that have a hydroxyl group at the β position. *In vivo* both are hydrolyzed to their pharmacologically active hydroxy acid forms, however, lactonization of the statin acids can also occur [21,22]. To determine if PONs would likely contribute the closure of the statin acids we investigated the statin acid lactonization activity of PON1 and rPON3. Since the open form of aliphatic δ-lactones is largely favored even at acidic pHs ([17,19], see above), lactonization experiments with the statins were performed at pH 5.5. rPON3 catalyzed the lactonization of LVA at an estimated rate of 8 nmol/min/mg (Fig. 4). Lactonization rates of SVA by rPON3 could not be accurately determined due to the co-elution and interference of tergitol (present in the enzyme preparation) with SV on the HPLC.

No lactonization of LVA or SVA (20 μM) by PON1 (6.25 μg/mL) could be detected. The LV hydrolytic activity of our PON1 preparation at pH 7.4 was very low, <1 nmol/min/mg. The previously reported statinase activity for PON1 was also below 1 nmol/min/mg [11]. Because the statin acid lactonizing activity under our conditions was much lower than the hydrolytic activity it is likely that lactonization activity with purified PON1 was below our detection limit.

To investigate further the potential of PON1 to lactonize statin acids we examined the ability of plasma from PON1 KO and wild type mice to catalyze the lactonization of LVA and SVA. The absence of PON1 in the KO plasma was verified by its lack of paraoxonase activity whereas the wild

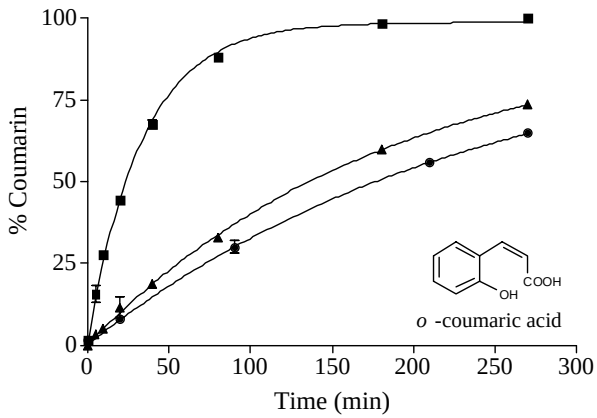


Fig. 3. Lactonization of *o*-coumaric acid by PON1 and rPON3. *o*-Coumaric acid (100 μM) was incubated in the presence of 1.25 μg/mL of PON1 (squares) or rabbit PON3 (triangles) or without PON (circles) as described in Section 2. Coumarin and *o*-coumaric acid were analyzed by HPLC as described in Section 2. Experiments without PON were performed in triplicate and the data is presented as the mean ± SD.

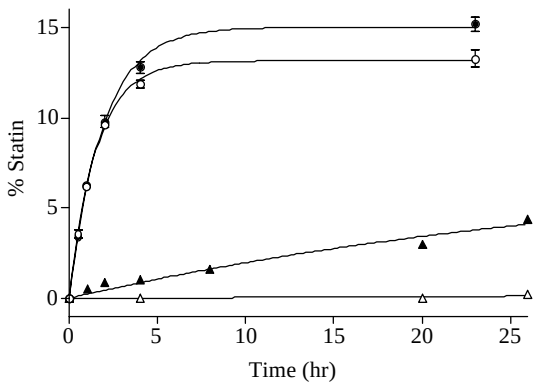


Fig. 4. Lactonization of statin acids (20 μM) by rPON3 and mouse plasma. LVA was incubated in the presence of 1.25 μg/mL rPON3 (closed triangles) and SVA was incubated in the presence of wild type (closed circles) or KO (open circles) mouse plasma at pH 5.5. Statin and statin acids were analyzed by HPLC as described in Section 2. No significant lactonization was detected in the absence of rPON3 or plasma (open triangles).

type plasma hydrolyzed paraoxon at a rate of 108 nmol/min/mL (at pH 8). Both wild type and KO plasma samples had comparable lactonizing activity with LVA and SVA (SVA lactonization shown in Fig. 4). No lactonization of LVA and SVA was detected after pre-incubation of the wild type and KO plasma with the serine esterase inhibitor PMSF. Because PON1 is not inhibited by PMSF, the results suggest that a PMSF sensitive esterase(s), presumably carboxylesterase(s), is the major contributor to LVA and SVA lactonization in the mouse plasma.

