Glutathione peroxidase-1 but not -4 is involved in the regulation of cellular 5-lipoxygenase activity in monocytic cells

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In contrast to neutrophils or B-lymphocytes, cells of the monocytic lineage like rat macrophages, human peripheral blood monocytes and Mono Mac 6 cells contain a strong inhibitor of 5-lipoxygenase (5-LO) activity, which scavenges hydroperoxides and inhibits 5-LO activity in broken-cell preparations in the absence of exogenously added thiols. Chromatographic purification of the inhibitor from the human monocytic cell line Mono Mac 6 and amino acid sequence analysis revealed that the inhibitory factor is glutathione peroxidase-1 (GPx-1). In contrast to the peroxidase activity of GPx-1, 5-LO inhibition by GPx-1 was supported by β-mercaptoethanol and there was no absolute requirement for millimolar concentrations of glutathione or dithiothreitol. These cofactor characteristics suggest that both activities address distinct catalytic properties of GPx-1. 5-LO inhibition by GPx-1 was not due to direct GPx–5-LO protein–protein interactions, since GPx-1 did not bind to immobilized 5-LO. Interestingly, 5-LO derived from granulocytes was significantly more resistant against GPx-1 inhibition than B-lymphocytic 5-LO, which correlates with the respective cellular 5-LO activities. In summary, the data suggest that, in addition to previously reported phospholipid hydroperoxide glutathione peroxidase (GPx-4), GPx-1 is an efficient inhibitor of 5-LO even at low thiol concentrations, and is involved in the regulation of cellular 5-LO activity in various cell types.

Key words: inflammation, leukotriene, B-lymphocyte, Mono Mac 6 cell.

INTRODUCTION

Leukotrienes are mediators of inflammatory responses that can be formed in a variety of cells of myeloid origin like granulocytes, monocytes/macrophages, mast cells and dendritic cells and in B-lymphocytic cells after stimulation. 5-Lipoxygenase (5-LO) catalyses the biosynthesis of leukotriene A_4 from arachidonic acid, which can be subsequently transformed into leukotriene B_4 or leukotriene C_4 by leukotriene A_4 hydrolase or leukotriene C_4 synthases, respectively [1,2]. In resting neutrophils, Mono Mac 6 cells and HL-60 cells, 5-LO is localized in the cytosol and it was shown for neutrophils that 5-LO translocates to the nuclear membrane after cell stimulation where it interacts with FLAP (5-LO-activating protein) [3–5]. The cellular capacity for leukotriene biosynthesis is upregulated during myeloid cell maturation. It has been shown that 5-LO mRNA and protein expression increases during differentiation *in vitro* of the human promyelocytic cell line HL-60 by DMSO or 1,25-dihydroxyvitamin D_3 (VD3) [5,6]. When expression of 5-LO protein and activity was studied in detail, it became evident that DMSO differentiation of HL-60 cells or differentiation of Mono Mac 6 cells with either transforming growth factor β1 (TGFβ) or VD3 induces expression of 5-LO protein but no or only low cellular 5-LO activity [7,8]. In HL-60 cells, addition of TGFβ is required for upregulation of cellular 5-LO activity during DMSO-induced differentiation [9]. Moreover, we could show that VD3 and TGFβ upregulate cellular leukotriene synthesis in DMSO-differentiated HL-60 cells by induction of a peroxidase-insensitive 5-LO catalytic activity [10]. B-lymphocytes and B-lymphocytic cell lines constitutively express 5-LO protein but there is no detectable enzyme activity after stimulation with ionophore and arachidonic acid. Recently, several groups including ours showed that the phospholipid hydroperoxide glutathione peroxidase (GPx-4) is responsible for the suppression of 5-LO activity in a variety of cell types like B-lymphocytes, A431 cells, RBL-2H3 cells and immature HL-60 cells [10–12]. In addition to their function in the regulation of cellular 5-LO activity, glutathione peroxidases (GPxs) also play an important role in the 5-LO inhibition by non-redox-type inhibitors. Recently, it was shown that the inhibitory potency of several non-redox-type 5-LO inhibitors strongly depends on GPxs and that increased peroxide levels lead to impaired efficacy of inhibitors like ZM 230487 or L-739,010 [13].

In contrast to neutrophils, cytosol from rat alveolar and peritoneal macrophages and from human peripheral blood monocytes contains a strong inhibitor of 5-LO activity [14,15]. Initial characterization of the inhibitor demonstrated that it is a protein that scavenges hydroperoxides. Accordingly, we observed a strong 5-LO inhibitory activity in the cytosol of Mono Mac 6 cells which inhibited 5-LO activity in broken-cell preparations in the absence of exogenously added thiols, as reported for various monocytic cell types [15]. Here, we show that the endogenous inhibitor is glutathione peroxidase-1 (GPx-1) and that this enzyme is a very effective suppressor of 5-LO activity in intact cells and in broken-cell preparations even at low concentrations of thiols.

