Evidence for the presence of phospholipid hydroperoxide glutathione peroxidase in human platelets: implications for its involvement in the regulatory network of the 12-lipoxygenase pathway of arachidonic acid metabolism

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The 12-lipoxygenase pathway of arachidonic acid metabolism in platelets and other cells is bifurcated into a reduction route yielding 12-hydroxyeicosatetraenoic acid (12-HETE) and an isomerization route forming hepoxilins. Here we show for the first time the presence of phospholipid hydroperoxide glutathione peroxidase (PHGPx) protein and its activity in platelets. The ratio of the activity of PHGPx to that of cytosolic glutathione peroxidase (GPx-1) was consistently found to be approx. 1:60 in platelets and UT7 megakaryoblasts. Moreover, short-lived PHGPx mRNA was detected in megakaryocytes but not in platelets. Carboxymethylation of selenium-containing glutathione peroxidases by iodoacetate, which results in the inactivation of PHGPx and GPx-1 without inhibition of 12-lipoxygenase, markedly altered the pattern of arachidonic acid metabolism in human platelets. Whereas the formation of 12-HETE was inhibited by 80 %, a concomitant accumulation of 12-hydroperoxyeicosatetraenoic acid (12-HpETE) by two orders of magnitude as well as the formation of hepoxilins A_3 and B_3 were observed. The formation of hepoxilins also occurred when 12-HpETE was added to untreated platelets. In selenium-deficient UT7 cells, which were devoid of GPx-1 but not of PHGPx, the reduction of 12-HPETE was retained, albeit with a lower rate than in control cells containing GPx-1. We therefore believe that both GPx-1 and PHGPx are involved in the regulatory network of the 12-lipoxygenase pathway in platelets and other mammalian cells. Moreover, the diminution of hydroperoxide tone in platelets incubated with arachidonic acid leads primarily to the formation of 12-HETE, whereas the increase in hydroperoxide tone (a situation found under oxidative stress or selenium deficiency or on incubation with 12-HPETE) partly diverts the 12-lipoxygenase pathway from the reduction route to the isomerization route, thus resulting in the formation of hepoxilins.

Key words: hepoxilin A_3, hydroxy-epoxy fatty acids, iodoacetate, trioxilins.

INTRODUCTION

Glutathione peroxidases are believed to have a prominent role in the defence against oxidative damage to cells [1,2]. However, much evidence has accumulated illustrating that glutathione peroxidases are also intimately integrated in the regulation of the metabolism of arachidonic acid in mammalian cells [3–7]. This role is derived from the fact that glutathione peroxidases are capable of reducing the hydroperoxy fatty acids emanating from dioxygenase-catalysed reactions (lipoxygenases or prostaglandin endoperoxide synthases) to the corresponding alcohols. Moreover, hydroperoxy-polyenoic fatty acids contribute to a large extent to the hydroperoxide tone of the cells, which is the sum of the steady-state concentrations of all hydroperoxides, and is determined by the balance of hydroperoxide-generating and hydroperoxide-consuming processes. At low concentrations hydroperoxy-polyenoic fatty acids trigger prostaglandin endoperoxide synthases [8] and lipoxygenases [9,10], and at higher concentrations promote the suicide inactivation and hydroperoxidase activities of these enzymes [11]. Consequently, any change in the cellular hydroperoxide tone might thus alter the arachidonic acid metabolism of mammalian cells. Because glutathione peroxidases belong to the important hydroperoxy-polyenoic fatty-acid-reducing enzymes, their role in regulating the hydroperoxide tone and, in turn, the synthesis of eicosanoids deserves special attention.

An important family of glutathione peroxidases is characterized by the presence of selenocysteine at the active site. Four members of this selenoenzyme family are known so far, differing in their primary and quaternary structures, in reaction specificities, and in the localizations [2]. For the regulation of intracellular eicosanoid metabolism, cytosolic glutathione peroxidase (GPx-1, also known as cGPx) and phospholipid hydroperoxide glutathione peroxidase (PHGPx) can be considered.

Abbreviations used: ETYA, 5,8,11,14-eicosatetraynoic acid; GPx-1, cytosolic glutathione peroxidase; 12-HETE, (12*S*)-hydroxy-(5*Z*,8*Z*,10*E*,14*Z*)-eicosatetraenoic acid; 12-HHTrE, 12-hydroxy-(5*Z*,8*E*,10*E*)-heptadecatrienoic acid; 13-HODE, (13*S*)-hydroxy-(9*Z*,11*E*)-octadecadienoic acid; 12-HpETE, (12*S*)-hydroperoxy-(5*Z*,8*Z*,10*E*,14*Z*)-eicosatetraenoic acid; HXA_3, hepoxilin A_3; HXB_3, hepoxilin B_3; IMEM, Iscove's minimal essential medium; PAPC, 1-palmitoyl-2-arachidonoyl-phosphatidylcholine; PAPC-OOH, 1-palmitoyl-2-[(15*S*)-hydroperoxy-(5*Z*,8*Z*,11*Z*,13*E*)-eicosatetraenoyl]-phosphatidylcholine; PHGPx, phospholipid hydroperoxide glutathione peroxidase; RT–PCR, reverse-transcriptase-mediated PCR; 8,9,12-TriHETrE, 8,9,12-trihydroxy-(5*Z*,10*E*,14*Z*)-eicosatrienoic acid; 8,11,12-TriHETrE, 8,11,12-trihydroxy-(5*Z*,9*E*,14*Z*)-eicosatrienoic acid.

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There is ample evidence for a crucial role of glutathione peroxidase in platelets in the arachidonic acid metabolism, which until now has been ascribed solely to GPx-1 [3,12]. To our knowledge, no evidence for the occurrence of PHGPx at the same time and its possible role in platelets has yet been presented.

PHGPx differs from GPx-1 in its ability to react not only with free hydroperoxy fatty acids but also with esterified ones, including those present in membrane phospholipids. Consequently, PHGPx possesses a pronounced affinity for binding to membranes [13] that are also the site of eicosanoid syntheses. Mammalian 5-, 12- and 15-lipoxygenases are known to translocate to intracellular membranes in activated cells [14–16], so that their primary metabolites, the hydroperoxy fatty acids, are formed in a membrane compartment and are therefore preferentially accessible for PHGPx. Thus PHGPx has been shown to have a greater role than GPx-1 in the regulation of the 5-lipoxygenase pathway of arachidonic acid metabolism in leukocytes. This conclusion was achieved by investigations *in vitro* and *ex vivo* with selenium-deficient cells and animals, subsequently replenished by selenium, to which the two enzymes revealed different response patterns [5]. The replenishment of selenium caused a rapid reappearance of PHGPx, but not of GPx-1, leading to inverse responses of the formation of 5-lipoxygenase products in leukocytes [5]. The crucial role of PHGPx in the control of leukotriene synthesis has recently been supported by another study, in which a stable transfectant of rat basophilic leukaemia cells overexpressing PHGPx was used [7].