3.4. Lactonization of long chain hydroxy fatty acids

Over the last two decades, a growing body of evidence suggests a physiological role for PON1 metabolism of oxidized lipids [31]. This led us to extend our investigations to two endogenous hydroxy fatty acids, 4-HDoHE and 5-HETE, which are derived from docosahexaenoic acid (22:6) and arachidonic acid (20:4), respectively, and are the result of enzymatic and non-enzymatic oxidations [32–34]. We estimated the initial rate of lactonization of 4-HDoHE at pH 6, conditions under which the γ-lactone should form in significant amounts. As shown in Table 2, at a substrate concentration of 5 μM both PON1 type Q and PON1 type R lactonized 4-HDoHE at approximately the same rate. Equilibrium assays with 4-HDoHE and 4-HDoHE lactone were not performed because the latter was not commercially available and could not be generated in sufficient quantities due to its instability during extended incubation times.

Table 2
Rates of lactonization of 4-HDoHE and hydrolysis of 5-HETE lactone

Sample	Arylesterase[a] (μmol/min/mL)	4-HDoHE lactonization[b] (nmol/min/mL)	5-HETE lactone hydrolysis[b] (μmol/min/mL)
PON1 Q	800[c]	2100 ± 200[c]	24.2 ± 4.4[c]
PON1 R	741[c]	2000 ± 400[c]	29.7 ± 2.1[c]
Human plasma 1	85	25.4 ± 5.3	5.3 ± 0.9
Human plasma 2	64	17.3 ± 3.8	4.4 ± 0.3
Human plasma 3	85	21.5 ± 4.9	5.5 ± 0.5
Mouse WT plasma	64	48.1 ± 3.9	5.9 ± 0.3
Mouse KO plasma	17	16.2 ± 1.5	0.45 ± 0.02

[a] Substrate (phenyl acetate) concentration 1 mM.
[b] The values are the average of three experiments ± SD.
[c] Activity/mg of PON1.

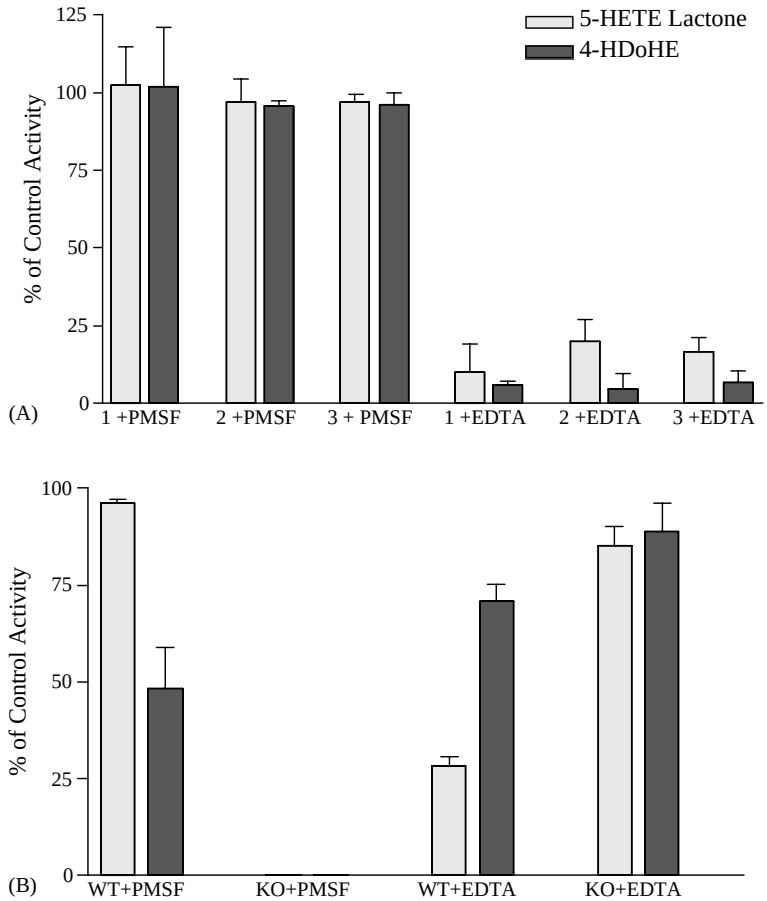


Fig. 5. Inhibition of 5-HETE lactone hydrolysis and 4-HDoHE lactonization by PMSF and EDTA in human plasma from three individuals numbered 1, 2 and 3 (A) and mouse plasma (B). Plasma was incubated in the presence of 2 mM PMSF for 2 hr or 20 mM EDTA for 16 hr and analyzed for 5-HETE lactonase and 4-HDoHE lactonizing activities as described in Section 2. The activities of the controls were similar to those given in Table 2.

5-HETE (20 μM) was also lactonized by PON1 (no spontaneous lactonization occurred under the assay conditions), but the apparent equilibrium value of the lactone at pH 6 was low (less than 4%) precluding any lactonization rate determinations. This was expected because generally aliphatic δ-lactones are relatively unstable and favor the open form ([17], see above). 5-HETE lactone (5 μM) was efficiently hydrolyzed by PON1 and, as with 4-HDoHE, no significant differences were found in the rates of hydrolysis by the Q and R types of PON1 (Table 2).