Abbreviations used: 5-LO, 5-lipoxygenase; TGFβ, transforming growth factor β1; VD3, 1,25-dihydroxyvitamin D_3; DTT, dithiothreitol; GPx, glutathione peroxidase; GPx-1, glutathione peroxidase-1; GPx-4, phospholipid hydroperoxide glutathione peroxidase; FCS, fetal calf serum; MALDI-TOF, matrix-assisted laser-desorption ionization–time-of-flight; 5-HPETE, 5-hydroperoxyeicosatraenoic acid; TRAP-1, TGFβ-receptor-I-associated protein-1.

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MATERIALS AND METHODS

Materials

RPMI 1640 medium was from Gibco and fetal calf serum (FCS) was obtained from Boehringer Mannheim. Insulin was a gift from Aventis (Frankfurt, Germany). VD3 was kindly provided by Dr H. Wiesinger (Schering AG, Berlin, Germany). Human TGFβ was purified from outdated platelets according to [16]. HPLC solvents and β-mercaptoethanol were from Merck (Darmstadt, Germany). Dithiothreitol (DTT), bovine GPx-1 (G6137), human GPx-1 (G4013), glutathione reductase and selenium dioxide were purchased from Sigma (Deisenhofen, Germany). GSH and NADPH were obtained from Serva (Heidelberg, Germany). Polyclonal anti-human GPx-1/catalase antibody was purchased from Dunn Labortechnik (Asbach, Germany).

Cells

Mono Mac 6 cells were kindly provided by Dr H. W. L. Ziegler-Heitbrock (Institute for Immunology, University of Munich, Munich, Germany) and cultured in RPMI 1640 as described in [17]. Cultures were seeded at a density of 3×10^5 cells/ml. BL41-E95A cells were kindly provided by Dr Hans-Erik Claesson (Karolinska Institute, Stockholm, Sweden) and maintained in RPMI 1640 medium containing 10 % (v/v) heat-inactivated FCS, 100 μg/ml streptomycin and 100 units/ml penicillin. Cultures were seeded at a density of 2×10^5 cells/ml. All cell lines were maintained in a humidified atmosphere of air at 37 °C and 6 % CO_2. For serum-free culture of both cell lines, serum was replaced by 5 μg/ml human transferrin, 10 μg/ml bovine insulin [18] and 1 μg/ml catalase. Cells were cultured in serum-free medium for at least 2 passages before experiments were started (1 passage corresponds to 3 or 4 days). Granulocytes were isolated from human peripheral blood buffy-coat preparations derived from healthy donors at St. Markus Krankenhaus (Frankfurt, Germany) as described previously [19].

Determination of 5-LO activity

Homogenates and 100000-***g*** supernatants were prepared and 5-LO activity was determined as described by Werz and Steinhilber [10]. 5-LO activity is expressed as ng of 5-LO metabolites/10^6 cells, which includes the all-*trans* isomers of leukotriene B_4, leukotriene B_4, and 5-hydroxy-6,8,11,14-eicosatetraenoic acid.

Determination of 5-LO inhibitory activity

As shown previously, 100000-***g*** supernatants of Mono Mac 6 cells dose-dependently inhibit the 5-LO activity of BL41-E95A 100000-***g*** supernatants in the presence of β-mercaptoethanol [20]. Thus inhibition of 5-LO activity of BL41-E95A 100000-***g*** supernatants was used to monitor inhibitory fractions from Mono Mac 6 preparations. Aliquots of the fractions obtained during chromatographic purification of the inhibitor were added to 1 ml of BL41-E95A 100000-***g*** supernatants (corresponding to 10^7 cells) and 5-LO activity was determined in the presence of 0.5 mM β-mercaptoethanol as described in [10].

Determination of GPx activity

Method A: GPx activity was measured by the indirect glutathione reductase-coupled method described by Wendel [21], and 1 unit of GPx activity was defined as the conversion of 0.5 μmol of NADPH to $NADP^+$ per min at 37 °C and 1 mM GSH. Cellular GPx activity is expressed as m-units/10^6 cells.

Method B: co-substrate specificity was determined by a slightly modified GPx assay described by Cha and Kim [22]. Reaction mixture (100 μl) containing 5 mM thiol and GPx (13 m-units) in PBS, pH 7.0, was incubated at 37 °C and the reaction was started by addition of 12.6 μl of cumene hydroperoxide (1.5 mM final concentration). After 1, 3, 5 and 7 min, 20 μl of the reaction mixture was added to 0.8 ml of a trichloroacetic acid solution (12.5 %, w/v) to stop the reaction. Subsequently, 0.2 ml of 10 mM $Fe(NH_4)_2(SO_4)_2$ and 0.1 ml of 2.5 M KSCN were added and the complex was developed for at least 3 min, giving a purple colour. Cumene hydroperoxide concentration was calculated after measuring the absorbance at 480 nm using a molar absorptivity of 8703. For each measurement, the non-enzymic reaction was assessed without GPx-1.

