Preferential inhibition of paraoxonase activity of human paraoxonase 1 by negatively charged lipids

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Abstract To determine the causes responsible for a preferential decrease of paraoxonase activity, which has been observed in the serum of patients with cardiovascular diseases, the inactivation or inhibition of paraoxonase 1 (PON1) by various endogenous factors was examined using paraoxon or phenyl acetate as a substrate. When purified PON1 was incubated with various endogenous oxidants or aldehydes, they failed to cause a preferential reduction of paraoxonase activity, suggesting no participation of the inactivation mechanism in the preferential loss of paraoxonase activity. Next, when we examined the inhibition of PON1 activity by endogenous lipids, monoenoic acids such as palmitoleic acid or oleic acid inhibited paraoxonase activity preferentially, in contrast to a parallel inhibition of both activities by polyunsaturated or saturated acids. Noteworthy, oleoylglycine inhibited paraoxonase activity, but not arylesterase activity, complying with the selective inhibition of paraoxonase activity. Moreover, such a selective inhibition of paraoxonase activity was also expressed by lysophosphatidylglycerol or lysophosphatidylinositol, but not by lysophosphatidylserine or lysophosphatidylcholine, indicating the importance of the type of head group. Furthermore, such a preferential or selective inhibition of paraoxonase activity was also observed with PON1 associated with HDL or plasma. These data suggest that some negatively charged lipids may correspond with PON1 associated with HDL or plasma. Preferential inhibition of paraoxonase activity of human paraoxonase 1 by negatively charged lipids. J. Lipid Res. 2004. 45: 2211–2220.

Supplementary key words paraoxonase 1 • oxidative inactivation • phospholipid • lysophospholipids

Paraoxonase 1 (PON1), which is associated with HDL (1–3), was initially identified for its ability to hydrolyze organophosphate compounds and aromatic carboxylic acid esters (1, 4–6). Although the potential interest of PON1 comes from the detoxification of toxic organophosphate compounds through its hydrolytic action (4, 7), it also has the ability to hydrolyze homocysteine thiolactone, which could pose potential harm by the homocysteinylation of some proteins (6, 8). In addition, some lactone compounds such as statin drugs or fatty acid lactone were found to be PON1 substrates (9, 10).

Recent interest in the enzyme has arisen from the idea that PON1 protects LDL and HDL from lipid peroxidation (1, 3, 11, 12). Animal models provide support for this contention, in that a lower serum level of PON1 is associated with a greater susceptibility of LDL to oxidation and an increased risk of atherosclerosis (13). Clinical investigations indicate that PON1 activity is lower in subjects with coronary vascular diseases than in control subjects (14–16). Thus, the serum level of PON1 activity has been considered as a predictor of cardiovascular disease in the early phase.

Two substrates, paraoxon and phenyl acetate, have been used in the routine assay of PON1 activity, because the same active site of PON1 is responsible for the hydrolysis of the two substrates (1, 5). Nevertheless, there is evidence that the binding sites for the two substrates do not overlap exactly with each other (17–19); paraoxonase activity is stimulated by 1 M NaCl, but arylesterase activity is not (18). Some synthetic compounds, such as PD 65950, differentiate between paraoxonase activity and arylesterase activity (19). Moreover, PON1 exhibits a substrate-dependent polymorphism; the PON1 Q/R 192 polymorphism imparts different catalytic activity toward paraoxon, in contrast to the same rate for the hydrolysis of phenyl acetate by both alloenzymes, Q isozyme and R isozyme (4, 20).

The importance of the substrate-specific feature of PON1 may be seen in the substrate-dependent alteration of serum PON1 activity in cardiovascular disease patients; the serum paraoxonase activity decreased significantly (~20%), in contrast to no change of serum arylesterase activity (21–23). Likewise, such a preferential decrease of paraoxonase activity has been demonstrated in some oxidative stress-associated diseases such as hypercholesterolemia, di-

Abbreviations: lysoPG, lysophosphatidylglycerol; PON1, paraoxonase 1.

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abates mellitus, or Alzheimer’s disease (24–26). The same phenomenon was also observed with smoking or advancing age, in which oxidative stress was predominant (8, 27).

