Cellular Glutathione Peroxidase as a Predominant Scavenger of Hydroperoxyeicosatetraenoic Acids in Rabbit Alveolar Macrophages

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Received April 12, 1999; Accepted June 22, 1999

The cytosol of rabbit lung alveolar macrophages contains a high amount of peroxidase, which reduces 5-hydroperoxyeicosatetraenoic acid (5-HPTE) to 5-hydroxyeicosatetraenoic acid (5-HETE) in the presence of glutathione. This peroxidase was purified 69-fold to homogeneity with overall recovery of activity of 18.5%. The molecular mass of the enzyme was approximately 80 kDa by gel filtration, and emerged as a single band at 23.1 kDa under reducing condition by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The amino-terminal sequence of the purified peroxidase was completely identical to the sequence deduced from cellular glutathione peroxidase (cGPx) gene of rabbit liver. No other activity that reduces 5-HPTE to 5-HETE was observed during purification. These results suggest that cGPx plays an important role in metabolism of lipid hydroperoxides, especially in HPTE, alveolar macrophages.

Key words cellular glutathione peroxidase; hydroperoxyeicosatetraenoic acid; purification; glutathione-S-transferase

Alveolar macrophages represent a first line of defense in the host-defense systems of the lung and have a high capacity for metabolism of arachidonic acid in physiological and pathological states.1,2 Leukotrienes are major products of arachidonic acid metabolism by alveolar macrophages,3,4 and important mediators involved in both host defense mechanisms and in inflammation, because of their potent effects on cell migration, muscle contraction, vascular permeability, and the release of lysosomal enzymes.5–7 Leukotriene is synthesized by 5-lipoxygenase, which catalyzes a two-step reaction from arachidonic acid to the unstable epoxide leukotriene A4 via 5-hydroperoxyeicosatetraenoic acid (5-HPETE).8,9 There is evidence that fatty acid hydroperoxides might activate 5-lipoxygenase.10,11 A requirement for fatty acid hydroperoxides in the activation of lipoxygenase suggests that the activity of 5-lipoxygenase might be regulated by its own product, namely 5-HPETE.12 In addition, HPETE was reported to possess several biological functions, for example, an inhibition of the neuronal Na+, K+-ATPase13 and induction of the expression of the activating protein 1 (AP-1) in vascular smooth muscle cells.14 Furthermore, HPETEs have deleterious effect of peroxide, which acts as a toxin or a possible inducer of apoptosis.15

However, HPETE peroxidase, which is responsible for the reduction of intracellular HPETE, remains undefined. HPETE can be reduced to hydroxyeicosatetraenoic acid (HETE) by lipid peroxidases such as glutathione peroxidases (GPx).16–18 Based on the substrate specificity, two types of GPx are generally recognized; selenium-dependent GPx19 and selenium-independent enzymes.20 The former is classified into two groups, (i) tetrameric enzymes, such as cellular glutathione peroxidase (cGPx)21 and (ii) monomeric enzyme, phospholipid hydroperoxide glutathione peroxidase (PHGPx).22 The latter is attributed to glutathione-S-transferase (GST) isoenzymes acting on a variety of organic hydroperoxides.23 Recently, Bjornstedt et al.24 demonstrated that thioredoxin reductase also contributes to the reduction of lipid hydroperoxide and hydrogen peroxide. Mashima et al.25 isolated apolipoprotein-1 as a hydroperoxide-reducing protein from human plasma. These previous results indicate that several kinds of peroxidase responsible for the reduction of HPETE are present, however few report have been made on the intracellular distribution of HPETE peroxidases.

In the present study, the HPETE peroxidase activity of several kinds of mammalian cells, including inflammatory cells, were determined. HPETE peroxidase in alveolar macrophages was purified and identified as cGPx, which was exclusively expressed as HPETE peroxidase in macrophages.

MATERIALS AND METHODS

Reagents Glutathione reductase and Triton X-100 were from Boehringer Mannheim (Mannheim, Germany). Bovine hemoglobin, a reduced form of glutathione (GSH), 1-chloro-2,4-dinitrobenzen (CDNB), N-acetyl-l-cystein, and L(+)-ascorbic acid were purchased from Wako (Osaka, Japan). Arachidonic acid was from Funakoshi (Tokyo, Japan). 10-N-Methyl-carbamoylmethylene (MCDP) was a kind gift from Kyowa Medix (Shizuoka, Japan). DEAE-cellulose DE52 was from Whatman (Maidstone, U.K.). Glutathione-Sepharose 4B, activated thiol-Sepharose, and a Superose column were obtained from Pharmacia (Uppsala, Sweden). NADPH, and 3-(N-morpholino)propanesulfonic acid (MOPS) were from Sigma (St. Louis, MO, U.S.A.). Polyvinylidene difluoride (PVDF) membranes were from Millipore (Bedford, MA, U.S.A.). All other chemicals were of the highest grade available.