Purification of a cytosolic 5-LO inhibitor from Mono Mac 6 cells

Cells were cultured in FCS medium supplemented with Se^{4+} (5 ng/ml). After cell harvest, a 100000-***g*** supernatant was prepared as described in [10] and applied directly to a Sephacryl S-200 column (1.6 cm × 62 cm) equilibrated with PBS/1 mM EDTA. The column was eluted at a flow rate of 0.5 ml/min and 3-ml fractions were collected and checked for inhibition of 5-LO activity of BL41-E95A supernatants. Inhibitory fractions were pooled and concentrated by ultrafiltration (YM 10 membrane, Amicon) to 2.5 ml. Then, the buffer of the concentrate was changed by gel-permeation chromatography on a prepacked Sephadex® G-25 column (PD-10 column, Pharmacia) which had been equilibrated with 0.005 M phosphate buffer (pH 8.0)/1 mM EDTA/2.5 mM β-mercaptoethanol (buffer DEAE-1). The resulting sample was further purified by anion-exchange chromatography using a Fractogel® EMD DEAE Tentakel column (Merck; bed volume, 5 ml) equilibrated with buffer DEAE-1. After the elution of unabsorbed material at 1.5 ml/min, the flow rate was increased to 2 ml/min and a linear gradient of 0–100 % 0.08 M phosphate buffer (pH 8.0)/1 mM EDTA/2.5 mM β-mercaptoethanol (over 13 min) was started. The UV absorbance was recorded at 280 nm. Fractions (2 ml) were collected and aliquots were tested for 5-LO inhibitory activity. Inhibitory fractions were combined and after addition of ammonium sulphate (1 M, final concentration) the solution was added to a RESOURCE™ PHE column (6.4 mm × 30 mm, Pharmacia) equilibrated with 0.05 M phosphate buffer (pH 7.4)/1 mM EDTA/1 M $(NH_4)_2SO_4$/2.5 mM β-mercaptoethanol (buffer Phenyl-Superose-1). After elution of the column with 11.5 ml of Phenyl-Superose-1 buffer at a flow rate of 0.8 ml/min, a linear gradient (gradient volume 20 ml) from 1 M ammonium sulphate to pure buffer [buffer Phenyl-Superose-1 without $(NH_4)_2SO_4$] was used to elute the inhibitory enzyme at a flow rate of 1 ml/min. The elution of the inhibitory enzyme was complete after an additional 8 min of elution with pure phosphate buffer. Fractions (2 ml) were collected and elution of the 5-LO inhibitor was monitored by inhibition of 5-LO activity of BL41-E95A supernatants. The active fractions were pooled, supplemented with $(NH_4)_2SO_4$ (1.2 M final concentration) and applied directly on to a RESOURCE™ ISO column (6.4 mm × 30 mm, Pharmacia) equilibrated with 0.05 M phosphate buffer (pH 7.0)/1 mM EDTA/2.5 mM β-mercaptoethanol containing 1.2 M $(NH_4)_2SO_4$ (buffer Isopropyl-Superose-1). Elution conditions, fraction collection and tests of 5-LO inhibition were the same as described for Phenyl-Superose chromatography. The inhibitory fractions were pooled and used for protein identification. All purification steps including centrifugation were performed at +4 °C. Prior to determination of 5-LO inhibitory activity, the buffer of each aliquot was changed to PBS/1 mM EDTA using Sephadex® G-25 columns (PD-10, Pharmacia) according to the manufacturer's instructions.

SDS/PAGE analysis

Protein purification was examined by reducing SDS/PAGE performed in 15 % slab gels (0.75 mm) according to [23]. Proteins were visualized by silver staining.

Protein analysis

For Western-blot analysis, SDS/PAGE was carried out as described above and blotting was done according to a previous protocol [24] using a Mini Trans-Blot® assembly (Bio-Rad). Amino acid sequencing was carried out after SDS/PAGE and in-gel trypsin digestion of the upper band of the 23-kDa doublet. Purification of the fragments and sequencing was performed as described in [25].

Matrix-assisted laser-desorption ionization–time-of-flight (MALDI-TOF) MS analysis was performed on a Voyager RPDE mass spectrometer (PerSeptive Biosystems, Framingham, MA, U.S.A.). The isolated and lyophilized protein was taken up in water, mixed with matrix solution (2,5-dihydroxybenzoic acid, 20 g/l), and spotted on to the probe slide. Following an air-dry step, spectra were obtained in the linear mode at an acceleration voltage of 25 kV. Carbonic anhydrase was used as external standard.

Partial purification of 5-LO

The 5-LO enzyme of BL41-E95A cells and granulocytes was partially purified by ATP-agarose affinity chromatography [26].

Data analysis

Statistical evaluation of the data was performed using Student's t test for unpaired observations. A P value of < 0.05 was considered significant.

RESULTS

Mono Mac 6 and BL41-E95A cells contain distinct selenium-dependent 5-LO inhibitors

Cellular 5-LO activity of BL41-E95A and Mono Mac 6 cells is strongly inhibited by selenium. Both cell lines show low cellular 5-LO activity after cultivation in FCS medium or in serum-free medium supplemented with 1 ng/ml Se^{4+} (13 nM) supplied as selenite (Figure 1). Transfer into serum-free medium for 2–3 passages lead to a strong increase in 5-LO activity of intact BL41-E95A and Mono Mac 6 cells. In BL41-E95A cells, 5-LO activity of the corresponding cell homogenates was hardly affected by the cellular selenium status.