Concerning the oxidative inactivation of PON1, it has been shown that PON1 activity was reduced during exposure to oxidized LDL or reactive oxygen species (28–32). Additionally, 4-hydroxy-2-nonenal, which gradually accumulates in the later stage of LDL oxidation, can also cause the inactivation of PON1 arylesterase activity (32). Despite these data, there is no information about whether the oxidative modification of PON1 at the active site, occurring in oxidative stress, may result in the preferential loss of paraoxonase activity of PON1. Alternatively, the substrate-dependent modulation of PON1 activity by endogenous factors, which could accumulate in oxidative stress-related diseases, may be related to the preferential reduction of paraoxonase activity of PON1. Such factors could be endogenous inhibitors or activators, which can affect the substrate-specific activity of PON1 under the assay conditions used. Previously, some lipids such as phosphatidylcholines were reported to stimulate PON1 arylesterase activity (33). Our recent studies showed that the arylesterase activity was inhibited competitively by polyunsaturated fatty acids at low concentrations (32, 34). Thus, the effect of lipids on PON1 arylesterase activity seems to differ according to the type of lipid. Likewise, the paraoxonase activity of PON1 could be affected by some lipids. However, there has been no attempt to examine the modulation of PON1 activity by lipids, especially with regard to a preferential inhibition of paraoxonase activity. Recently, there have been reports (35–38) that the level of negatively charged lipids is enhanced in lipoproteins in the oxidative stress-associated state. In some oxidative stress-related diseases, the preferential reduction of paraoxonase activity has been commonly observed (24–26). In this regard, it would be interesting to determine how the lipids, especially negatively charged lipids, affect two activities, paraoxonase and arylesterase activities, of PON1 with the aim of finding the causes responsible for the preferential loss of paraoxonase activity.

In this study, first, we examined the inactivation of PON1 activities by various endogenous factors, such as reactive oxygen species, lipoxidation, and glycoxidation products, which could be generated under oxidative stress in vivo. Second, various endogenous lipids, including fatty acids, phospholipids, and lysophospholipids, were tested for the preferential inhibition of paraoxonase activity.

### EXPERIMENTAL PROCEDURES

#### Materials

All materials, including lipids, were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted. H$_2$O$_2$ (30%) and 4-hydroxy-2-nonenal were from Junsei Chemical Co. (Tokyo, Japan) and Calbiochem Co. (La Jolla, CA), respectively. Phospholipids and lysophospholipids were provided by Avanti Polar Lipids, Inc. (Alabaster, AL). Metal ions, including cupric sulfate and ferrous sulfate, were of analytical grade. Oleoyl sulfate was kindly supplied by Dr. T. R. Holman (Department of Chemistry and Biochemistry, University of California, Santa Cruz).

#### Purification of PON1 from human plasma

PON1 was purified from human plasma by chromatographic procedures according to a slight modification of the published procedures (5, 34, 39). The purified PON1 was kept in 25 mM Tris buffer containing 1 mM Ca$^{2+}$ at 4°C. The purified enzyme possessed a specific activity of $\sim$1,018 and 0.552 nmol/min/mg protein in the hydrolysis of phenyl acetate and paraoxon, respectively. The purified PON1, used mainly for this study, was found to belong to phenotype AB group, based on the dual substrate method (19).

#### Assay of PON1

Arylesterase activity of PON1 was measured by adding enzyme solution to 0.5 ml of 50 mM Tris buffer, pH 7.4, containing 1 mM CaCl$_2$ and 10 mM phenyl acetate, and the rate of generation of phenol was monitored at 270 nm as described before (5, 34). For the inhibition study, the concentration of phenyl acetate was adjusted to 1 mM unless otherwise described. Paraoxonase activity was determined by measuring the change of absorbance at 405 nm in 50 mM Tris buffer (pH 7.4) containing 1 mM CaCl$_2$ and 1 mM paraoxon as described before (5). One unit of arylesterase activity is expressed as one micromole of phenol produced from the hydrolysis of phenyl acetate per minute, and one unit of paraoxonase activity is expressed as one nanomole of p-nitrophenol produced from paraoxon per minute.

#### Oxidative inactivation of PON1

PON1 (5 arylesterase units/ml) was preincubated with ascorbate (0.5 mM) in the presence of 1 μM Ca$^{2+}$ or 2 μM Fe$^{2+}$ in 0.1 ml of 10 mM PBS buffer (pH 7.4) containing 1 mM Ca$^{2+}$ at 38°C as described (34), and then the aliquot (20 μl) was subjected to arylesterase or paraoxonase activities. Separately, PON1 was preincubated with H$_2$O$_2$ or NaOCl in 0.1 ml of 10 mM PBS buffer (pH 7.4) containing 1 mM Ca$^{2+}$ at 38°C.

#### Inactivation of PON1 by 4-hydroxy-2-nonenal or methylglyoxal

PON1 was preincubated with 4-hydroxy-2-nonenal (1 mM) in 0.1 ml of 10 mM PBS buffer (pH 7.4) containing 1 mM Ca$^{2+}$ at 38°C, and then the aliquot (20 μl) was subjected to arylesterase or paraoxonase activities. Separately, PON1 was preincubated with 6 mM methylglyoxal, a modifier of lysine residue (40).