5-HPETE was prepared from arachidonate by potato lipoxigenase26 and purified by normal phase HPLC using an isocratic mobile phase of hexane, 2-propanol and acetic acid (97 : 3 : 0.01) as eluent on a Lichrosorb Si-60 column (Merck, Darmstadt, Germany).

Assays of Enzymatic Activities Rabbit lung alveolar macrophages were obtained as described previously.27 Mac-
rophages (5×10⁶ cells) were collected after washing with ice-cold phosphate-buffered saline and sonicated in 15 ml of 10 mM Tris–HCl buffer (pH 7.4) containing 1.5 M NaCl. The homogenate was centrifuged at 105,000×g for 60 min, and the supernatant (cytosol) was used for assays of enzymatic activity. The methylene blue method was applied for the standard assay of HPETE peroxidase. An aliquot of the supernatant was incubated for 10 min at 37°C in the reaction mixture containing 10 nmol 5-HPETE, 0.5 mM GSH, 5 mM EDTA, and 2.5% 2-propanol in 0.8 ml of 0.1 M Tris–HCl (pH 7.6). The reaction was stopped by the addition of methanol, and the total lipids were extracted by the method of Bligh and Dyer. The lipid extract was dried under a stream of nitrogen gas, and the residue was dissolved in 0.1 ml of 2-propanol. Then, 1 ml of MCDP reagent, containing 40 μM MCDP, 67.5 mg/ml bovine hemoglobin, and 0.1% Triton X-100 in 0.1 M MOPS pH 6.0, was added to the lipid extract and incubated at 30°C for 20 min. HPETE was determined by absorbance at 675 nm. This method detects both GSH-dependent and -independent reduction of HPETE. GST activity was measured spectrophotometrically by monitoring the formation of the conjugate of reduced GSH and CDNB at 340 nm.

Purification of HPETE Peroxidase Purification steps were carried out at 4°C unless otherwise stated. The cytosol of rabbit lung alveolar macrophages was prepared as described above, and dialyzed overnight against 100 mM phosphate buffer (pH 7.4). The dialyzed supernatant was applied to a DEAE-cellulose column (1.0×20 cm) equilibrated in 10 mM phosphate buffer (pH 7.4). A linear gradient of increasing NaCl to 0.6 M was applied at a flow rate of 0.5 ml/min. The fractions containing the HPETE peroxidase activity were pooled and dialyzed overnight against 50 mM Tris–HCl buffer (pH 8.0).

In the next step, the pooled fractions were applied to a GSH-Sepharose 4B column (1.0×5 cm) equilibrated with the same buffer. The activity of HPETE peroxidase was recovered in flow-through fractions with 50 mM Tris–HCl buffer (pH 8.0). The flow rate throughout was 0.5 ml/min. The flow-through fractions were applied directly to a hydroxyapatite column (1.0×20 cm) equilibrated with 10 mM phosphate buffer (pH 6.7). A linear gradient of increasing phosphate to 0.2 M was applied at 0.2 ml/min and then the column was washed with 0.3 M phosphate buffer (pH 6.7).

After concentrating with Centricon-30 (Amicon, Beverly, MA, U.S.A.), the active fractions were diluted with ten volumes of 1.5 M potassium phosphate (pH 7.6), and loaded on an activated thiol-Sepharose column. Chromatography was carried out as described by Re et al., with slight modification, namely, the use of 10 mM GSH as ligand. The flow rate throughout was 0.1 ml/min. The above sample was applied to the column (1.0×20 cm) equilibrated with 1.5 M potassium phosphate buffer (pH 7.6), and then eluted with 0.5 M potassium phosphate buffer (pH 7.6). After washing, most of the HPETE peroxidase activity was eluted with 10 mM GSH in the buffer. The fractions containing the HPETE peroxidase activity were again concentrated with Centrinon-30, and dialyzed overnight against distilled water for amino-terminal sequencing.

Amino-Terminal Sequencing of HPETE Peroxidase Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was run under a reducing condition according to Laemmli in 12.5% acrylamide gel. Electrophoretic onto a PVDF membrane was performed as described previously. After electrophoretic, the amino-terminal sequence of HPETE peroxidase was cut from membrane and determined with a model 473A sequencer (Applied Biosystems, Foster City, CA, U.S.A.).

Quantitation of Proteins Concentrations of proteins were determined with BCA protein assay reagent (Pierce, Rockford, IL, U.S.A.) with bovine serum albumin as the standard.