However, in contrast to BL41-E95A cells, 5-LO activity in Mono Mac 6 cell homogenates strongly depended on the cell culture conditions. Cultivation of Mono Mac 6 cells in the presence of FCS or selenium strongly suppressed 5-LO activity in the cell homogenates whereas serum-free culture conditions resulted in high 5-LO activity.

Thus in contrast to GPx-4 that was previously identified as endogenous inhibitor in HL-60 cells and BL41-E95A cells [10], the selenium-dependent Mono Mac 6 inhibitor does not require addition of millimolar concentrations of thiols as co-substrate in order to inhibit 5-LO activity in cell homogenates or 100000-*g* supernatants (Figure 1) [10]. Determination of the apparent molecular size by gel-permeation chromatography revealed that the Mono Mac 6 inhibitor is larger than 66 kDa and has a much higher molecular mass than the 5-LO inhibitor (GPx-4) described previously in BL41-E95A cells (Figure 2). Thus it was of interest

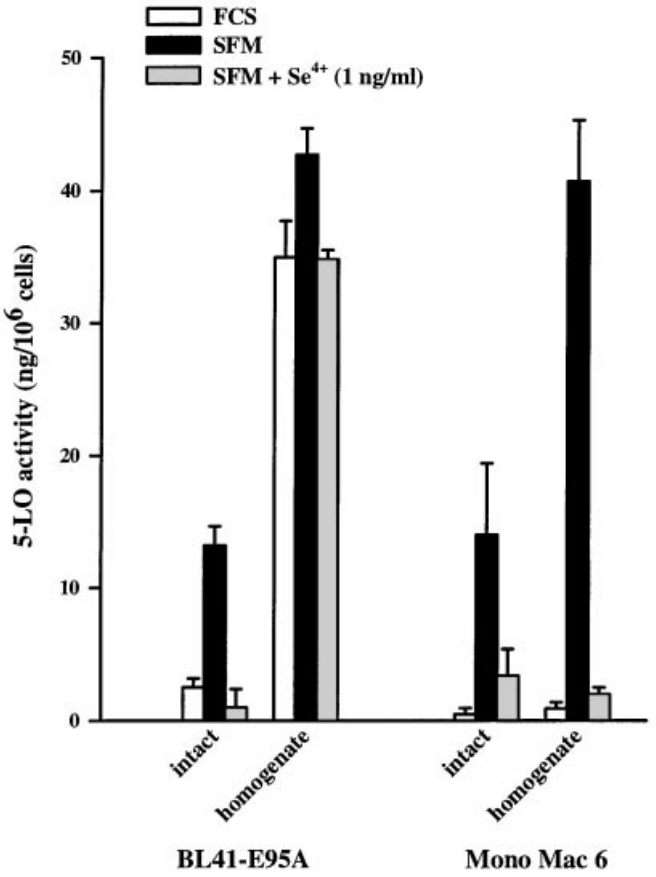

Figure 1 Selenium-dependent inhibition of 5-LO activity of intact cells and cell homogenates from BL41-E95A and Mono Mac 6 cells

Cells were cultured under the indicated culture conditions for 2 or 3 passages (SFM, serum-free medium). Mono Mac 6 were differentiated for 2 days with TGFβ (1 ng/ml). Then, 5-LO activity of intact cells and cell homogenates was determined in the absence of exogenously added thiols. Results are expressed as means $\pm$ S.E.M. of three independent experiments.

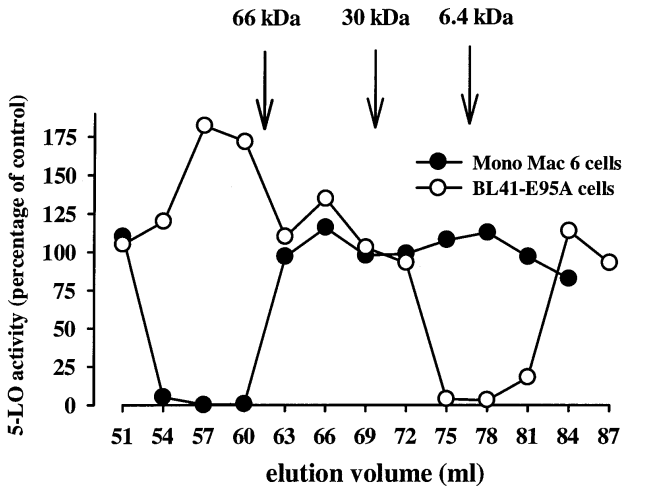

Figure 2 Gel-permeation chromatography of the endogenous 5-LO inhibitor from Mono Mac 6 and BL41-E95A cells

Each 100000-*g* supernatant was applied to a Sephacryl S-200 column, eluted as described in the Materials and methods section, and inhibition of 5-LO activity by the fractions was determined. Data are representative of at least two independent experiments.