#### Inhibition of PON1 activity by fatty acids or oleoyl derivatives

PON1 (1 arylesterase unit/ml, 0.022 μM) was incubated with phenyl acetate (1 mM) or paraoxon (1 mM) in the presence of each fatty acid in 0.5 ml of 50 mM Tris buffer (pH 7.4) containing 1 mM Ca$^{2+}$ at 25°C, and the formation of product was monitored as described above. Separately, the activity of PON1 was determined in the presence of each derivative containing oleoyl moiety. Fatty acids and oleoyl derivatives, dissolved in ethanol, were included in the reaction mixture, so that the final concentration of ethanol was 0.5–1%. Under the conditions used, the binding of the lipid compound (oleic acid or linoleic acid) to the assay cuvette (quartz) was negligible, based on an enzyme-based assay (41) using a nonesterified fatty acid assay kit (Wako Chemicals).

#### Inhibition of PON1 activity by phospholipids or lysophospholipids

PON1 (1 arylesterase unit/ml) was incubated with phenyl acetate or paraoxon in the presence of each phospholipid or lysophospholipid in 0.5 ml of 50 mM Tris buffer (pH 7.4) containing

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1 mM Ca\(^{2+}\) at 25°C. Phospholipids, dissolved in ethanol, were included in the reaction mixture (final concentration of ethanol, 0.5–1%), and lysophospholipids were dissolved in 25 mM Tris buffer (pH 7.4). The possible binding of lysophosphatidylglycerol (lysoPG) to the assay cuvette was negligible.

**Combinational effect of two different lipids on PON1 activity**

PON1 (1 arylesterase unit/ml) was incubated with phenyl acetate or paraoxon in the presence of two different lipids in 0.5 ml of 50 mM Tris buffer (pH 7.4) containing 1 mM Ca\(^{2+}\) at 25°C, and the formation of product was monitored as described above.

**Inhibition of HDL-PON1 or plasma-PON1 by oleic acid, lysoPG, or their combination**

HDL-PON1 (1 arylesterase unit/ml), prepared as previously described (34), was incubated with paraoxon or phenyl acetate in the presence of oleic acid, monooleoyl-lysoPG (1 or 3 μM), or their combination in 0.5 ml of 50 mM Tris buffer (pH 7.4) containing 1 mM Ca\(^{2+}\). Separately, plasma (10 or 25 μl) was incubated with paraoxon or phenyl acetate in the presence of lipid inhibitor as described above.

**Other analyses**

Inhibition of PON1 by fatty acids was carried out by incubating PON1 with paraoxon or phenyl acetate in the presence of each fatty acid in 0.5 ml of 50 mM Tris buffer (pH 7.4) containing 1 mM Ca\(^{2+}\) at room temperature, and the inhibition pattern of fatty acids was analyzed by Lineweaver-Burk plot (34).

**RESULTS**

**Effect of endogenous oxidants or aldehydes on PON1**

To determine whether the oxidative inactivation of PON1 may be responsible for the preferential reduction of paraoxonase activity, PON1 was exposed to various endogenous oxidants in 10 mM PBS buffer containing 1 mM Ca\(^{2+}\), and the remaining activity was determined using paraoxon or phenyl acetate as a substrate. First, when PON1 was preincubated with ascorbate/Cu\(^{2+}\), which has been found to modify the histidine residue of PON1 (32, 34), both arylesterase activity and paraoxonase activity were inactivated to a similar extent (Table 1). A similar inactivation of both activities was also observed after exposure to the ascorbate/Fe\(^{2+}\) system, another inactivating oxidant (32, 34). Then, when PON1 was exposed to H\(_2\)O\(_2\) or NaOCl, it was found that paraoxonase activity was less sensitive to NaOCl than was arylesterase activity, whereas H\(_2\)O\(_2\) inactivated both activities to a similar extent (Table 1). Taken together, these oxidative systems failed to inactivate paraoxonase activity preferentially. Subsequently, we turned to the inactivation of PON1 by either 4-hydroxy-2-nonenal, a modifier of cysteine and histidine residues (42), or methylglyoxal, a modifier of lysine residue (40), which are generated during in vivo oxidative stress. As shown in Table 1, 4-hydroxy-2-nonenal, which was reported to inactivate PON1 (32), caused a parallel loss of paraoxonase activity and arylesterase activity. In addition, the same degree of inactivation of both activities was also observed with methylglyoxal. Thus, a preferable inhibition of paraoxonase activity of PON1 was not found with lipoxidation or glycoxidation products. All of these data indicate that the inactivation of PON1 by endogenous species, which are generated during in vivo oxidative processes, may not be related to the preferential loss of PON1 activity. In an attempt to provide further support for this, PON1 was exposed to various amino acid modifiers, such as N-bromosuccinimide, diethylpyrocarbonate, and p-hydroxymercuribenzoate, which had been observed to inactivate PON1 activity (43). However, none of these modifiers showed the substrate-dependent inactivation of PON1 activity (data not shown).