These observations prompted us to examine whether PHGPx also participates in the regulation of the 12-lipoxygenase pathway in human platelets. This metabolic pathway is bifurcated at the level of the primary lipoxygenase product, (12*S*)-hydroperoxy-(5*Z*,8*Z*,10*E*,14*Z*)-eicosatetraenoic acid (12-HpETE), which can be reduced to (12*S*)-hydroxy-(5*Z*,8*Z*,10*E*,14*Z*)-eicosatetraenoic acid (12-HETE) or isomerized to hepoxilins. Both 12-HETE and hepoxilins are biologically active eicosanoids with a distinct array of biological actions [17–19]. Because glutathione peroxidases and as yet unidentified hepoxilin-forming catalysts in platelets compete for the same substrate, the rate of formation of hepoxilins necessarily depends directly on the overall glutathione peroxidase activity and consequently on the hydroperoxide tone of platelets. Here we provide the first evidence for the occurrence of PHGPx in human platelets and its possible role in the 12-lipoxygenase pathway.

MATERIALS AND METHODS

Cell preparations

Human platelets were prepared from healthy donors as described elsewhere [20], with minor modifications. Unless stated otherwise, washed platelets were suspended in PBS containing 0.25 % BSA and 0.1 % glucose without Ca^{2+} to a final concentration of $(3–4) \times 10^9$ cells/ml.

Human megakaryocytes were isolated from bone marrow aspirates by the protocol of Tanaka et al. [21]. In brief, 6 ml of bone marrow aspirate from healthy volunteers was diluted with an equal volume of MK medium [22]. The cell suspension was layered over two different solutions of Percoll (Pharmacia) with densities of 1.050 and 1.020 g/ml. Megakaryocytes were present in the fraction heavier than 1.020 g/ml and lighter than 1.050 g/ml Percoll [23]. The megakaryocytes were purified by using a monoclonal antibody against the human platelet glycoprotein IIb/IIIa complex together with magnetic beads (Boehringer, Mannheim, Germany) coated with anti-mouse IgG antibody (Sigma, Munich, Germany). The biotinylated secondary antibody was used to coat the streptavidin-conjugated magnetic beads. The separation was performed with a Dynal magnetic particle concentrator (MPC 1; Dynal, Oslo, Norway).

UT7 cells were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Braunschweig, Germany) and grown in Iscove's minimal essential medium (IMEM) containing 10 % (v/v) fetal calf serum and 10 ng/ml granulocyte/macrophage colony-stimulating factor for control experiments. A431 cells were also obtained from DSMZ and grown in Dulbecco's modified Eagle's medium containing 10 % (v/v) fetal calf serum to 100 % confluence for obtaining lysates for the measurement of enzyme activities. The cells were harvested by treatment with trypsin and washed twice with PBS. Selenium-deficient UT7 cells were obtained by an adapted protocol as used by others for obtaining selenium-deficient rat basophilic leukaemia cells [5]. In brief, the cells were grown for 20 days in IMEM containing 0.75 % (v/v) fetal calf serum and 10 ng/ml granulocyte/macrophage colony-stimulating factor; the doubling time was 66 h. For partial replenishment of selenium, the cells were incubated for a further 2 h with IMEM containing 10 % (v/v) fetal calf serum and 0.95 μM sodium selenite.

A431 cells, platelets and UT7 cells were lysed by sonication in 10 mM Tris/HCl buffer, pH 7.4, containing 10 μg/ml leupeptin and 0.1 mM PMSF. The homogenates were centrifuged at 10000 ***g*** for 10 min at 4 °C to separate them into supernatant and pellet fractions.

For separation of PHGPx and GPx-1, the supernatant of platelet lysate was passed through a Sephadex G-100 Superfine column. As detected by measuring glutathione peroxidase activity with H_2O_2 as substrate, GPx-1 was eluted immediately after the void volume, whereas a second well-defined activity peak coincided with both PHGPx activity (see the next section) and the 23 kDa protein fraction.

Assay of glutathione peroxidase activities

The specific substrate used for PHGPx was 1-palmitoyl-2-[(15*S*)-hydroperoxy-(5*Z*,8*Z*,11*Z*,13*E*)-eicosatetraenoyl]-phosphatidylcholine (PAPC-OOH), which was prepared as described previously [24]. In brief, 1-palmitoyl-2-arachidonoyl-phosphatidylcholine (PAPC) (780 μg) was mixed with 6 ml of 10 mM sodium deoxycholate and 13 ml of 0.2 M sodium borate, pH 9.0, under sonication for 5 min. Dioxygenation was started by the addition of 500 μg of soybean lipoxygenase (grade I; Sigma) per 10 ml sample at room temperature and stopped after 10 min by the addition of methanol/chloroform/water (2:1:1, by vol.). The products were separated by reverse-phase HPLC on a Nucleosil 1005 C_8 column with the solvent system methanol/acetonitrile/water (90:6:4, by vol.) containing 20 mM choline chloride.

PHGPx activity was measured by the method of Imai et al. [7] with a few modifications. The reaction mixture contained 0.1 M Tris/HCl, pH 7.4, 5 mM EDTA, 1.5 mM NaN_3, 3 mM GSH, 0.25 mM NADPH, 1 unit of glutathione reductase and 0.1 % (v/v) Triton X-100. The reaction was started by the addition of 0.25 mM H_2O_2 or 20 μM PAPC-OOH. For the specific measurement of GPx-1 activity, 0.25 mM t-butyl hydroperoxide was used as substrate. 12-HpETE reductase activity was measured with 20 μM 12-HpETE. The reactions were followed at 25 °C at 340 nm (UV max kinetic microplate reader; Molecular Devices, Sunnyvale, CA, U.S.A.) and corrected for a blank.

Western blot analyses

A rabbit polyclonal antibody raised against a peptide fragment of human PHGPx (residues 116–130) was obtained from FZB

Biotechnik GmbH (Berlin, Germany). A rabbit polyclonal antibody against pig PHGPx was kindly supplied by Dr M. Maiorina (Padova, Italy) and a monoclonal antibody against recombinant human PHGPx (*sec*46*cys* mutant) by Dr H. Kühn (Berlin, Germany). For Western blot analysis, the washed platelets were lysed by sonication; precipitation with 10 %-satd $(NH_4)_2SO_4$ was performed to remove the membranes completely. The platelet lysate, together with A431 cell lysate as a positive control, were subjected to SDS/PAGE [12 % (w/v) gel]. After semi-dry transfer, the blot was blocked with 3 % (w/v) BSA. Alkaline-phosphatase-conjugated goat anti-rabbit IgG (dilution 1:1000) was used as the secondary antibody. The colour development was performed with 5-bromo-4-chloroindol-3-yl phosphate/Nitro Blue Tetrazolium (Sigma).