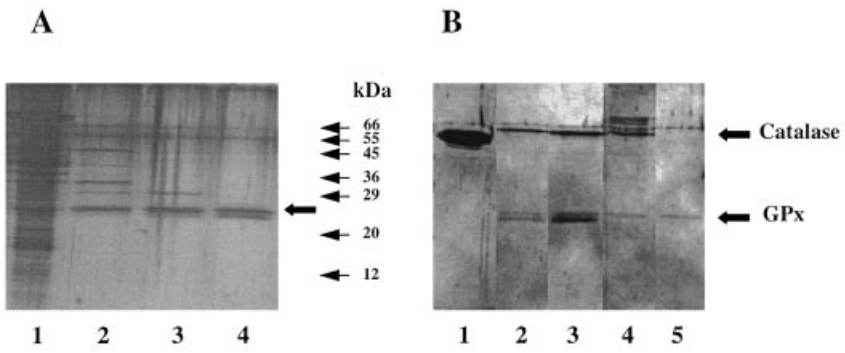

Figure 3 SDS/PAGE analysis (A) and Western-blot analysis (B) of the 5-LO inhibitor

Concentrated protein samples from different stages of purification were electrophoresed by reducing SDS/PAGE (15% gel). (**A**) Lane 1, sample from DEAE chromatography; lane 2, sample from Phenyl-Superose chromatography; lane 3, sample from Isopropyl-Superose chromatography; lane 4, bovine GPx. (**B**) Lane 1, catalase; lanes 2 and 3, human GPx-1; lane 4, sample from DEAE chromatography; lane 5, sample from Isopropyl-Superose chromatography. In (**A**) the position of human GPx-1 is indicated with a bold arrow.

to identify the selenium-dependent inhibitor in Mono Mac 6 cells.

Purification of the 5-LO inhibitor and identification as GPx-1

Purification was performed as described in the Materials and methods section and the obtained fractions were tested for 5-LO inhibition and GPx activity. It became apparent that the 5-LO inhibitory activity co-eluted with the GPx activity during all chromatographic purification steps (results not shown).

Purification of the 5-LO inhibitor by chromatography on EMD DEAE, Phenyl-Superose and Isopropyl-Superose was monitored by SDS/PAGE analysis (Figure 3A, lanes 1–3). After the final Superose column-chromatography step only two protein bands were detectable following silver staining. One band was very faint, and the other, with a molecular mass of approx. 23 kDa, co-migrated in the gel with bovine GPx-1 (Figure 3A, lane 4).

Since experimental data indicated that the inhibitory enzyme might be a GPx distinct from GPx-4 but with a similar size to GPx-1, Western-blot analysis was performed using an antibody against human GPx-1/catalase (Figure 3B). The antibody recognized catalase (Figure 3B, lane 1), human GPx-1 reference (Figure 3B, lanes 2 and 3, two different concentrations) and the isolated protein band displaying the same mobility as GPx-1 (Figure 3B, lanes 4 and 5). These results strongly suggested that the enzyme responsible for 5-LO inhibition in Mono Mac 6 cells is GPx-1. It was interesting to note that the antibody recognized a double band when the human GPx-1 standard was analysed. The same double band was also detected in the SDS/PAGE analysis of the isolated enzyme in some of the enzyme preparations from Mono Mac 6 cells (results not shown). A possible explanation for the occurrence of a double band with identical immunological properties is that the second band represents a proteolytically processed form of GPx-1 as described previously [27]. The molecular masses of the double band of the isolated inhibitor were 22087 and 20290 Da, as determined by MALDI-TOF MS. Unfortunately, the precision of the MALDI MS analysis was limited since only rather broad signals could be obtained for these two peaks in the mass spectrum (results not shown). However, the data fit well with the calculated molecular mass of GPx-1 and a truncated form generated by proteolysis at a cleavage site between amino acids 15 and 16, giving molecular masses of 21768 and 20350 Da, respectively [28].

Table 1 Cellular GPx activities of Mono Mac 6, HL-60 and BL41-E95A cells

Cells were cultured under the indicated culture conditions for 2 passages. Differentiation of Mono Mac 6 and HL-60 cells was induced by addition of TGFβ (1 ng/ml) and VD3 (50 nM) for 4 days. GPx activity of the 100000-***g*** supernatants was determined with the GPx assay (method A, see the Materials and methods section) and the results are expressed as m-units/10^6 cells (means $\pm$ S.E.M.; $n = 3$).

	GPx activity (m-units/10^6 cells)		
	Mono Mac 6		
Cell-culture conditions	Undifferentiated	Differentiated	BL41-E95A
With serum	6.5 $\pm$ 1.1	8.2 $\pm$ 0.3	0.6 $\pm$ 0.3
With serum + 1 ng/ml Se^{4+}	20.1 $\pm$ 3.4	28.0 $\pm$ 1.3	1.7 $\pm$ 0.1
Serum-free medium	1.8 $\pm$ 0.3	1.3 $\pm$ 0.3	0.3 $\pm$ 0.1

For final identification of the isolated enzyme, amino acid sequence analysis was performed. The protein was found to have a blocked N-terminus and was therefore submitted to in-gel trypsin digestion. Chromatographic separation of the fragments and sequencing of three peptides gave the sequences DYTQMNELQR, NEEILNSLKYVR and FLVGPDGVPLRR, corresponding to amino acids 53–62, 87–98 and 165–176 of GPx-1.