**Inhibition of PON1 activity by endogenous lipids**

In the subsequent study, we turned to the substrate-dependent inhibition of PON1 by endogenous lipids, which are suspected to be present in blood samples. To this end, the arylesterase and paraoxonase activities of PON1 were determined in 50 mM Tris buffer (pH 7.4) containing 1 mM Ca\(^{2+}\) for the designated times, and then the aliquot (20 μl) was taken for the assay of remaining activity using phenyl acetate (10 mM) or paraoxon (1 mM) as described in Experimental Procedures. Data are expressed as means ± SD of triplicate assays, presented as a percentage of control activity.

**TABLE 1. Inactivation of PON1 activity by endogenously generated oxidants or aldehydes**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration</th>
<th>Time</th>
<th>Enzyme Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mM/min/μM</td>
<td></td>
<td>Paraoxonase</td>
</tr>
<tr>
<td>Ascorbate/Cu(^{2+})</td>
<td>0.5/0.001</td>
<td>30</td>
<td>22.2 ± 1.6</td>
</tr>
<tr>
<td></td>
<td>0.5/0.005</td>
<td>60</td>
<td>16.6 ± 2.3</td>
</tr>
<tr>
<td>Ascorbate/Fe(^{2+})</td>
<td>0.5/0.002</td>
<td>10</td>
<td>78.9 ± 5.4</td>
</tr>
<tr>
<td></td>
<td>2.0/0.06</td>
<td>30</td>
<td>55.2 ± 3.9</td>
</tr>
<tr>
<td>H(_2)O(_2)</td>
<td>2</td>
<td>30</td>
<td>82.7 ± 3.6</td>
</tr>
<tr>
<td>NaOCl</td>
<td>1</td>
<td>90</td>
<td>78.7 ± 4.9</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>90</td>
<td>38.9 ± 3.9</td>
</tr>
<tr>
<td>4-Hydroxy-2-nonenal</td>
<td>1</td>
<td>10</td>
<td>59.1 ± 2.1</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>90</td>
<td>40.9 ± 3.9</td>
</tr>
<tr>
<td>Methylglyoxal</td>
<td>6</td>
<td>60</td>
<td>62.5 ± 4.3</td>
</tr>
</tbody>
</table>

Paraoxonase 1 (PON1; 5 arylesterase units/ml; 0.11 μM) was incubated with various oxidants or aldehydes in 0.1 ml of 10 mM PBS buffer (pH 7.4) containing 1 mM Ca\(^{2+}\) for the designated times, and then the aliquot (20 μl) was taken for the assay of remaining activity using phenyl acetate (10 mM) or paraoxon (1 mM) as described in Experimental Procedures.
Inhibition of PON1 activity by fatty acids

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Concentration (μM)</th>
<th>Paraoxonase Activity %</th>
<th>Arylesterase Activity %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Caproic</td>
<td>30</td>
<td>85.7 ± 2.5</td>
<td>88.5 ± 5.6</td>
</tr>
<tr>
<td>Caprylic</td>
<td>30</td>
<td>52.6 ± 5.4</td>
<td>55.3 ± 4.5</td>
</tr>
<tr>
<td>Lauric</td>
<td>30</td>
<td>71.9 ± 0.7</td>
<td>65.9 ± 2.7</td>
</tr>
<tr>
<td>Myristic</td>
<td>30</td>
<td>98.9 ± 0.4</td>
<td>95.6 ± 4.6</td>
</tr>
<tr>
<td>Palmitic</td>
<td>30</td>
<td>102.8 ± 0.2</td>
<td>98.5 ± 5.8</td>
</tr>
<tr>
<td>Palmitoleic</td>
<td>10</td>
<td>46.6 ± 4.1</td>
<td>65.4 ± 5.2</td>
</tr>
<tr>
<td>Oleic</td>
<td>10</td>
<td>54.4 ± 3.2</td>
<td>69.5 ± 1.9</td>
</tr>
<tr>
<td>Linoleic</td>
<td>10</td>
<td>30.3 ± 2.0</td>
<td>32.1 ± 2.2</td>
</tr>
<tr>
<td>Arachidonic</td>
<td>10</td>
<td>44.5 ± 3.6</td>
<td>41.4 ± 4.2</td>
</tr>
</tbody>
</table>

PON1 (1 arylesterase unit/ml) was incubated with phenyl acetate (1 mM) or paraoxon (1 mM) in the presence of each fatty acid (10 or 30 μM) in 0.5 ml of 50 mM Tris buffer (pH 7.4) containing 1 mM CaCl₂ at 25°C, and the formation of product was monitored as described in Experimental Procedures. Data are expressed as means ± SD of triplicate assays, presented as a percentage of control activity. 