The findings with 4-HDoHE and 5-HETE lactone prompted us to investigate to what extent the metabolism of these two lipids in human and mouse plasma is dependent on PON1. 4-HDoHE was lactonized by the human and mouse plasma samples (Table 2). In the human samples, the lactonization rates correlated with the arylesterase activity, which is almost completely accounted for by PON1 in human plasma. In the PON1 KO mouse plasma, the 4-HDoHE lactonizing activity was only about one-third that found in the wild type plasma suggesting that PON1 does contribute to this activity in the wild type plasma, although other esterases also contribute to the lactonization of 4-HDoHE.

5-HETE lactone was effectively hydrolyzed in the human and wild type mouse plasma samples and, as with 4-HDoHE lactonization, 5-HETE lactone hydrolysis correlated with the arylesterase activity in the human samples (Table 2). The 5-HETE lactone hydrolytic activity in the KO mouse plasma was about 13-fold lower than the wild type mouse plasma suggesting that this activity is predominantly due to PON1 in the wild type plasma.

Inhibition studies with PMSF and the PON1 inhibitor EDTA were also performed to estimate the contribution of PON1 to the metabolism of these two substrates in the plasma samples. The paraoxonase activity of the EDTA-treated plasma samples was reduced by 95–97% indicating an almost complete inhibition of PON1, whereas there was no significant inhibition of paraoxonase activity in the PMSF-treated samples (data not shown). As shown in Fig. 5A, there was no decrease in 5-HETE lactone hydrolytic activity or 4-HDoHE lactonizing activity in the human plasma samples treated with PMSF. Conversely, both activities were inhibited from 80 to 95% in the EDTA-treated samples suggesting that PON1 is the major esterase catalyzing these activities in the human plasma samples.

The 4-HDoHE lactonizing activity in the wild type mouse plasma was inhibited by about 50% when treated with PMSF and about 30% when treated with EDTA (Fig. 5B). This is consistent with the rates shown in Table 2 (wild type vs. KO) that indicate both PON1 and a carboxylesterase(s) substantially contribute to this activity in the wild type plasma. The 5-HETE lactone hydrolytic activity in the wild type plasma was not affected by treatment with PMSF but inhibited by approximately 72% when treated with EDTA (Fig. 5B). This is also consistent with the results given in Table 2 (wild type vs. KO) that suggest PON1 is the major contributor to this activity in the wild type plasma. In the KO plasma, both activities appeared to be catalyzed by a carboxylesterase(s) due to the complete inhibition by PMSF and lack of significant inhibition by EDTA (Fig. 5B).

4. Discussion

Ring opening can have dramatic effects on the physical properties and biological effects of lactone drugs and endogenous compounds, and this has prompted continual investigation of esterases involved in lactone hydrolysis. However, there has been much less investigation of the reversibility of enzymatic lactone hydrolysis. We now report that PON1, as well as rPON3, can also catalyze the reverse reaction (lactonization) of hydroxy acids. Using γ-hydroxyheptanoic acid and *o*-coumaric acid as model substrates we found that lactonization by PON1 is dependent on calcium and can be stimulated by the phospholipid DLPC, as has previously been shown for the lactonase activities. Although the mechanism of stimulation by phospholipids is not understood, it at least compensates for activity lost due to the addition of detergents during the purification of PON1 [29]. These similar characteristics support the supposition that the lactones and hydroxy acids both bind the same catalytic center of the enzyme.

Generally, our results support the hypothesis that the hydroxy acid of any lactone substrate can also be lactonized by PON1, within a pH range over which the enzyme is functional, as long as the hydroxy acid/lactone ratio is greater than its equilibrium value. However, comparison of the lactonization rates of different hydroxy acids must not only take into account the catalytic efficacy of the enzyme but also the equilibrium substrate/product ratio (or the fraction of initial substrate that can be lactonized). The rates are dependent upon the thermodynamic drive for the reaction, which decreases as the fraction of the initial substrate that can be metabolized decreases, i.e. a higher equilibrium substrate/product ratio. This is exemplified in Fig. 1 with γ-hydroxyvaleric acid. At pH 6.5, where only 14.2% of the hydroxy acid is ‘‘available’’ for lactonization, the rate of lactonization by PON1 is lower than at pH 6 where 34.5% of the hydroxy acid can be lactonized. Accordingly, hydroxy acids with ‘‘favorable’’ lactone/hydroxy acid equilibrium ratios such as γ-hydroxyvaleric acid, γ-hydroxyheptanoic acid, 4-HDoHE and *o*-coumaric acid were all effectively lactonized by PON1.