Mono Mac 6 cells have much higher GPx activities than BL41-E95A cells

Analysis of GPx-1 and GPx-4 mRNA expression by reverse transcriptase PCR showed that both genes are expressed in lymphocytes and in undifferentiated and differentiated myeloid cells [10]. To elucidate whether there are differences in cellular peroxidase activity between Mono Mac 6 and BL41-E95A cells and whether there are changes in cellular GPx activity related to Mono Mac 6 cell differentiation, GPx activity was determined in undifferentiated cells and in Mono Mac 6 cells differentiated in the presence of VD3 (50 nM) and TGFβ (1 ng/ml) for 4 days (Table 1). The data demonstrate that undifferentiated Mono Mac 6 cells show at least an 11-fold-higher GPx activity than BL41-E95A cells.

Addition of selenium (1 ng/ml) as selenite elevated GPx activity of all investigated cell lines about 3-fold, whereas serum-

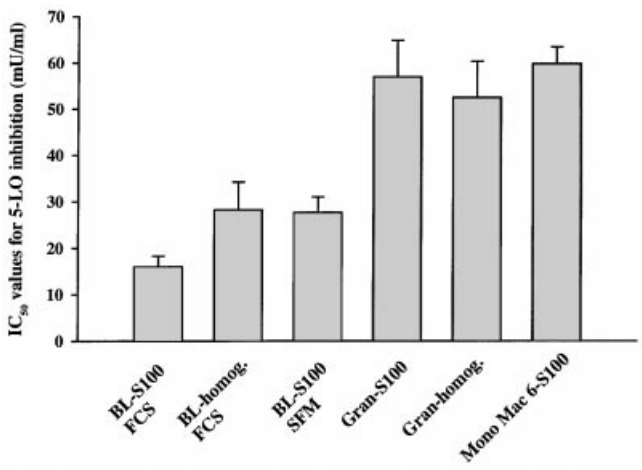

Figure 4 Inhibition of 5-LO activity of various cell types by undifferentiated Mono Mac 6 100000-*g* supernatants

IC_{50} values for inhibition of 5-LO activity were determined in the presence of 0.5 mM β-mercaptoethanol. Values given are means $\pm$ S.E.M. for at least three independent experiments. S100, 100000-***g*** supernatant; SFM, serum-free medium; Gran, granulocytes.

free culture conditions led to very low cellular GPx activities. Differentiation of Mono Mac 6 cells did not result in significant changes in cellular GPx activity (Table 1).

Cell-dependent differences in stability of 5-LO catalytic activity against GPx-1

We have shown previously that upregulation of cellular 5-LO activity during differentiation of myeloid cells is due to a reduced sensitivity of the 5-LO catalytic activity against inhibition by peroxidases [10]. Therefore, it was of interest to compare the inhibitory effect of the Mono Mac 6 100000-***g*** supernatant on 5-LO derived from BL41-E95A, granulocyte and differentiated Mono Mac 6 cells (Figure 4). Undifferentiated Mono Mac 6 cells used as the GPx source were grown in FCS medium containing 0.1 ng/ml Se^{4+} for 4 days. Granulocytes were isolated as described in the Materials and methods section, and BL41-E95A cells were cultured either for 4 days in FCS medium or for 2 passages in serum-free medium. Mono Mac 6 cells grown under serum-free conditions for 1 passage were differentiated by addition of VD3 (50 nM) and TGFβ (1 ng/ml) for 4 days [8]. IC_{50} values for 5-LO inhibition were determined from dose-response curves obtained by addition of increasing concentrations of GPx-1 derived from 100000-***g*** supernatants of undifferentiated Mono Mac 6 cells to broken-cell preparations of BL41-E95A cells, TGFβ/VD3-differentiated Mono Mac 6 cells and granulocytes in the presence of 0.5 mM β-mercaptoethanol. As can be seen from Figure 4, higher GPx activities are required for inhibition of granulocyte or Mono Mac 6 5-LO than for B-lymphocytic 5-LO. Thus as shown previously for GPx-4, 5-LO derived from differentiated myeloid cell lines or granulocytes is significantly more resistant towards peroxidase inhibition than B-lymphocytic 5-LO (Figure 4).

Inhibition of 5-LO by GPx-1 is not due to direct protein–protein interactions

In order to investigate whether 5-LO inhibition by GPx-1 might be due to direct interactions between GPx-1 and 5-LO, 5-LO from BL41-E95A (grown in FCS medium) and granulocyte 100000-***g*** supernatants was immobilized on ATP-agarose columns and the columns were washed in order to remove unbound materials. Then, GPx-1 from Mono Mac 6 100000-***g*** supernatants (obtained from cells grown in FCS medium supplemented with 5 ng/ml Se^{4+}) was applied to each column. The resulting pass-through fraction was collected and three washing steps were performed as described for 5-LO isolation and collected. Then, 5-LO was eluted as described above (5-LO fraction). GPx activity was measured in the pass-through fraction, the three wash fractions and the 5-LO fraction. Interestingly, more than 98 % of the GPx activity was recovered in the pass-through fraction and the first washing fraction but no GPx activity co-eluted with 5-LO from the ATP-agarose column. The data suggest that there is no direct binding of GPx-1 to 5-LO.

