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Identification of a lipoxygenase inhibitor in A431 cells as a phospholipid hydroperoxide glutathione peroxidase

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Abstract An endogenous lipoxygenase inhibitor, purified from the cytosol of human epidermoid carcinoma A431 cells, was analyzed by N-terminal microsequencing and mass spectrometric analysis. The inhibitor was purified by SDS-PAGE, then subjected to in-gel CNBr cleavage and trypsin digestion. The N-terminal sequence data obtained from a 6–8 kDa band of in-gel CNBr cleavage and the three isolated peptides of in-gel trypsin digestion, and the C-terminal peptide sequence from matrix-assisted laser desorption ionization mass spectrometry matched the sequence of human phospholipid hydroperoxide glutathione peroxidase. The purified inhibitor exhibited peroxidase activity using phosphatidylcholine hydroperoxides as the substrate. We therefore concluded that the lipoxygenase inhibitor present in A431 cells was a phospholipid hydroperoxide glutathione peroxidase.

Key words: Lipoxygenase inhibitor; Microsequencing; PHGPx; A431 cell

1. Introduction

Oxidative stress is associated with a disturbance in the reactive oxygen species-antioxidant balance in favor of the former [1,2]. Many of the reactive oxygen species are oxygen-free radicals which are involved in the oxidative damage in a number of physiological and pathophysiological phenomena and processes as diverse as inflammation, aging, carcinogenesis, drug action and drug toxicity. The reactive oxygen species may be formed from polyunsaturated fatty acids either by an enzymatic or by a nonenzymatic oxygenation. Reduction of the reactive oxygen species can be achieved either by the inhibition of lipid oxygenation enzymes or by the interception of free radicals by antioxidants and some antioxidation enzymes such as superoxide dismutase, catalase and glutathione peroxidase.

Lipoxygenase and fatty acid cyclooxygenases are the major enzymes involved in the metabolism of polyunsaturated fatty acids including arachidonic acid [3]. In studying the regulation of arachidonate metabolism in human epidermoid carcinoma A431 cells, we previously reported a putative inhibitor of 12-lipoxygenase, which masked the biosynthesis of 12(*S*)-hydroxyeicosatetraenoic acid (12(*S*)-HETE) in intact cells [4]. A similar observation was also reported in primary culture of ovine tracheal epithelial cells [5]. The putative inhibitor in A431 cells was purified to a 22 kDa protein whose inhibitory activity was regulated by the cellular reduction-oxidation (redox) conditions [6]. In addition to 12-lipoxygenase, the inhibitor also inhibited the activities of 5-lipoxygenase and fatty acid cyclooxygenases in a cell-free system [6]. In the present study, we performed the sequence analysis of the purified inhibitor protein by a high sensitivity microsequencer and mass spectrometer, and found that the sequence data, obtained from a band of in-gel CNBr cleavage and the three peptides isolated from in-gel trypsin digestion, and the C-terminal peptide sequence matched the cDNA sequence of human phospholipid hydroperoxide glutathione peroxidase (PHGPx). The purified inhibitor exhibited peroxidase activity using phosphatidylcholine hydroperoxides as the substrate. We therefore concluded that the lipoxygenase inhibitor present in human epidermoid carcinoma A431 cells is PHGPx.

2. Materials and methods

2.1. Materials

Pre-made polyacrylamide slab gels (8×10 cm, 16 or 18%) using Tris-glycine gradient system were purchased from Novex Inc. (San Diego, CA, USA). CNBr, phosphatidylcholine, soybean lipoxygenase, GSH, NADPH, glutathione reductase and purified glutathione peroxidase (GSHPx-1) from bovine erythrocytes were from Sigma Chemical Co (St. Louis, MO, USA). Sequencing grade trypsin was supplied by Boehringer Mannheim (Mannheim, Germany).

2.2. Purification of inhibitor

The inhibitor in cytosol of A431 cells was purified by a series of column chromatographies using CM Sephadex C-50, Sephadex G-100 SF and Mono P columns according to the methods described previously [6]. Elution of the inhibitor in the purification chromatographies was monitored by the 12-lipoxygenase activity assay [6].