The inability to detect lactonization of the statin acids with the PON1 preparation is likely due the formation of statins below our detection limit (see Section 3). rPON3, which has high statinase activity [14], did lactonize LVA (Fig. 4). However, the 10-fold lower LVA lactonizing activity, compared to the previously reported lactonase activity, is probably at least partially due to the instability of the δ-lactone which results in a comparatively lower

thermodynamic drive for lactonization. A decreased substrate affinity, catalytic efficiency and/or enzyme stability at pH 5.5 may also contribute to the lower activity.

The relatively low lactone/hydroxy acid equilibrium ratio of unstable lactones like the statins and 5-HETE lactone suggests that lactonization of their hydroxy acids at physiological pH values will be negligible. However, significant lactonization of statin acids occurs *in vivo* [21,22] and 5-HETE lactone, presumably derived from the 5-lipoxygenase product 5-HETE, has been detected in activated human B-lymphocytes [35]. These findings suggest the presence of mechanisms *in vivo* that facilitate the energetically unfavorable lactonization of stable hydroxy acids. Lactones can be orders of magnitude more lipophilic than their hydroxy acids [23]. Once formed the lipophilic lactone may rapidly be removed from the local environment of the lactonizing enzyme by diffusing through membranes, allowing lactonization to continue. Alternatively, a higher energy conjugate of the hydroxy acid could provide the energy necessary for enzymatic or spontaneous lactonization. Simvastatin acid was shown to undergo CoASH-dependent formation of its lactone in mouse liver preparations [36]. Statins can also be glucuronidated and the glucuronides then undergo spontaneous lactonization [37]. Acyl glucuronidation is not uncommon [38], and derivatization and lactonization of hydroxy fatty acids in a manner analogous to that described for the statins could presumably occur *in vivo*. Interestingly, we found that mouse plasma carboxylesterase(s) can also lactonize the statin acids (Fig. 4). Because these esterases can hydrolyze the CoA derivatives of various fatty acids [39] it would be reasonable to propose that they may catalyze the lactonization of hydroxy acid–CoA conjugates.

4-HDoHE, and particularly 5-HETE lactone, were very active substrates for purified PON1 types R and Q (Table 2) and the inhibition assays in the human plasma samples argue strongly that PON1 is a major contributor to both activities (Fig. 5). PON3 is present in human plasma [40], although the levels appear to be significantly lower that PON1 (D.I. Draganov unpublished observations). We are currently investigating the ability of human PON3 to metabolize 5-HETE lactone and 4-HDoHE.

The 5-HETE lactonase activity in the mouse plasma also appears to be mostly due to PON1. However, carboxylesterase(s) makes a greater contribution to 4-HDoHE lactonization in the mouse plasma even though PON1 still contributes significantly to this activity (Table 2, Fig. 5B). Approximately 20% of both the 4-HDoHE lactonizing and 5-HETE lactone hydrolytic activities could not be accounted for by PMSF and EDTA sensitive esterase activity in the wild type plasma (Fig. 5B). It's possible that inhibition by PMSF may not be complete and/or other PMSF and EDTA insensitive esterase(s) may make minor contributions to these activities in the wild type plasma.

As mentioned above, 5-HETE lactone and 4-HDoHE have been detected in biological systems although there has been little investigation of their biological activities. Conversely, 5-HETE is generated in a wide variety of cell types and elicits a plethora of biological effects [41]. The significance of PON1's ability to metabolize 4-HDoHE and 5-HETE/5-HETE lactone is not clear. However, the ubiquity of bioactive long chain unsaturated fatty acids with γ and δ oxygen functional groups suggests that PON1 could play an important role in modulating the activities of this class of compounds. Endogenous 5,6-epoxides of arachidonic acid exhibit a broad range of activities and can readily lactonize spontaneously [42,43]. Although not reported *in vivo*, 5,6-dihydroxyeicosatrienoic acid lactone was a potent dilator of canine coronary arterioles [43]. We found that this lactone is hydrolyzed by PON1 (Dr. Philip Stetson, personal communication) and *in vivo* PON1 could regulate the level or distribution of such lactones. γ- and δ-iodolactone, derived from docosahexaenoic acid and arachidonic acid, respectively, are synthesized in thyroid follicles and δ-iodolactone inhibits signal transduction pathways induced by local growth factors [44]. The structural similarity between the iodolactones and PON1's other substrates indicate that they will likely be hydrolyzed by the enzyme and suggests a role for PON1 in thyroid metabolism.

The ability of paraoxonases to catalyze hydroxy acid lactonization extends the potential contribution of this family of enzymes to the metabolism of drugs and endogenous compounds. Also, lactonization of hydroxy acids by rPON3 and carboxylesterase(s) indicates that this may be a property of lactonases that has not generally been given much attention. Future studies will focus on the ability of PON1, PON2 and PON3 to metabolize these and other oxidized fatty acid substrates.

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