Fig. 1. Inhibition of paraoxonase 1 (PON1) activity by linoleic acid or oleic acid. PON1 (1 arylesterase unit/ml) was incubated with phenyl acetate (1 mM) or paraoxon (1 mM) in the presence of linoleic acid (0.3–10 μM; closed symbols) or oleic acid (0.3–10 μM; open symbols) in 0.5 ml of 50 mM Tris buffer (pH 7.4) containing 1 mM CaCl₂ at 25°C, and the formation of product was monitored as described in Experimental Procedures. Data are expressed as means ± SD of triplicate assays, presented as a percentage of control activity. Triangles, arylesterase activity; squares, paraoxonase activity. Conc, concentration.

Differential inhibition of paraoxonase activity by oleic acid (3 μM) was found in the presence of linoleic acid (0.3 μM); remaining activities of paraoxonase and arylesterase were estimated as 49.0% and 63.2% of control, respectively. Based on these results, monoenoic acids such as palmitoleic acid or oleic acid are suggested to be preferential inhibitors of paraoxonase activity.

To determine the binding site for preferential inhibitors of paraoxonase activity, the inhibitory effect of linoleic acid or oleic acid on paraoxon-hydrolyzing activity was analyzed by Lineweaver-Burk plot. As shown in Fig. 2A, linoleic acid expressed a competitive inhibition at lower concentrations (1 or 4 μM), although it tended to show a mixed type of inhibition at higher concentrations. Meanwhile, oleic acid at lower concentrations (0.3 or 1 μM) inhibited paraoxonase activity in a pattern similar to a noncompetitive inhibition (Fig. 2B), although at higher concentrations it tended to inhibit PON1 competitively. These results led to the notion that the preferential inhibition of paraoxonase activity by oleic acid may be ascribed to its binding to a site, a so-called peripheral site, distant from the active center of PON1. Thus, it is supposed that the peripheral site, responsible for the preferential inhibition, may be close to a paraoxon binding subsite rather than a phenyl acetate binding subsite.

To determine the structural requirement for the preferential inhibition of PON1, the inhibitory effect of various oleoyl moiety-containing derivatives on PON1 activity was determined. As demonstrated in Table 3, a preferable inhibition of paraoxonase activity was also seen by oleoylglycerine or oleoylsulfate, similar to the observation with oleic acid. In contrast, other oleoyl derivatives devoid of a negative charge failed to distinguish between the two activities. In another study (Fig. 3), two oleoylated derivatives, oleoylglycerine and oleoylsulfate, demonstrated a similar pattern of substrate-dependent inhibition of PON1 activity; both compounds at the concentrations used inhibited paraoxonase activity significantly. In particular, oleoylglycerine expressed a remarkable inhibition of paraoxonase activity with a maximal inhibition of ~27%, while expressing no inhibition of arylesterase activity, in agreement with the selective inhibition of paraoxonase activity. Thus, it seems that the type of oleoylated lipid inhibitor, preferential or selective, may be determined by the type of negatively charged moiety. In this regard, endogenous phospholipids, negatively charged, were tested for the preferential or selective inhibition of paraoxonase activity. However, negatively charged phospholipids, such as dioleoylphosphatidylglycerol, soybean phosphatidylinositol or dimyristoylphosphatidic acid, failed to inhibit PON1 activity significantly. Thus, negatively charged lysophospholipids were tested for the inhibition of PON1 activity. As shown in Fig. 4, monooleoyl-lysoPG expressed a significant inhibition of paraoxonase activity at micromolar levels; monooleoyl-lysoPG expressed ~16% and 24% inhibition at 3 and 10 μM, respectively. However, increasing monooleoyl-lysoPG concentration over 10 μM failed to further enhance the degree of inhibition, implying that the maximal inhibition of paraoxonase activity by lysoPG may be limited to ~24%. A similar but lower inhibition was also demonstrated by soybean lysophosphatidylinositol. In contrast, monomyristoyl-lyso phosphatidic acid (lysoPA), monooleoyl-lysophosphatidylcholine, or monopalmitoyl-lysophosphatidylserine showed no remarkable inhibition of paraoxonase activity. Thus, the structure of the head group seems to be crucial for the inhibition of paraoxonase activity by lysophospholipids. Separately, when lysophospholipids were tested for the inhibition of arylesterase activity of PON1, none of these lysophospholipids showed a remarkable inhibition. Thus, the inhibitory effect of lysophospholipids seems to be site selective.
phospholipids had a remarkable inhibition of arylesterase activity. Rather, the arylesterase activity was enhanced to some extent (~15%) by monooleoyl-lysoPG at micromolar levels (Fig. 4).