Reverse-transcriptase-mediated PCR (RT–PCR)

The total RNA was isolated with a Trizol RNA isolation protocol (Gibco BRL) or an RNeasy® mini kit (Qiagen). Reverse transcription was performed with Expand reverse transcriptase (Boehringer Mannheim) and oligo(dT) primer. The total RNA (4 μg) was reverse-transcribed at 42 °C for 90 min. For amplification of PHGPx cDNA the oligonucleotides 5′-TGTGCGCGCTCCATGCACGAGT-3′ and 5′-AAATAGTGGGGCAGGTCCTTCTCT-3′, obtained from TIB Biomol (Berlin, Germany), were used as the primer set. Platelet-type 12-lipoxygenase cDNA was amplified with the primer set 5′-CTGGCCCCAGAAGATCTGATC-3′ and 5′-GATGATCTACCTCCAAATATG-3′ and cDNA for β-actin with various primer sets supplied by Stratagene (La Jolla, CA, U.S.A.). PCR was performed with 1 μl of the cDNA reaction sample with 1.5 mM $MgCl_2$ and 1 unit of *Taq* polymerase (AB Technologies, Berlin, Germany) on a Unoblock thermal cycler (Biometra, Göttingen, Germany). After initial denaturation for 2 min at 94 °C, 30 cycles at 94 °C for 30 s, 68 °C (PHGPx and β-actin) and 60 °C (12-lipoxygenase) for 30 s were performed, followed by 72 °C for 1 min. The PCR products were separated on 2 % (w/v) agarose gel; DNA was stained with ethidium bromide.

For estimation of the rate of breakdown of PHGPx mRNA in differentiated UT7 cells, the cells (10^6/ml) were treated with 10 μM PMA (Sigma) for 72 h to induce differentiation to megakaryocytes in a thromboprotein-like manner [25]. Thereafter the cells were treated with 10 ng/ml actinomycin D (Sigma) and were harvested at various intervals for the determination of the amount of PHGPx mRNA. The quantification of PHGPx mRNA was done by semi-quantitative RT–PCR, with β-actin mRNA as standard.

HPLC analysis of 12-lipoxygenase metabolites from arachidonic acid

Straight-phase HPLC analyses of arachidonic acid metabolites were performed by the use of a LC10AT instrument equipped with an SPD-M10A diode array detector (Shimadzu, Duisburg, Germany) on a Supelco-SIL column (250 mm × 4.6 mm, 5 μm particle size). The mobile phase was n-hexane/propan-2-ol/acetic acid (100:2:0.1, by vol.) with a flow rate of 1.0 ml/min.

For analysis, washed human platelets were pretreated with 2 mM iodoacetate for 10 min at 37 °C and then incubated with 130 μM arachidonic acid for another 10 min. These treatments did not produce damage to plasma membranes as judged by the Trypan Blue exclusion test. (13*S*)-Hydroxy-(9*Z*,11*E*)-octadecadienoic acid (13-HODE) was added as an internal standard at the end of the reaction. The reactions were stopped by the addition of precooled solvent mixture diethyl ether/methanol/1 M monosodium citrate (135:15:1, by vol.) containing 2,6-di-t-butyl-4-hydroxytoluene (5 mg/l). After phase separation, the aqueous phase was re-extracted three times with diethyl ether. The combined organic phases were dried over anhydrous Na_2SO_4, evaporated in a nitrogen stream and dissolved in the HPLC solvent (see above). This extraction procedure was examined by recovery experiments with defined amounts of 12-HpETE, 12-HETE, 13-HODE and arachidonic acid and proved to be appropriate. In contrast, when citric acid was used instead of monosodium citrate for the reaction stop, as frequently proposed in the literature, we observed marked losses of the eicosanoids and in particular 12-HpETE, which was degraded to compounds absorbing at 280 nm, presumably 12-oxo acids [26], as judged by corresponding HPLC runs (results not shown). In our hands, these artifacts were avoided by the use of monosodium citrate.

To detect the hepoxilins directly by HPLC analysis, the eicosanoids were esterified with 9-anthranyldiazomethane (ADAM) as described by Demin et al. [27], with some modifications. The derivatives were prepared with 10 μg of ADAM (Research Organics, Cleveland, OH, U.S.A.) in diethyl ether at room temperature in the dark with constant stirring. The reaction mixture was dried down, reconstituted in solvent and injected on a Novapak C_{18} column (250 mm × 4.6 mm, 4 μm particle size) (VDS Optilab, Berlin, Germany). The mobile phase was methanol/acetonitrile/water (90:6:4, by vol.).

GC–MS analysis of hepoxilins

GC–MS was performed on a Varian Saturn 4D GC–MS–MS system equipped with a Supelco DB5-MS column (30 m × 0.25 mm; the thickness of the separating material within the column was 0.25 μm). The temperature programme was started at 150 °C, increasing to 250 °C within 10 min at a rate of 10 °C/min, and kept at 250 °C for a further 10 min. The temperatures of the injector and transfer lines were 230 °C and 220 °C respectively.

For analysis of hepoxilins, the platelets were incubated with 100 μM arachidonic acid in the presence or absence of 2 mM iodoacetate or with 50 μM 12-HpETE for 30 min at 37 °C. The reaction was stopped by the addition of 5 vol. of diethyl-ether-saturated 10 mM HCl and the mixture was left to stand for 30 min at room temperature. The acid-hydrolysed extracts were then evaporated under a nitrogen stream and then reconstituted in 50 μl of methanol. By this treatment hepoxilin A_3 (HXA_3) was found to be quantitatively hydrolysed to a mixture of 8,9,12-trihydroxy-(5*Z*,10*E*,14*Z*)-eicosatrienoic acid (8,9,12-TriHETrE) and 8,11,12-trihydroxy-(5*Z*,9*E*,14*Z*)-eicosatrienoic acid (8,11,12-TriHETrE), which is in agreement with other reports [3,28]. The fatty acids were converted into their methyl esters by treatment with 300 μl of diazomethane in diethyl ether for 15 min at room temperature in the dark. Thereafter the samples were evaporated to dryness. Hydroxy groups were silylated by the addition of 30 μl of trimethylsilylimidazole in dry pyridine (TriSil-Z®; Pierce) under nitrogen and heating for 5 min at 60 °C. After evaporation, the samples were reconstituted in 10 μl of dodecane. A 1 μl aliquot of this solution was injected for GC–MS analysis. Hepoxilin B_3 (HXB_3) turned out to be stable under these conditions, as the corresponding hydrolysis product 10,11,12-trihydroxy-(5*Z*,8*Z*,14*Z*)-eicosatrienoic acid could not be detected. HXA_3 was quantified from the peak areas of the mass fragments at *m*/*z* 243 and 213, characteristic of 8,9,12-TriHETrE and 8,11,12-TriHETrE respectively (see Figure 6C). HXB_3 was quantified from the peak area of the mass fragment at *m*/*z* 119 (see Figure 6B); this mass fragment was very sensitive and gave more reproducible results in recovery experiments than the other

characteristic mass fragments such as those at m/z 269 and 282. Calibration curves were obtained for hepoxilins with authentic standards. The recovery of added standards was more than 85% (results not shown).