Table 2 Effects of thiols on 5-LO inhibition by Mono Mac 6 100000-*g* supernatants and bovine GPx-1

IC_{50} values for 5-LO inhibition by Mono Mac 6 100000-***g*** supernatants were determined in the presence of 0.5 mM of the indicated thiol, those of bovine GPx-1 were determined with 1 mM thiol. Endogenous low-molecular-mass thiols of the 100000-***g*** supernatants were removed by gel filtration on a PD-10 column (Pharmacia). IC_{50} values for 5-LO inhibition are expressed as means obtained from at least two independent dose-response curves ($\pm$ S.E.M.). n.d., not determined.

	IC_{50} value (m-units/ml)	
Cofactor	GPx from Mono Mac 6 100000-***g*** supernatants	Bovine GPx-1
GSH	11.5 $\pm$ 0.5	10.7 $\pm$ 2.7
DTT	20 $\pm$ 10	14.2 $\pm$ 3.6
β-Mercaptoethanol	35 $\pm$ 5	51.8 $\pm$ 12.1
Without thiol addition	215 $\pm$ 15	84 $\pm$ 2.5
Thiol removal	280 $\pm$ 30	n.d.

Thiol specificity of GPx-1

GPx-1 accepts GSH and to a lesser extent DTT as co-substrate, but not β-mercaptoethanol [29,30]. However, in our experiments, 5-LO inhibition by GPx-1 could also be observed in the presence of β-mercaptoethanol (e.g. Table 2). Interestingly, the presence of 2.5 mM β-mercaptoethanol was essential for the recovery of 5-LO inhibitory activity during DEAE, Phenyl- and Isopropyl-Superose column chromatography. No 5-LO inhibition could be detected after any of these purification steps if they were performed in the presence of 0.5 mM β-mercaptoethanol. Furthermore, 5-LO inhibition was more effective in the presence of elevated concentrations of β-mercaptoethanol [IC_{50} values for 5-LO inhibition were 16 $\pm$ 2.3 and 8.5 $\pm$ 1.9 m-units/ml in the presence of 0.5 and 2 mM β-mercaptoethanol, respectively (means $\pm$ S.E.M.; $n = 3$)]. Similar results were obtained with bovine GPx [IC_{50} values were 27.7 $\pm$ 1.8 and 17.7 $\pm$ 4.4 m-units/ml, respectively, ($n = 3$)].

The effects of thiols (GSH, DTT and β-mercaptoethanol) on 5-LO inhibition by GPx are shown in Table 2. Inhibition of 5-LO activity was determined in 100000-***g*** supernatants of BL41-E95A cells which were kept in serum-free medium for 2 passages in order to inhibit endogenous selenium-dependent peroxidases. 100000-***g*** Supernatants of Mono Mac 6 cells that were cultured in FCS medium supplemented with 5 ng/ml Se^{4+} were used as a GPx source. IC_{50} values for 5-LO inhibition by Mono Mac 6-derived GPx-1 were determined in the presence or absence of 0.5 mM thiol and the IC_{50} values for bovine GPx were obtained in the presence of 1 mM thiol. As can be seen from Table 2, 5-

LO inhibition by the peroxidases was most effective in the presence of GSH and only 3-fold-higher GPx concentrations are required with β-mercaptoethanol. 5-LO inhibition still occurred in the absence of added thiols although 5–10-fold-higher GPx concentrations were required. Removal of endogenous GSH and other low-molecular-mass thiols by gel filtration on PD-10 columns (Pharmacia) equilibrated with PBS/1 mM EDTA (Table 2) slightly increased the IC_{50} values from 215 to 280 m-units/ml, indicating that even traces of thiol slightly enhance the inhibitory activity of GPx-1.

Next, the acceptance of thiols by GPx-1 for peroxidase activity was investigated. GPx-1 activity was determined with GPx assay method B in the presence of 5 mM GSH, DTT, β-mercaptoethanol or β-mercaptoethanol in combination of 10 μM GSH, respectively. GPx activity could only be detected in the presence of GSH (0.4 nmol of peroxide/min per m-unit) or DTT (0.1 nmol of peroxide/min per m-unit) but not with β-mercaptoethanol, even in the presence of low concentrations (10 μM) of GSH, thus confirming the data found in the literature [29,30]. Therefore, it can be concluded that β-mercaptoethanol does not serve as co-substrate for the peroxidase activity of GPx-1 but that it is able to support 5-LO inhibition by GPx-1.