In an attempt to determine the property of the binding site responsible for the selective inhibition of paraoxonase activity, we examined the combinational effect of oleoylglycine and monooleoyl-lysoPG, both of which inhibited paraoxonase activity selectively. When the concentration of oleoylglycine was fixed at 3 μM and that of monooleoyl-lysoPG was varied (3–30 μM), the inhibitory effect of oleoylglycine on paraoxonase activity was not affected further by monooleoyl-lysoPG at any concentration (Fig. 5A). This indicates that total inhibition exerted by the combination does not exceed the maximal inhibition (27%) achieved by the respective lipid (Figs. 3, 4). Meanwhile, the arylesterase activity was further enhanced by the inclusion of monooleoyl-lysoPG in a concentration-dependent manner (up to 15%), reflecting the stimulatory effect of monooleoyl-lysoPG on arylesterase activity in the presence of oleoylglycine. Next, we examined the combinational effect of oleic acid and monooleoyl-lysoPG. For this purpose, the concentration of oleic acid was fixed at 0.3, 1.0,

**TABLE 3. Inhibition of PON1 activity by oleoyl derivatives**

<table>
<thead>
<tr>
<th>Oleic Acid Derivative</th>
<th>Concentration μM</th>
<th>Paraoxonase %</th>
<th>Arylesterase %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oleoylglycine</td>
<td>3</td>
<td>72.5 ± 1.5</td>
<td>100.2 ± 9.9</td>
</tr>
<tr>
<td>Oleamide</td>
<td>10</td>
<td>104.2 ± 2.8</td>
<td>103.8 ± 7.5</td>
</tr>
<tr>
<td>Oleoyl ethyl ester</td>
<td>3</td>
<td>82.9 ± 0.4</td>
<td>86.5 ± 2.5</td>
</tr>
<tr>
<td>Ricinoleic acid</td>
<td>10</td>
<td>75.5 ± 1.4</td>
<td>75.8 ± 4.7</td>
</tr>
<tr>
<td>Oleoyl cerebroside</td>
<td>10</td>
<td>99.6 ± 2.9</td>
<td>106.5 ± 5.2</td>
</tr>
<tr>
<td>Oleoyl sulfate</td>
<td>3</td>
<td>78.7 ± 4.5</td>
<td>93.8 ± 5.1</td>
</tr>
</tbody>
</table>

PON1 (1 arylesterase unit/ml) was incubated with phenyl acetate (1 mM) or paraoxon (1 mM) in the presence of oleoyl derivative (3 or 10 μM) in 0.5 ml of 50 mM Tris buffer (pH 7.4) containing 1 mM Ca²⁺ at 25°C, and the formation of product was monitored as described above.
Inhibition of paraoxonase activity by lysophospholipids. PON1 (1 arylesterase unit/ml) was incubated with paraoxon (1 mM) in the presence of each lysophospholipid (0.1–10 μM) in 0.5 ml of 50 mM Tris buffer (pH 7.4) containing 1 mM CaCl₂ at 25°C, and the formation of product was monitored as described for Fig. 1. Data are expressed as means ± SD of triplicate assays. Open triangles, monooleoyl-lysoPG; closed triangles, soybean lysophosphatidylglycerol (lysoPG); closed squares, monomyristoyl-lysoPA; closed circles, monooleoyl-lysophosphatidylcholine; open squares, monopalmitoyl-lysophosphatidylserine. Conc, concentration.

Fig. 4. Inhibition of paraoxonase activity by lysophospholipids. PON1 (1 arylesterase unit/ml) was incubated with paraoxon (1 mM) in the presence of each lysophospholipid (0.1–10 μM) in 0.5 ml of 50 mM Tris buffer (pH 7.4) containing 1 mM CaCl₂ at 25°C, and the formation of product was monitored as described for Fig. 1. Data are expressed as means ± SD of triplicate assays. Open triangles, monooleoyl-lysoPG; closed triangles, soybean lysophosphatidylglycerol (lysoPG); closed squares, monomyristoyl-lysoPA; closed circles, monooleoyl-lysophosphatidylcholine; open squares, monopalmitoyl-lysophosphatidylserine. Conc, concentration.