RESULTS

PHGPx activity and PHGPx protein occur in human platelets

To detect PHGPx activity specifically in human platelets, we applied the peroxidized phospholipid PAPC-OOH as substrate in a coupled spectrophotometric assay with glutathione reductase. As shown in Figure 1(A), a dose-dependent activity was measured with human platelet cytosol that was completely blocked by the carboxymethylating agent iodoacetate (curve d), indicating that the activity was due to a selenoenzyme. This activity was reproducible with various platelet preparations from two different donors (Figure 1, Table 1). Because GPx-1 is not capable of reacting with PAPC-OOH, the activity observed could be exclusively attributed to PHGPx. Separation of the platelet lysate into supernatant and pellet fractions revealed that approx. 12% of the PHGPx activity was present in the pellet, whereas GPx-1 activity was found almost exclusively in the cytosol, as expected (results not shown).

To establish further the occurrence of PHGPx protein in platelets, we performed Western blot analyses with three different antibodies against PHGPx: a selective polyclonal antibody raised against a peptide fragment of human PHGPx, a monoclonal antibody against human recombinant PHGPx (*sec*46*cys* mutant) and a polyclonal antibody against pig PHGPx used previously by Dr Ursini's group in Padova. Besides platelet cytosol, we also examined as respective positive and negative controls a lysate from A431 cells, in which the presence of PHGPx has been clearly established [29], and human red-blood-cell cytosol, which is a rich source of GPx-1 [1] but not of PHGPx. With all three selective anti-PHGPx antibodies we observed a strong immunoreactive band at a molecular mass of approx. 23 kDa in A431 cells and platelets but not in red blood cells. Figure 2 shows the corresponding experiment with the antibody against pig PHGPx as an example.

In further experiments we addressed the contribution of PHGPx to the reduction of 12-HpETE, the primary product of the arachidonate 12-lipoxygenase pathway, in platelet cytosol. For this purpose we fractionated the platelet cytosol through a Sephadex G-100 Superfine column. By this method, two glutathione peroxidase fractions representing GPx-1 (approx. 90 kDa) and the later-eluting PHGPx (approx. 23 kDa), which were active towards H_2O_2, were obtained (results not shown). The fractions were tested for glutathione-dependent 12-HpETE reductase activity. Both glutathione peroxidases proved to be

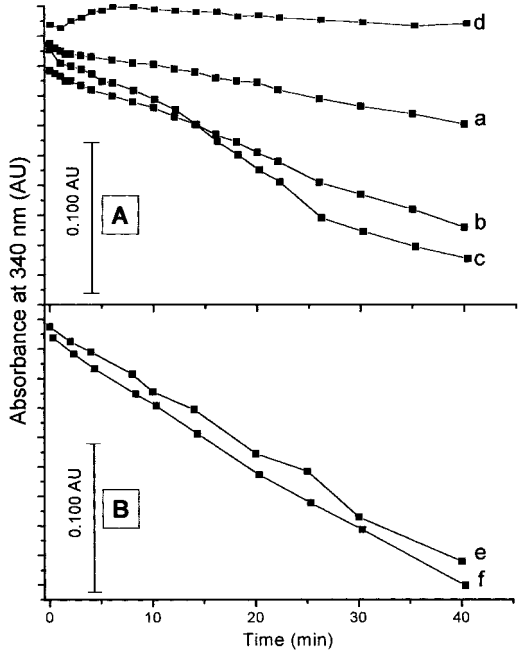

Figure 1 Demonstration of PHGPx activity in the cytosol of human platelets

Activity was measured at 25 °C with PAPC-OOH as substrate, as described in the Materials and methods section, with supernatant from lysates of platelets isolated from two male donors: non-smoker, 29 years of age (**A**); smoker, 33 years of age (**B**). The amounts of platelet supernatant in the assay corresponded to a total platelet protein value (Lowry method, Bio-Rad D_c protein assay kit) of 0.10 (curve a), 0.25 (curve b), 0.51 (lanes c and d), 0.33 (curve e) and 0.40 mg (curve f). In curve d the supernatant was preincubated with 2 mM iodoacetate for 10 min at 37 °C. Curves a–d were obtained with lysate from a single platelet preparation to examine the dose dependence of the assay system, whereas curves e and f were obtained with two different platelet preparations from the other donor. AU, absorbance units.

Table 1 GPx-1 and PHGPx activities in various cells

Cell lysates were obtained as described in the Materials and methods section. Activities of GPx-1 and PHGPx were measured with H_2O_2 and PAPC-OOH respectively. The reactions were followed for at least 20 min (see Figure 1). Each value is the sum of activities measured in the supernatant and the resuspended pellet after correction for blanks. The protein content of the cell suspensions was determined by the Lowry method with the Bio-Rad D_c protein assay kit. Results are means ± S.E.M.; the number of experiments is indicated in parentheses.

	Activity (nkat/mg of protein)	
Cell source	GPx-1	PHGPx
Human platelets	68.4 ± 6.4 (2)	1.4 ± 0.2 (4*)
UT7 cells	63.4 ± 4.4 (5)	1.2 ± 0.1 (2)
UT7 cells, Se-deficient	0.18†	1.8†

* Two different preparations from each of two donors.
† Average of two determinations from a single experiment.

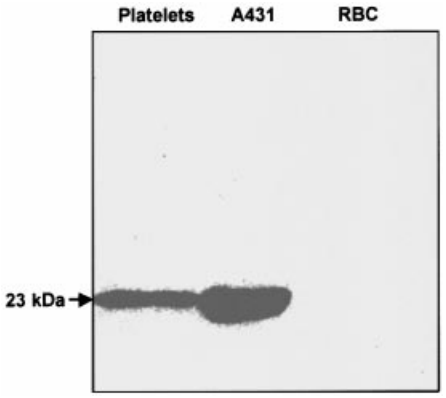

Figure 2 Western blots using PHGPx antibody with lysates of platelets, A431 cells and red blood cells

Human platelet cytosol was prepared as described in the Materials and methods section; Western blots were performed with a polyclonal antibody against pig PHGPx. A431 cells and human red blood cells (RBC) were used as positive and negative controls respectively.

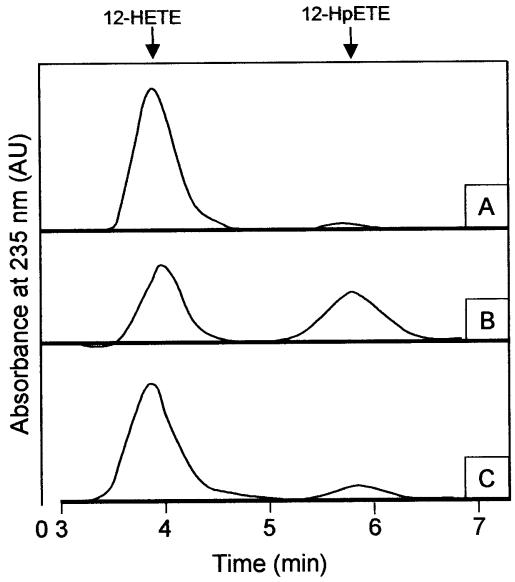

Figure 3 Reduction of 12-HpETE by UT7 cells with and without selenium deficiency

Deficiency and replenishment of selenium in UT7 cells were performed as described in the Materials and methods section. Control cells (3.5×10^6) (**A**) and selenium-deficient cells (**B**, **C**) were incubated with 5 μM 12-HpETE at 37 °C for 10 min (**A**, **B**) or 30 min (**C**). After the reaction had been stopped, the metabolites were separated by straight-phase HPLC as described in the Materials and methods section. AU, absorbance units.