2.3. SDS-PAGE and electroblotting

Sample was separated by reducing SDS-PAGE using 16% slab gels with an operation voltage set at 125 V for 90 min. Proteins or peptides separated on the gel were immediately transferred onto a PVDF membrane by an electroblot apparatus. Protein band on the blot, visualized by Coomassie blue staining, was cut out for direct N-terminal sequence analysis. For in-gel digestion experiments, samples were run on reducing SDS-PAGE as described and the Coomassie blue-stained protein bands were excised and washed with distilled water and placed in a 1.5 ml centrifuge vial for subsequent treatment.

2.4. In-gel trypsin digestion and CNBr cleavage

Procedures for in-gel trypsin digestion were similar to those reported by Merewether et al. [7] and the drying step for the gel bands was performed according to the methods described by Hellman et al. [8]. Following the digestion, the digest was extracted twice with 150 μl of 50% acetonitrile in 0.1% trifluoroacetic acid (TFA). Each extraction proceeded about 30 min with gentle mixing. The combined fraction was partially dried for peptide mapping.

For in-gel CNBr cleavage, the excised protein band was allowed to dry under conditions similar to those of in-gel trypsin digestion. The dried gel was then rehydrated with 10 μl of 70% formic acid containing CNBr at a concentration of 200 mg/ml. The partially rehydrated gel was then transferred to a 0.5 ml vial. Approximately another 25 μl of the above-mentioned CNBr solution was added to cover the gel. The sample was then incubated at 25°C for 20 h. The digest was then extracted twice with 150 μl of 50% acetonitrile in 0.1% TFA. Each extraction proceeded about 30 min with gentle mixing. The combined

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fraction was completely dried and reconstituted into 30 μl of SDS-PAGE sample buffer. Electrophoresis was performed using 18% slab mini-gel and the separated peptides on the gel were transferred onto PVDF membrane for N-terminal sequence analysis.

2.5. Micro HPLC peptide mapping

The in-gel tryptic digestion sample was separated by reverse-phase HPLC using a micro-liquid chromatograph (Model 1090, Hewlett Parkard, CA, USA) equipped with a diode array detector and a PC-based workstation for data processing. A microbore C4 reverse-phase column (1.0 mm×10 cm, Synchrom, CA, USA) was equilibrated with 98% mobile phase A/2% mobile phase B at a flow rate of 0.1 ml/min. HPLC mobile phase A consisted of 0.1% TFA in HPLC-grade water and mobile phase B was 0.1% TFA in 90% HPLC-grade acetonitrite. The separation of the peptide mixture was performed by a 70 min linear gradient from the initial equilibration condition (see above) to 40% mobile phase A/60% mobile phase B at a flow rate of 0.1 ml/min. The peptide peaks were detected by UV absorbance at both 215 and 280 nm. Peptide fractions were manually collected for further analysis.

2.6. Microsequencing and mass spectrometric analysis

N-terminal amino acid sequence analysis was performed on either an automatic Procise sequencer or a modified high sensitivity Procise sequencer (Applied Biosystem Inc., Foster City, CA, USA) using sequencing programs recommended by the vendor with slight modification. The on-line PTH amino acid separation was performed using either a narrow bore C18 column (2.1 mm×25 cm) for the Procise sequencer or a microbore C18 column (0.8 mm×25 cm) for the high sensitivity modified Procise sequencer under conditions recommended by the vendor. In the latter case, the detection and quantification of PTH amino acids can be obtained at 50 femtomole level using microbore HPLC separation.

Matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) of peptides was performed on a Kompact MALDI IV mass spectrometer (Kratos Analytical, Ramsey, NJ, USA) equipped with a curved field reflectron and a precursor ion gate according to Jones et al. [9]. Aliquots of sample (0.7 μl) and matrix solution (0.4 μl of α-cyano-4-hydroxycinnamic acid; 33 mM in acetonitrile/methanol/water, 5:3:2 v/v) were properly mixed and spotted onto the probe slide. Following an air-dry step, the sample was ready for analysis. Spectra were obtained in either the linear mode, at an acceleration voltage of 20 kV, or the reflector mode, with the linear voltage at 20 kV and the ion mirror set at 21 kV. The MALDI IV laser power can be adjusted from a relative scale (from 0 to 180) set by the manufacturer, in which the threshold of detection ranged from 45 to 65 in the linear mode and from 85 to 105 in the reflectron mode for postsource decay of a selected molecular ion. The naming system of fragment ions uses the traditional a, b, and c series of ions if the charge is retained on the N-terminus as well as x, y, and z series of ions if the charge is on the C-terminus, which are followed by a subscript number indicating the position of the fragmentation in the peptide backbone [10].