DISCUSSION

Despite reports (16, 23, 25–27, 44–46) on the preferential loss of paraoxon-hydrolyzing activity in serum of persons with oxidative stress-associated pathological signs, there have been no reports concerning causes for the preferential loss of paraoxonase activity. Even the substrate-dependent polymorphism could not account for the preferential reduction of serum paraoxonase activity in cardiovascular disease patients versus controls (21, 47). Our present results may provide one possible explanation for a preferential decrease of PON1 activity, which has been observed in the assay conditions routinely used. There are two possible mechanisms for the substrate-dependent alteration of PON1 activity: one is that PON1, modified by acquired factors such as reactive oxygen species or reactive aldehydes, may distinguish between two substrates, phenyl acetate and paraoxon; the other is that paraoxonase and arylesterase activities may be discriminated by endogenous inhibitors, which are present in the HDL particle or se-
rum sample. Earlier studies have shown that PON1 could be inactivated by endogenously generated oxidants (12, 14, 31, 32, 48). In the present study, however, the differential substrate recognition was not addressed by any PON1 molecules modified by the oxidants except HOCl, which caused a greater reduction of arylesterase activity than paraoxonase activity. Thus, it is unlikely that the inactivation of PON1 by endogenous oxidant species is related to the preferential loss of paraoxonase activity. This might be supported by additional findings that a parallel inactivation of both activities was observed after treatment with general modifiers of tryptophan (43), histidine (49), or cysteine residues (12, 14, 34), which had been observed to inactivate PON1. The same may also apply to the coincident inactivation of both activities by 4-hydroxy-2-nonenal, a lipid peroxidation product, as a modifier of cysteine and histidine residues (42, 50). Likewise, a parallel inactivation was also observed with methylglyoxal, a glycoxidation product, as a modifier of the lysine residue (40). Thus, it is more likely that the structural modification of PON1 by reactive oxidants or endogenously produced aldehydes is not related to the preferential inactivation of PON1 activity.

With regard to the inhibition of PON1, previous studies showed that the arylesterase or paraoxonase activity of PON1 was inhibited by divalent metal ions, phosphate compounds, or fatty acids (18, 34, 51, 52). However, there has been no report concerning the substrate-specific inhibition of PON1 activity. The present data demonstrate that monounsaturated fatty acids such as oleic acid at micromolar levels express a preferential inhibition of PON1 activity, either purified or HDL-associated. Even plasma paraoxonase activity was preferably inhibited by oleic acid. Thus, it is likely that monoenoic acids such as oleic acid or palmitoleic acid might be among the physiological factors responsible for the preferential inhibition of paraoxonase activity. Assuming that the serum concentration of monoenoic acids amounts to $100 \text{ mM}$ (53) and that the serum sample is normally diluted 20- to 50-fold in the assay system, the concentration of oleic acid in the routine assay condition may be sufficient to show a preferential inhibition of paraoxonase activity. Furthermore, the amount of nonesterified fatty acids in serum is enhanced in some diseases such as metabolic syndrome (54), in which lipase activities are enhanced. Because albumin is a tight binder of free fatty acids in serum, the serum level of available monoenoic acids would be affected by the saturation state of the fatty acid binding site in albumin or the distribution of nonesterified fatty acids. Noteworthy, the inhibitory effect of fatty acids on paraoxonase activity differed according to the structure of the fatty acid: linoleic acid at micromolar concentrations showed a competitive inhibition, whereas oleic acid showed a pattern similar to a noncompetitive inhibition. Additional studies using the dilution or gel filtration methods indicate that PON1 interacts reversibly with lipid compounds under the conditions used (data not shown). Because substrate-dependent inhibition of PON1 was exhibited by oleic acid, but not linoleic acid, it is supposed that the preferential inhibition of paraoxonase activity might be ascribed to binding to a peripheral site distinct from the active center. This might be analogous to the previous observation (34, 52) that the prevention by

Fig. 5. Combinational effect of two different lipids on PON1 activity. A: PON1 (1 arylesterase unit/ml) was incubated with phenyl acetate or paraoxon in the presence of oleoylglycine and monooleoyl-lysoPG in 0.5 ml of 50 mM Tris buffer (pH 7.4) containing 1 mM Ca$^{2+}$ as described for Fig. 4; the concentration of monooleoyl-lysoPG was varied (1–30 $\mu$M), whereas that of oleoylglycine was fixed at 3 $\mu$M. Closed squares, paraoxonase; closed triangles, arylesterase. B: PON1 (1 arylesterase unit/ml) was incubated with paraoxon in the presence of oleic acid and monooleoyl-lysoPG as described for A; the concentration of monooleoyl-lysoPG was varied (1–30 $\mu$M), whereas that of oleic acid was fixed at 0.3 $\mu$M (closed circles), 1 $\mu$M (closed squares), or 3 $\mu$M (closed triangles). C: PON1 was incubated with phenyl acetate in the presence of oleic acid and monooleoyl-lysoPG; the concentration of monooleoyl-lysoPG was varied (1–30 $\mu$M), whereas that of oleic acid was fixed at 0.3 $\mu$M (closed circles), 1 $\mu$M (closed squares), or 3 $\mu$M (closed triangles). Data are expressed as means ± SD of triplicate assays. Conc, concentration.
oleic acid of the oxidative inactivation of PON1 may be ascribed to its binding to a site separate from the active center. Nevertheless, it is not ruled out that some part of oleic acid inhibition may be attributable to its binding to the PON1 active center, because it also inhibited arylesterase activity. In this regard, oleic acid may be called a preferential inhibitor of paraoxonase activity. Thus, oleoylglycine may correspond to a selective inhibitor of paraoxonase activity, in that it inhibited only paraoxonase activity but not arylesterase activity.