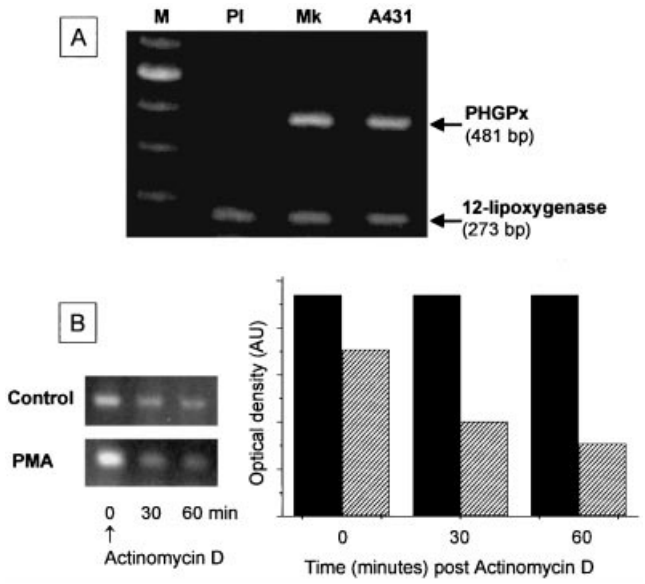

Figure 4 RT–PCR detection of the messengers for PHGPx and 12-lipoxygenase in human platelets and megakaryocytes (A), and rates of breakdown of PHGPx mRNA in untreated and PMA-treated UT7 cells (B)

(**A**) Shown are lanes containing molecular mass standard mixture (M), platelets (PI), megakaryocytes (Mk) and A431 cells (positive control). After separate PCRs for PHGPx and 12-lipoxygenase, the reaction mixtures were pooled and subjected to electrophoresis. For experimental conditions see the Materials and methods section. (**B**) UT7 cells were treated with 10 μM PMA for 3 days along with the corresponding untreated controls. The levels of PHGPx mRNA and β-actin mRNA were determined by RT–PCR as described in the Materials and methods section. Further transcription was stopped by the addition of actinomycin D (10 ng/ml); the levels of the mRNA species were estimated as a function of time. The column pairs in the right panel show the relative levels of PHGPx mRNA normalized for the level of β-actin mRNA for control cells (filled columns) and PMA-treated cells (hatched columns). AU, absorbance units.

active; PHGPx contributed to an activity of 0.19 nkat/mg of protein, whereas GPx-1 revealed a value of 11.3 nkat/mg of protein. However, it should be stressed that these model estimates do not reflect the physiological conditions in intact cells, in which translocation of the 12-lipoxygenase to the membrane compartment occurs. We also compared the glutathione peroxidase activities of platelets with those of the human megakaryocyte cell line UT7. Interestingly, the two species of cells did not significantly differ from each other with respect to their activities of GPx-1 and PHGPx (Table 1). Because PHGPx has been reported to be responsible for strong suppression of the platelet-type 12-lipoxygenase [29], we inferred that the 12-HpETE reductase activity in platelets cannot be solely ascribed to GPx-1; a putative role of PHGPx must therefore be seriously considered.

From the data in Table 1 it is evident that UT7 cells exhibit activities of GPx-1 and PHGPx in the same range as in platelets. To evaluate the distinct role of PHGPx in the 12-lipoxygenase pathway, we cultured selenium-deficient and partly replenished cells. Because it is known that GPx-1 is much more sensitive to selenium deficiency than PHGPx and that PHGPx is restored much more rapidly on replenishment with selenium [2,5], we applied this strategy (see the Materials and methods section) and obtained UT7 cells that exhibited almost no measurable GPx activity with t-butyl hydroperoxide as substrate but showed approx. 130% of the PHGPx activity of the control cell preparation. Because UT7 cells do not express 12-lipoxygenase, we analysed the reduction of 12-HpETE to 12-HETE in selenium-deficient and control UT7 cells. Strikingly, the selenium-deficient cells were active in reducing 12-HPETE to 12-HETE, albeit at a lower rate than the control cells (Figure 3). This observation showed clearly that PHGPx substituted for GPx-1, which was in fact absent from these cells.

Occurrence of short-lived PHGPx mRNA in human megakaryocytes

We analysed PHGPx mRNA in human megakaryocytes by RT–PCR with a specific primer set for PHGPx and obtained a positive reaction. The lysate of A431 cells was used as a positive control. The identity of the amplification product for PHGPx was established by automated nucleotide sequencing and comparison with the human gene (results not shown). In contrast, we failed to detect this amplification product under identical conditions in platelet lysates, although positive results were obtained for platelet 12-lipoxygenase mRNA (Figure 4A) and β-actin mRNA (results not shown). Because platelets lost their nucleus during megakaryopoiesis, we inferred that the absence of sizeable PHGPx mRNA in platelets could be simply a consequence of a shorter lifetime of PHGPx mRNA than that of other mRNA species. To verify this assumption we investigated the breakdown rates of PHGPx messengers in the human megakaryoblast cell line UT7 before and after differentiation by phorbol ester to megakaryocytes (see the Materials and methods section). For this purpose, transcription was stopped by the addition of actinomycin D, and the time course of the level of PHGPx mRNA was followed (Figure 4B). Surprisingly, the half-life of PHGPx mRNA was approx. 3 h in undifferentiated cells, which declined to approx. 0.5 h in differentiated cells. This observation gave a plausible explanation for the near absence of PHGPx mRNA in circulating platelets.

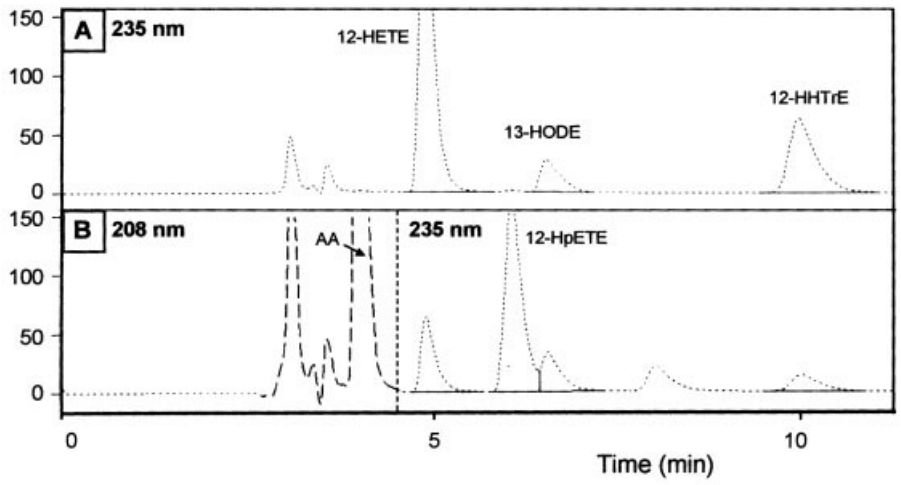

Figure 5 Representative profiles of UV-detectable arachidonic acid metabolites synthesized by untreated (A) and iodoacetate-treated (B) human platelets