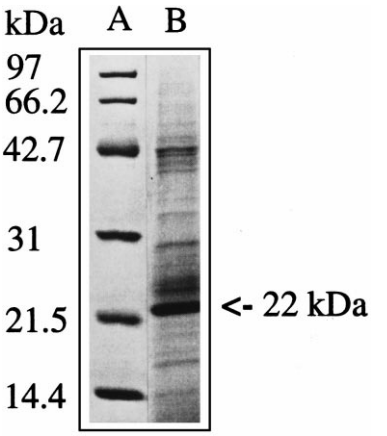

Fig. 1. SDS-PAGE and electroblotting of purified protein. Sample (3 μg of protein) purified by a series of column chromatographies including CM Sephadex C-50, Sephadex G-100 SF and Mono P was analyzed by 16% SDS-PAGE, and then transferred onto a PVDF membrane. Proteins were visualized by Coomassie blue staining. Positions of molecular mass markers are given on lane A and the position of the purified 22 kDa protein is given on lane B.

2.7. Activity assay of PHGPx

Phospholipid hydroperoxides were prepared by incubating phosphatidylcholine with soybean lipoxygenase, followed by separation with a Sep-Pak C_{18} cartridge according to the methods described by Maiorino et al. [11]. PHGPx activity was measured by recording the disappearance of absorbance of NADPH at 340 nm in the presence of GSH, glutathione reductase and phosphatidylcholine hydroperoxides as previously described [11]. Briefly, the reaction mixture in a final volume of 1 ml contained 3 mM GSH, 0.1 mM NADPH, 1.2 units of glutathione reductase, 5 mM EDTA, 0.12% (v/v) Triton X-100 and sample of PHGPx in 100 mM Tris buffer (pH 7.4). The enzymatic reaction was started by the addition of 10∼30 μM phosphatidylcholine hydroperoxides. One unit of activity was defined by the reduction of 1 nmol/min at 37°C.

3. Results

3.1. N-terminal block of the 22 kDa protein

When compared to the standard proteins, the Coomassie blue-stained intensity of a 22 kDa protein band blotted onto PVDF membrane shown in Fig. 1, suggested that the sample amount is between 0.5–1.0 μg (or 20–45 pmol range). This PVDF blot was cut out and subjected to high sensitivity mi-

Table 1
Sequence yield of an in-gel CNBr-digested peptide

Cycle	Amino acid identified	
	Major sequence (fmol recovered)[a]	Secondary sequence[b]
1	Lys (320)	Val
2	Ile (200)	Asn
3	Gln (200)	Leu
4	Pro (120)	Asp
5	Lys (100)	–
6	Gly (100)	Tyr
7	Lys (80)	Arg
8	Gly (80)	Gly
9	Ile (70)	Phe
10	Leu (60)	Val

A 6–8 kDa band, obtained from in-gel CNBr digestion of 22 kDa protein on PVDF membrane was analyzed by a high sensitivity protein sequencer (see Section 2).
[a]Sequence yields in femtomole were background corrected.
[b]The yield was not calculated.