RESULTS AND DISCUSSION

Activities of HPETE peroxidase, which reduced 5-HPETE, were measured in the cytosol prepared from macrophages and neutrophils (Table 1). The activities of alveolar macrophages of rabbit and rat were higher than those of neutrophils and rat basophilic leukemia cells (RBL-1 and RBL-2H3). Metabolites of 5-HPETE were identified by the normal phase HPLC after the incubation of 5-HPETE with the cytosol of alveolar macrophages (Fig. 1). The HPETE peroxidase activity in the cytosol is known to require GSH or other thiols for its activity. GSH was absolutely required for the full activation of this peroxidase of rabbit alveolar macrophages, however, other reducing agents such as cysteine were able to activate the peroxidase. The results of enzyme assay are presented in Table 1.

### Table 1. HPETE Peroxidase Activity Observed in the Cytosol Fraction of Inflammatory and Related Cells

<table>
<thead>
<tr>
<th>HPETE Peroxidase (units/mg protein)</th>
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<tbody>
<tr>
<td>Rabbit lung alveolar macrophage</td>
<td>4.25</td>
</tr>
<tr>
<td>Rat lung alveolar macrophage</td>
<td>2.50</td>
</tr>
<tr>
<td>Rat peritoneal neutrophil</td>
<td>1.63</td>
</tr>
<tr>
<td>RBL-1</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>RBL-2H3</td>
<td>0.08</td>
</tr>
</tbody>
</table>

1 unit is defined as 1 pmol HPETE reduced/min.
The cytosol from rabbit alveolar macrophages was prepared as described in Materials and Methods. (A) The HPETE peroxidase activity was measured with or without GSH at the concentration of 0.5 mM. (B) Effect of several reducing reagents on the HPETE peroxidase activity. All of reagents were added at the concentration of 0.5 mM. GSH, reduced form of GSH; Cys, cysteine; NAC, N-acetylcysteine; VC, vitamin C.

The purification was performed from the cytosol of rabbit alveolar macrophages since high activity of GSH-dependent peroxidase was found in the cell (Table 1). Cytosol was applied on a GSH-Sepharose column (Fig. 3). Activity of HPETE peroxidase did not bind to the column and was recovered in the flow-through fraction. GST should tightly bind to this column and be eluted from the column in the presence of GSH. No significant peak of the peroxidase was observed by the elution with the mobile phase containing 10 mM of GSH, suggesting that HPETE peroxidase might be GPx, but not GST. The HPETE peroxidase activity was purified through a 4-step purification (Table 2), and the activity was observed as a single peak through these procedures. The overall purification gave a 69-fold purification of HPETE peroxidase.

The molecular weight of the purified HPETE peroxidase, obtained by non-denatured gel filtration on a Superose column, was approximately 80000 daltons (Fig. 4A). The purified preparation emerged as a single band at 23.1 kDa, when SDS-PAGE was run under a reducing condition (Fig. 4B).

The yield of each purification step described in Table 2 is within normal range, and thus no remarkable evidence of activation or inactivation of cGPx was suggested throughout the purification. The Charcot-Leyden crystal protein, which possesses lyso phospholipase activity, was also purified with only about ten-fold purification. This protein comprises an estimated 7% to 10% of total eosinophil protein. These findings indicate that cGPx is enriched in alveolar macrophages.

Rabbit alveolar macrophages have a high capacity for metabolism of arachidonic acid and produce leukotriene B4 as the major product. 5-HETE is the critical intermediate in the biosynthesis of leukotrienes, in addition to being a biologically active lipid hydroperoxide. In this study, we showed that cGPx was the predominant enzyme reducing 5-HETE.
in rabbit alveolar macrophages, which suggests the possible role of this enzyme in the regulation of lipoxygenase activity in alveolar macrophages. Alveolar macrophages are directly exposed to a high concentration of inspired O₂ and scavenge inhaled pathogens and elicit immune responses. 43 Hyperoxic exposure of these host defense reactions causes production of reactive oxygen species and lipid peroxidation in lung. 44,45 Therefore, alveolar macrophages are exposed to oxidative stress, and the antioxidant enzymes must play a crucial role in the function. Antioxidant enzymes include GPx, catalase and superoxide dismutase (SOD). In alveolar macrophages exposed to hyperoxia, GPx was enhanced significantly, whereas total SOD activities showed no significant difference and the cell content of reduced GSH and the activity of catalase were lower in hyperoxia than in normoxia. 46 The predominant expression of this enzyme suggests that cGPx also plays a major role in the cell defense system of alveolar macrophages.

Acknowledgement  The authors thank Akiko Maru, Miyuki Murata and Yuki Shirai for their expert technical assistance.

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