Washed human platelets (3.2×10^8 cells/ml) were pretreated with 2 mM iodoacetate for 10 min at 37 °C. They were then incubated with 130 μM arachidonic acid for a further 10 min (**B**) along with the controls (**A**). Other conditions were as described in Table 2. After the reaction had been stopped, lipid extraction and straight-phase HPLC separations were performed as described in the Materials and methods section. The absorbance of the metabolites was monitored at the wavelengths at which the peak areas exhibited the highest sensitivities with the HPLC solvent used (235 nm for conjugated diene-containing metabolites, 208 nm for arachidonic acid). The fractions indicated were identified by co-migration with authentic standards as well as by their UV spectra.

Table 2 Patterns of UV-absorbing arachidonic acid metabolites of untreated and iodoacetate-treated human platelets

Platelet suspension (1.6×10^8 cells in 0.5 ml) was incubated with 130 μM arachidonic acid for 10 min at 37 °C. The reaction was stopped and the metabolites were then separated by straight-phase HPLC as described in the Materials and methods section. Their amounts were quantified by measurement of the peak area against calibration curves obtained with authentic standards. 13-HODE was used as an internal standard. For further details see the experimental conditions in the legend to Figure 6. Results are means ± S.E.M. for separate experiments with platelet preparations from different donors. The number of donors is given in parentheses.

	Quantity (nmol)		
Metabolite	Without iodoacetate	With iodoacetate	Effect of iodoacetate
Arachidonic acid	32.1 ± 2.7 (4)	35.9 ± 2.5 (4)	None
12-HpETE	0.1 ± 0.1 (6)	10.5 ± 1.6 (6)	+100-fold
12-HETE	8.6 ± 1.9 (6)	3.5 ± 0.5 (6)	−81%
12-HHTrE	4.7 ± 1.3 (6)	0.9 ± 0.2 (6)	−81%

Table 3 Effect of iodoacetate on the reduction of exogenous 12-HpETE in human platelets and UT7 cells

Cell suspensions (1.8×10^8 platelets or 1.8×10^6 UT7 cells, in 0.5 ml of medium) were pretreated with 2 mM iodoacetate and/or 10 μM ETYA for 10 min at 37 °C and then incubated with 20 μM 12-HpETE for a further 10 min. The eicosanoids were extracted and separated by straight-phase HPLC by monitoring A_{235} as described in the Materials and methods section. Each value is the sum of 12-HpETE and 12-HETE recovered; results are means for duplicate experiments.

	Pretreatment		Reaction products (%)	
Cells	Iodoacetate	ETYA	12-HpETE	12-HETE
Platelets	−	−	0	100
	+	−	64.7	35.3
	−	+	0	100
	+	+	61.6	38.3
UT7	−	−	0	100
	+	−	53	47

Taken together, our results suggest that PHGPx is expressed at early stages of megakaryopoiesis and that PHGPx protein, but not its mRNA, perseveres in mature platelets.

Effect of iodoacetate on the arachidonic acid metabolism in human platelets

To study the role of selenium-dependent glutathione peroxidases GPx-1 and PHGPx in the eicosanoid metabolism of human platelets, the cells were pretreated with 2 mM iodoacetate and subsequently reacted with 130 μM arachidonic acid. Iodoacetate has been reported to inhibit GPx-1 and PHGPx activities in A431 cells strongly without affecting the platelet-type 12-lipoxygenase at this concentration [29,30]. The HPLC patterns of UV-detectable metabolites of platelets with and without treatment with iodoacetate are shown in Figure 5. As expected, the control cells produced mainly the 12-lipoxygenase product 12-HETE and the cyclo-oxygenase product 12-hydroxy-(5*Z*,8*E*,10*E*)-heptadecatrienoic acid (12-HHTrE), whereas only traces of 12-HpETE could be detected under these conditions (Figure 5A, Table 2). Moreover, the synthesis of 12-HETE and 12-HHTrE could be blocked by pretreating platelets with 5,8,11,14-eicosatetraynoic acid (ETYA), an inhibitor of cyclo-oxygenases and lipoxygenases (results not shown). However, pretreatment of platelets with iodoacetate resulted in a huge accumulation of 12-HpETE with a concomitant drastic decrease in the formation of 12-HETE (Figure 5B, Table 2). However, the sum of the two products of the 12-lipoxygenase pathway was only slightly diminished. Iodoacetate also inhibited the formation of 12-HHTrE to some extent.