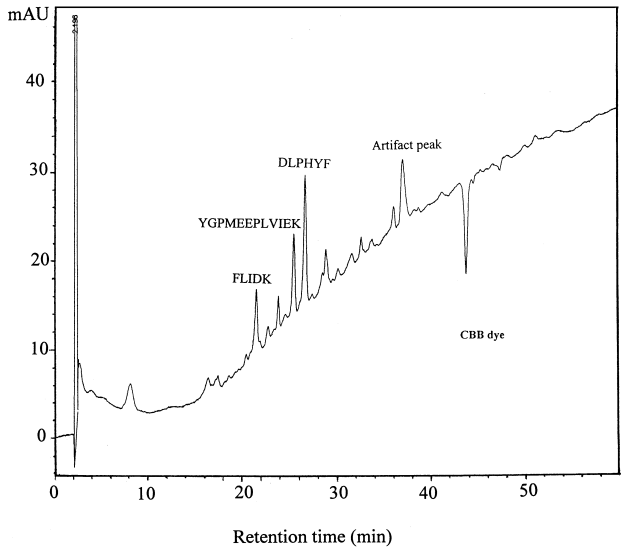

Fig. 2. Micro HPLC peptide mapping. The in-gel trypsin-digestive sample was separated by reverse-phase HPLC using a micro-liquid chromatography [7]. The peptide fractions were collected for micro-sequencing. The sequence data of three peptides are indicated respectively. CBB dye indicates the remaining Commassie blue dye.

crosequencer analysis. However, no clear signal above 0.2 pmol level at the first sequencing cycle could be detected and no sequence could be identified in the following cycles (data not shown). This low sequencing yield indicated this protein may be N-terminally blocked. Another 22 kDa protein blot was subsequently subjected to CNBr cleavage and sequencing of the cleaved products. Several clear mixed signals at 1–2 pmol levels were detected, clearly indicating that the protein was indeed blocked at its N-terminus. The nature of the blockage is unknown.

3.2. *In-gel CNBr cleavage, isolation and sequence analysis of CNBr peptides*

In a separate experiment, a second sample was also subjected to SDS-PAGE. The 22 kDa protein described above was isolated by cutting out two gel bands. These two bands were combined and subjected to in-gel CNBr digestion. The CNBr digested peptides were separated on a 18% reducing SDS-PAGE gel then blotted onto PVDF. The peptides near 22 kDa, 10 kDa, 6–8 kDa were barely visible after staining (data not shown). The 6–8 kDa band was then sequenced by high sensitivity sequencer, a major sequence and a minor sequence were detected with sequencing yield listed in Table 1.

3.3. *In-gel trypsin digestion, peptide mapping and sequence analysis*

Fig. 2 shows a peptide map from micro HPLC of a tryptic peptide mixture obtained from in-gel trypsin digestion of two 22 kDa protein bands. Several peaks were detected at 215 nm. A negative peak near 44 min retention time was the remaining Coomassie blue dye. About one third of the fraction from four peaks was directly sequenced. Three peaks with retention times at 21 min, 25 min and 27 min showed very clean sequences as indicated in Fig. 2. One peak with retention time at 38 min was detected to have no sequence signals and no mass spectrum, suggesting it might be an artifact peak. Several other small peaks were not analyzed because some of these peaks may be related to trypsin peptides derived from trypsin autolysis during digestion.

3.4. *Mass spectrometric analysis*

The obtained amino acid sequence of a tryptic peptide at 27 min retention time did not contain either a lysine or arginine at the C-terminus, indicating that it may be a C-terminal peptide of the protein. This was further confirmed by MALDI-MS analysis shown in Fig. 3. By MALDI-MS analysis in a linear mode, the peptide was determined to have a protonated, averaged mass of 792.2 dalton (Fig. 3A) which matched the expected mass of 791.9 dalton. Following post-source decay of the selected molecular ion (i.e. 792.2 dalton) in reflectron mode, a number of fragments were generated and can be assigned to various b and y series of sequence ions as well as several internal fragment ions with a standard error of ± 0.3 dalton (Fig. 3B). The determined sequence (Fig. 3C) completely matched the sequence obtained from automatic Edman degradation described above.

3.5. *Sequence identity to human PHGPx*

With a BLASTP search of the SWISS-PROT database, the sequence data, obtained from the 6–8 kDa band of in-gel CNBr cleavage (Table 1) and the three isolated peptides of