A common property of preferential or selective inhibitors of paraoxonase activity is that their action may occur at the same peripheral site, which is fit for the accommodation of some negatively charged lipids. Consistent with this, negatively charged lysophospholipids such as monooleoyl-lysoPG or soybean phosphatidylinositol inhibited paraoxonase activity selectively. However, an oleoyl group in the structure of lysophospholipids is not necessary for the selective inhibition, because a similar degree of selective inhibition was expressed by both monooleoyl-lysoPG and monomyristoyl-lysoPG (data not shown). Instead, the structural importance of the head group is evident from the different inhibitory potency among negatively charged lysophospholipids; neither lysophosphatidylserine nor lysoPA had an inhibitory effect. In addition, dioleoylphosphatidylglycerol, a negatively charged phospholipid, failed to inhibit paraoxonase activity, suggesting that the peripheral binding site may exhibit a size limitation. The possible link of such an action of preferential or selective lipid inhibitors to micelle formation is excluded, because there is no relationship between their inhibitory potency and their critical micellar concentration (c.m.c.) values (55, 56); for example, linoleic acid, with a lower c.m.c. value, had no preferential inhibition of paraoxonase activity, in contrast to a preferential inhibition by oleic acid showing a higher c.m.c. value. Likewise, lysophosphatidylcholine or lysophosphatidylserine showed no inhibition despite their low c.m.c. values (57). Furthermore, the concentration-dependent inhibition by oleic acid or lysoPG showed a saturation pattern well below their c.m.c. values, a characteristic feature expressed by a monomer portion during micelle formation (55, 57). For the efficient inhibition of serum PON1 activity, the total amount of negatively charged lipids in serum may be important. According to previous reports (35, 36), the level of negatively charged lipids was increased in HDL of ATP-binding cassette transporter A1-deficient mice or Tangier disease patients. The lipolytic modification of lipoproteins by secretory phospholipase A2 (58) or platelet-activating factor acetylhydrolase (59) caused an increase in the negative charge of lipoprotein. Additionally, the negatively charged lysophospholipids are generated during the hydrolysis of membrane phospholipids by phospholipase A2 in activated cells (37). Thus, it seems that the level of negatively charged lipids may be enhanced in various oxidative stress-associated diseases. Although the physiological significance of the preferential inhibition of paraoxonase activity needs further study, the present data indicate that negatively charged lipids such as oleic acid or lysoPG may be responsible for the preferential reduction of paraoxonase activity.

Fig. 6. Inhibition of HDL-PON1 or plasma-PON1 by oleic acid, lysoPG, or their combination. A: HDL-PON1 (final concentration, 1 arylesterase unit/ml; 0.12 mg protein/ml) was incubated with paraoxon (closed symbols) or phenyl acetate (open symbols) in the presence of each lipid (1–30 μM) in 0.5 ml of 50 mM Tris buffer (pH 7.4) containing 1 mM Ca²⁺ at 25°C. Squares, oleic acid; triangles, monooleoyl-lysoPG. B: HDL-PON1 (1 arylesterase unit/ml) was incubated with paraoxon or phenyl acetate in the presence of the combination of oleic acid and monooleoyl-lysoPG; the concentration of monooleoyl-lysoPG was varied (1–30 μM), whereas that of oleic acid was fixed at 3 μM. Squares, paraoxonase; triangles, arylesterase. C: Plasma (10 μl), containing 1 arylesterase unit, was added to the above buffer containing paraoxon (closed symbols) or phenyl acetate (open symbols), and the hydrolysis of substrate was determined in the presence of oleic acid and monooleoyl-lysoPG as described for B. Squares, oleic acid; triangles, monooleoyl-lysoPG. Data are expressed as means ± SD of triplicate assays, presented as a percentage of control. Conc, concentration.
in the routine assay system. Further study on the relationship between the paraoxonase activity and the amount of negatively charged lipids in HDL particles of different oxidative status may reveal the biochemical significance of negatively charged lipids in HDLs subjected to oxidative stress. This work was financially supported by the Korean Research Foundation (KRF-2003-042-C20506).

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