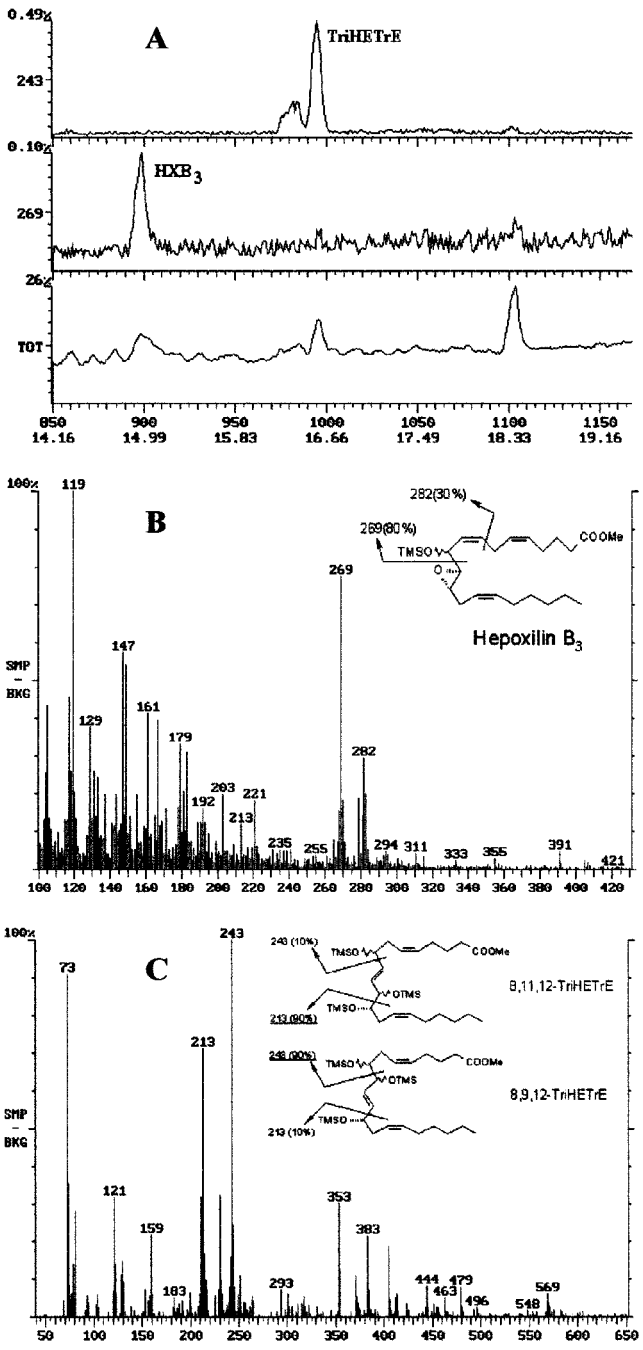

Figure 6 Hepoxilin formation in iodoacetate-treated human platelets

Washed human platelets were pretreated with 2 mM iodoacetate for 10 min at 37 °C and then incubated either for 30 min with 100 μM arachidonic acid or for 10 min with 50 μM 12-HpETE. After the reaction had been stopped with diethyl-ether-saturated 10 mM HCl, the organic phase was separated and dried down under a nitrogen stream. The residue was reconstituted in 50 μl of methanol and analysed for hepoxilins by GC–MS as described in the Materials and methods section. (**A**) Representative total-ion-current chromatogram of the *O*-trimethylsilyl derivatives of the methyl esters of a 8,9,12-TriHETrE/8,11,12-TriHETrE mixture and of HXB_3 methyl ester. (**B**) Electron-impact mass spectrum of the *O*-trimethylsilyl derivative of HXB_3 methyl ester. (**C**) Electron-impact mass spectrum of the *O*-trimethylsilyl derivatives of the methyl esters of a 8,9,12-TriHETrE/8,11,12-TriHETrE mixture. For further details see Table 4.

Pretreatment of platelets or UT7 cells with iodoacetate also suppressed the conversion of exogenous 12-HpETE into 12-HETE (Table 3). The strong inhibition by iodoacetate of the formation of 12-HETE from both exogenous and endogenously formed 12-HpETE underlines the prominent role of selenium-containing glutathione peroxidases GPx-1 and PHGPx in this reaction. However, the occurrence of a small but significant proportion of iodoacetate-resistant 12-HETE formation (Tables 2 and 3) suggests the existence of alternative catalysts. An involvement of ETYA-sensitive lipohydroperoxidase activity of the 12-lipoxygenase in intact platelets, which we observed in model experiments with pure human recombinant platelet 12-lipoxygenase (P. Shankaranarayanan, M. Sutherland and S. Nigam, unpublished work), could nevertheless be ruled out because pretreatment of the platelets with ETYA did not affect the reduction of 12-HpETE in the presence or absence of iodoacetate (Table 3).

Hepoxilin formation in iodoacetate-treated platelets

The inhibition by iodoacetate of the conversion of 12-HpETE to 12-HETE led us to consider an iodoacetate-induced diversion of the 12-lipoxygenase pathway of arachidonic acid metabolism partly or completely (depending on the extent of hydroperoxide tone) from the reduction pathway to the isomerization pathway, the latter yielding hydroxy-epoxy derivatives of arachidonic acid, the so-called hepoxilins [19]. To verify this we used a different analytical procedure in which the reaction was stopped by diethyl-ether-saturated 10 mM HCl and the products were analysed by GC–MS after the preparation of appropriate derivatives (see the Materials and methods section). Under these conditions synthetic HXA_3 was completely hydrolysed to a mixture of 8,9,12-TriHETrE and 8,11,12-TriHETrE, whereas HXB_3 remained unchanged as judged from corresponding recovery experiments. With this methodology we observed the formation of hepoxilins from 100 μM arachidonic acid in human platelets pretreated with 2 mM iodoacetate as well as from 20 or 50 μM 12-HpETE with untreated platelets, whereas we failed to detect of hepoxilin formation from arachidonic acid in untreated platelets within the limits of this method (Figure 6, Table 4). The pattern of the products identified reflected the formation of a mixture of HXA_3 and HXB_3. This observation is fully in agreement with earlier results of Bryant et al. [3], who treated platelets with glutathione-depleting agents, which suppress glutathione peroxidases by a different method. Notably, 2 mM NaCN, which has been shown to inhibit the pseudolipohydroperoxidase activity of haemoglobin, including the formation of hydroxy-epoxy fatty acids from hydroperoxy fatty acids [31], did not inhibit hepoxilin formation in platelets from arachidonic acid after pretreatment with iodoacetate or exogenous 12-HpETE (Table 4), thus excluding the involvement of non-enzymic haemin catalysis in hepoxilin formation in platelets.

With the same analytical method we also found hepoxilin formation in UT7 cells if treated with 20 μM 12-HpETE (0.4 nmol of HXA_3/10 min per 10^6 cells). Pretreatment of the cells with 2 mM iodoacetate, which inhibited both GPx-1 and PHGPx, enhanced the formation of hepoxilins by one order of magnitude (4.3 nmol HXA_3/10 min per 10^6 cells), again demonstrating that hepoxilin formation is strongly controlled by the selenium-containing glutathione peroxidases GPx-1 and PHGPx.

The formation of hepoxilins from exogenous arachidonic acid in iodoacetate-treated human platelets but not in untreated platelets was confirmed in independent experiments by separating the 9-anthranylmethyl esters of the arachidonic acid metabolites with the help of reverse-phase HPLC (see the Materials and methods section). In these experiments the addition of 1,1,3-trichloropropene oxide was necessary to inhibit the epoxide

Table 4 Hepoxilin formation in human platelets

Platelets were incubated in glucose-free PBS for the durations indicated at 37 °C with arachidonic acid in 1.0 ml (3.2×10^9 cells) or with 12-HpETE in 0.5 ml (1.6×10^9 cells). In samples containing ETYA and/or iodoacetate, the platelets were preincubated for 10 min at 37 °C with these compounds before the addition of substrate. Eicosanoid analyses by GC–MS were performed as described in the Materials and methods section. The amounts of hydrolysis products of HXA_3 and HXB_3 were quantified by peak area by using a calibration curve with authentic standards. Results are means ± S.E.M. for four separate experiments.

Substrate	Concentration (μM)	Incubation period (min)	Inhibitors	8,9,12-TriHETrE + 8,11,12-TriHETrE (nmol)	HXB_3 (nmol)
Arachidonic acid	100	30	None	< 0.1*	< 0.4*
			2 mM iodoacetate	0.4 ± 0.1	1.5 ± 0.1
			2 mM iodoacetate + 2 mM cyanide	1.0 ± 0.1	2.0 ± 0.1
			2 mM iodoacetate + 10 μM ETYA	< 0.1*	< 0.4*
12-HpETE	50	10	None	1.6 ± 0.3	3.0 ± 0.3
			10 μM ETYA	0.9 ± 0.3	1.2 ± 0.3
	20	10	None	0.4 ± 0.1	Present†
			10 μM ETYA	0.3 ± 0.1	Present†
			2 mM cyanide	0.4 ± 0.1	Present†
			10 μM ETYA + 2 mM cyanide	0.3 ± 0.1	Present†

* Not detectable qualitatively by GC–MS under the conditions used.
† Quantification was not possible owing to the small amount of HXB_3.

hydrolase activity of platelets, thus preventing the hydrolysis of HXA_3 to trioxilins (results not shown).