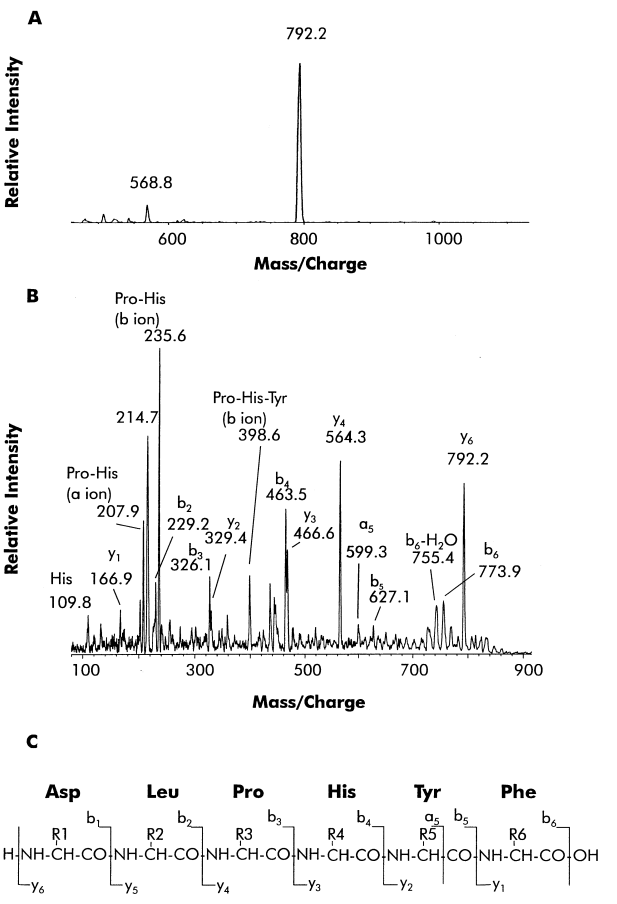

Fig. 3. MALDI-MS analysis of tryptic peptide at 27 min retention time. A: MALDI-MS analysis in linear mode for molecular mass measurement of the peptide (see Fig. 2). B: MALDI-MS postsource decay for fragmentation of the 792.2 dalton molecular ion. The assigned b, and y fragment ions, and a_5 ion, and internal fragments are indicated. C: The assigned peptide sequence using the obtained fragment ions shown in panel B.

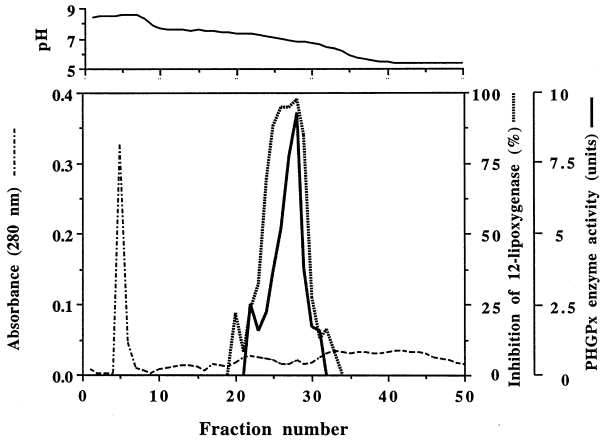

Fig. 4. PHGPx activity of purified inhibitor. The concentrated sample (997 μg of protein) obtained from column chromatographies of CM Sephadex C-50 and Sephadex G-100 SF was applied to a column (HP 5/20) of Mono P equilibrated with running buffer (50 mM ammonium acetate/1 mM DTT, pH 8). The proteins were eluted with elution buffer (Polybuffer 96 with 1:20 dilution in the presence of 1 mM DTT, pH 5.3). The flow rate was 0.5 ml/min. Each fraction, with a volume of 1 ml, was collected, and 30 μl of each fraction was used in the 12-lipoxygenase activity inhibitory assay [6] and 100 μl of each fraction was used in the PHGPx activity assay, respectively.

in-gel trypsin digestion (Fig. 2), matched the protein sequence of human PHGPx [12]. The obtained C-terminal peptide sequence also matched the C-terminus of human PHGPx, indicating that both proteins contain an identical C-terminus.

3.6. PHGPx activity in purified inhibitor

The PHGPx activity of the lipoxygenase inhibitor obtained from a series of column chromatographies including CM Sephadex G-50, Sephadex G-100 SF and Mono P was studied. The inhibitor was purified to 22 kDa by this series of column chromatographies [6]. The purified inhibitor exhibited PHGPx activity in accordance with its 12-lipoxygenase inhibitory activity (Fig. 4). At this purification stage, PHGPx accounted for 90–100% of the proteins on SDS-PAGE [6], and the specific activity of PHGPx was approximately 6.2 μmol/min/mg at 37°C. We have previously reported that the 12-lipoxygenase inhibitory activity of inhibitor was inactivated by iodoacetate [6]. Treatment of the purified inhibitor with 2 mM iodoacetate for 5 min also completely abolished the PHGPx activity (data not shown). Under the present assay conditions, no PHGPx activity was observed with 10 μg of purified glutathione peroxidase (GSHPx-1) of bovine erythrocytes (data not shown), indicating that the peroxidase assay was specific to PHGPx.