DISCUSSION

In previous studies, GPx-4 was shown to be an efficient endogenous inhibitor of cellular 5-LO activity in a variety of cell types, like BL41-E95A cells, in the presence of thiols. In contrast to this, rat and human monocytic cells and the human monocytic cell line Mono Mac 6 contain a cytosolic, selenium-dependent 5-LO inhibitor that does not require the presence of millimolar concentrations of thiol for inhibition of 5-LO activity (Figure 1) [14,15]. Gel-filtration chromatography revealed that this inhibitor has a higher molecular mass than the previously characterized GPx-4-like inhibitor in BL41-E95A cells [10]. No 5-LO inhibition was detected in fractions corresponding to the molecular mass of GPx-4 (18 kDa). This demonstrated that this enzyme is not involved in regulation of 5-LO activity in Mono Mac 6 cells. Purification, Western-blot analysis and protein sequencing led to the identification of GPx-1 as endogenous 5-LO inhibitor in Mono Mac 6 cells. In accordance to this, it was found that Mono Mac 6 cells possess a cellular GPx-1 activity that is at least 10-fold higher than in BL41-E95A cells (Table 1), giving a reasonable explanation for the observed differences in the 5-LO inhibitory activity in the cytosols of these cell lines. The 5-LO inhibitory potency in crude cell homogenates and in fractions obtained during purification correlated with the presence of GPx-1 and was practically identical to GPx-1 enzyme preparations, which were pure according to SDS/PAGE analysis (Table 2 and Figure 3A, lane 4), indicating that GPx-1 is the protein responsible for 5-LO inhibition.

Interestingly, cellular GPx activity of Mono Mac 6 cells does not change during differentiation of the cells with TGFβ and VD3 (Table 1). This is in contrast to the changes in 5-LO activity observed after differentiation of Mono Mac 6 cells with TGFβ and VD3. As shown before, differentiation of Mono Mac 6 cells by TGFβ plus VD3 is required for upregulation of cellular 5-LO activity whereas differentiation of the cells with TGFβ alone leads to prominent expression of 5-LO protein but extremely low activity in intact cells and homogenates ([8] and Figure 1). One possible explanation, which is supported by recombination experiments with cytosols and 5-LO enzyme preparations from different sources [8,10] and by differences in peroxidase sensitivity of 5-LO enzymes derived from cells with high and low cellular 5-LO activity (Figure 4) is that VD3 and TGFβ induce a peroxidase-insensitive 5-LO catalytic activity. This could be due to post-translational modifications of the 5-LO protein or to interactions with other proteins induced or activated by TGFβ and VD3. Recently, TGFβ-receptor-I-associated protein-1 (TRAP-1) partial cDNA was identified in a yeast two-hybrid screening system and the expressed protein was shown to physically interact with 5-LO [31]. On the other hand, TRAP-1 specifically interacts only with the activated form of the type-I TGFβ receptor [32]. Therefore, a function of TRAP-1 could be the association of the 5-LO protein to the activated TGFβ receptor, thereby providing a link between TGFβ receptor activation and the observed effects of TGFβ and TGFβ/VD3 on cellular 5-LO activity and/or peroxidase sensitivity.

No physical interaction was observed between GPx-1 and 5-LO protein from granulocytes and BL41-E95A cells, which suggests that suppression of cellular 5-LO activity by peroxidases is rather due to their effects on cellular peroxide tone than to specific 5-LO/GPx interactions.

In contrast to GPx-4, GPx-1 inhibits 5-LO activity in the absence of millimolar concentrations of thiols (Figure 1). Moreover, β-mercaptoethanol, which does not significantly serve as co-substrate for the peroxidase reaction (see the Results section) supports 5-LO inhibition by GPx-1 (Table 2), indicating that GPx-1 does not require high turnover of peroxides for 5-LO inhibition. This could be due to the circumstance that 5-LO inhibition can be achieved by keeping the peroxide concentration in the assay mixture under a certain level, thus prolonging the lag phase and preventing the onset of the lipoxygenase reaction. Due to the low 5-hydroperoxyeicosatraenoic acid (5-HPETE) production during the lag phase, 5-LO inhibition by GPx rather requires rapid reaction rates than a high turnover of substrate, e.g. 5-HPETE. In its reduced selenol form, GPx-1 rapidly reduces peroxides to the respective alcohols, whereas the active-site selenol is oxidized to selenenic acid [33]. In subsequent steps, the active-site selenenic acid of GPx is reduced to its selenol form by oxidation of thiols, preferentially GSH. This mechanism was confirmed in a recent study where it was shown that selenols like selenocysteine efficiently reduce lipid hydroperoxides like 15-HPETE to alcohols [34]. Interestingly, the selenocysteine residue of GPx-1 is expressed at the surface, allowing easy access of substrates and thus high reaction rates with peroxides [33]. The lack of an absolute requirement for thiols for 5-LO inhibition and the loss of the 5-LO inhibitory activity of GPx-1 upon purification in thiol-free buffer can thus be explained by the assumption that the selenol form of GPx-1 is the species responsible for 5-LO inhibition and that the suppression of the onset of the 5-LO catalytic activity by GPx-1 rather requires rapid reduction of hydroperoxides than a high turnover of substrate.

In summary, we have shown that GPx-1 is an efficient, endogenous inhibitor of cellular 5-LO activity when it is expressed in sufficient quantities. This might be of relevance for the regulation of cellular 5-LO activity in various cell types and for the pharmacology of peroxidase-dependent non-redox inhibitors of 5-LO.

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