4. Discussion

Two pieces of evidence were obtained from this study indicating that the 22 kDa lipoxygenase inhibitor in A431 cells was a human PHGPx. Firstly, the peptides isolated from the in-gel CNBr cleavage and trypsin digestion of the 22 kDa inhibitor so far matched the cDNA sequence of human PHGPx cloned from human testis cDNA library [12]. In addition, the obtained C-terminal peptide sequence from MALDI-MS also matched the C-terminus of human PHGPx. In the N-terminal sequence analysis, the N-terminal blockage was observed in the 22 kDa inhibitor, which was also reported in porcine PHGPx [13]. Secondly, the purified lipoxygenase inhibitor exhibited PHGPx activity when phosphatidylcholine hydroperoxides were used as the substrate. Treatment of the purified protein with iodoacetate completely abolished the phospholipid hydroperoxide-reduced activity.

PHGPx, a selenium-dependent glutathione peroxidase, belongs to glutathione peroxidase family which consists of five isozymes. They are: (a) the classical cellular glutathione peroxidase GSHPx-1 [14], (b) the phospholipid hydroperoxide glutathione peroxidase PHGPx [11], (c) the plasma glutathione peroxidase GSHPx-P [15], (d) the gastrointestinal glutathione peroxidase GSHPx-GI [16], and (e) the epididymal glutathione peroxidase GSHPx-5 [17]. Among these isozymes, PHGPx is unique in the substrate specificity because it can interact with lipophilic substrates including the peroxidized phospholipids and cholesterol and reduce these hydroperoxide to hydroxide compounds [18,19]. Several pieces of evidence have been provided to indicate the inhibition of lipoxygenases and fatty acid cyclooxygenase activities by PHGPx. The present results together with our previous results [6] clearly indicate that PHGPx inhibits the enzymic activities of 12-lipoxygenase, 5-lipoxygenase and cyclooxygenases in a cell-free system. Schnurr et al. [20] recently reported that PHGPx inhibits 15-lipoxygenase activity in vitro and indicated that the inhibition is probably due to the reduction of hydroperoxy lipids necessary as an activator for the 15-lipoxygenase activity. The in vitro activation of the fatty acid cyclooxygenase [21] and the soybean lipoxygenase [22–24] by fatty acid hydroperoxides has been studied in detail. Although limited information is available on the mechanism of hydroperoxide activation of mammalian lipoxygenases, reduction of arachidonate hydroperoxides, which are the initial products of arachidonic acid catalyzed by lipoxygenases and fatty acid cyclooxygenase, might be a common mechanism for the inhibition of enzyme activity by PHGPx.

In A431 cells with epidermal growth factor-induced expression of 12-lipoxygenase and fatty acid cyclooxygenase, when challenged with calcium ionophore A23187, prostaglandin E_2 formation increased by 3-fold but 12(*S*)-HETE remained unchanged [4]. These results suggest that 12-lipoxygenase, but not fatty acid cyclooxygenase, was down-regulated by the inhibitor, PHGPx, in intact cells. Localization of fatty acid cyclooxygenase in the lumina of endoplasmic reticulum and nuclear envelope [25–28] may prevent fatty acid cyclooxygenase from interacting with the cytosolic PHGPx in intact A431 cells. In another intact cell system, PHGPx was also reported to be the primary enzyme for the inhibitory regulation of 5-lipoxygenase activity in RBL-1 cells [29]. These results provide a significant evidence indicating that the activity of lipoxygenases can be regulated by the presence of PHGPx in intact cells. Brigelius-Floh et al. [30] recently reported that overexpression of PHGPx in a human endothelial cell line inhibits interleukin-1-induced nuclear factor kappaB activation. However, information on the physiological role of PHGPx is still very limited and needs further studies.

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