DISCUSSION

In the present study we have provided for the first time several lines of evidence for the occurrence of PHGPx protein and activity in human platelets: (1) enzymic activity with the specific substrate PAPC-OOH, (2) co-chromatography of this activity with the approx. 23 kDa fraction on a Sephadex G-100 column, (3) inhibition of this activity by iodoacetate, (4) a positive approx. 23 kDa band in Western blots with three different PHGPx-selective antibodies, and (5) the detection of authentic human PHGPx mRNA in megakaryocytes, the progenitor cells of platelets. The presence of PHGPx in platelets implies its possible involvement in the 12-lipoxygenase pathway in these cells. Although we found that PHGPx contributes to approx. 2% of the total 12-HpETE reductase activity of platelet cytosol, in intact cells its share might be significantly higher, because both 12-lipoxygenase and PHGPx tend to be bound to intracellular membranes, where the eicosanoid metabolism occurs preferentially. Interestingly, the reported results with GPx-1-deficient mice are not in line with the assumption that the reduction of 12-HpETE could be solely attributed to GPx-1 [32]. Thus Funk and co-workers observed no phenotypic changes in the GPx-1 knock-out mice [33]. In addition, tissues of these knock-out mice exhibited neither a lower rate of consumption of exogenously added H_2O_2 nor increased lipid peroxidation in comparison with those of wild-type mice. More importantly, they observed that platelets from GPx-1-deficient mice, when incubated with arachidonic acid at lower concentrations (25 μM), did not inhibit the formation of 12-HETE at all. However, on incubation with a higher concentration of arachidonic acid (75 μM), platelets from GPx-1-deficient mice still generated 12-HETE, albeit to a smaller extent, with a concomitant increase in polar products in comparison with those of wild-type mice. However, no trace of 12-HpETE was detected in the incubation of either concentration of arachidonic acid with platelets from either GPx-1-deficient or wild-type mice. The more polar products were probably epoxy alcohols and trihydroxy derivatives. Funk and co-workers [33] suggested that GPx-1 has a very limited contribution to the cellular antioxidant mechanism under physiological conditions and that platelets can rapidly metabolize 12-HpETE without functional GPx-1. In contrast, our results with human platelets treated with iodoacetate, which inhibits GPx-1 and PHGPx, showed an almost complete suppression of 12-HETE formation, indicating the importance of PHGPx as a 12-HpETE reductase. This discrepancy between the the two sets of observations could be explained by the fact that, in the absence of GPx-1, PHGPx is predominantly responsible for the reduction of 12-HpETE. Our results with selenium-deficient UT7 cells, which were selectively devoid of GPx-1 but still possessed a capacity to reduce 12-HpETE, albeit at a lower rate than the control cells, strongly supported this assumption (Figure 3). If Funk and co-workers had used double knock-out mice, deficient in GPx-1 and PHGPx, they would have found a similar pattern of 12-lipoxygenase metabolites of arachidonic acid to that obtained by us with iodoacetate-treated platelets.

Although iodoacetate can modify proteins by different methods, it is particularly effective in inactivating enzymes that contain a selenol [34]. Iodoacetate proved to be most appropriate for the investigation of the 12-lipoxygenase pathway in platelets because it did not inhibit the 12-lipoxygenase activity. This was ascertained by the observation that the sum of major 12-lipoxygenase metabolites analysed and the amount of unmetabolized arachidonic acid (see Table 2 and Figure 6) were not changed significantly under the conditions used. The insensitivity of the platelet 12-lipoxygenase towards iodoacetate is in fair agreement with the data of Chang and co-workers [29,30], who used A431 cell lysates. Unfortunately, no inhibitor of PHGPx with a higher specificity than iodoacetate, in particular with selectivity between various types of glutathione peroxidase, is yet available.

Our results also support the assumption that selenoenzymes modulate the arachidonic acid metabolism of platelets by lowering the hydroperoxide tone. Their inactivation led to a marked accumulation of 12-HpETE, the metabolism of which was consequently switched towards the formation of hepoxilins. However, in the absence of iodoacetate the extent of hepoxilin formation was below the limit of our detection method, which clearly showed that 12-HpETE was primarily reduced to 12-HETE by GPx-1 and PHGPx. Thus the selenocysteine-containing glutathione peroxidases turned out to be an effective enzymic system for controlling hepoxilin synthesis. To our knowledge, this role of glutathione peroxidases in the biosynthesis of hepoxilins has not previously been addressed.

The absence of PHGPx mRNA from platelets is seemingly due to the relatively short half-life of this mRNA, as shown with UT7 cells stimulated by phorbol ester (Figure 4). The putative short half-life of PHGPx mRNA suggests that the expression of this enzyme is a well-regulated process. In some cells the expression of PHGPx seems to be closely related to the expression of lipoxygenases. Thus Schnurr et al. demonstrated in A549 cells and in human monocytes an up-regulation of 15-lipoxygenase and a concomitant down-regulation of PHGPx by interleukin 4 [35]. This example indicates that lipoxygenases and PHGPx are coupled to each other not only metabolically but also with regard to the regulation of their gene expression. Because platelet-type 12-lipoxygenase and PHGPx, but not GPx-1, share a marked tendency to bind to membranes under certain conditions [15], which permits their joint localization at intracellular membranes, an important role in eicosanoid biosynthesis can be derived for PHGPx. In addition, in A431 cells the platelet-type 12-lipoxygenase has been reported to be up-regulated by epidermal growth factor [36], and PHGPx is generally capable of modulating eicosanoid metabolism by lowering the hydroperoxide tone, which has been demonstrated *in vivo* [5], in intact cells [5,7], in sub-cellular systems [6] and with isolated enzymes [6,37]. Therefore, it is reasonable to assume that PHGPx is integrated in a fine-tuning network of the regulation of the 12-lipoxygenase pathway of arachidonic acid metabolism in certain cells.

Hepoxilins, in particular HXA_3, exert a number of biological effects on mammalian cells such as secretion of hormones and other membrane transport processes, and activation of second messenger systems (for review see ref. [19]). Moreover, we recently observed that non-esterified HXA_3 is a potent chemoattractant for neutrophils [38]. In platelets, HXA_3 has been reported to inhibit the ADP-induced aggregation and to mediate a regulatory volume decrease [39]. Therefore, it is reasonable to assume, that the diversion of 12-lipoxygenase pathway from the reduction route to the isomerization route in severe oxidative stress could constitute a compensatory mechanism to maintain the functional integrity of platelets under these conditions. HXA_3 has also been reported to induce the heat-shock protein HSP72 in human neutrophils [40]. Since heat-shock proteins have been suggested to protect cells from reactive oxygen species as well [41], the latter effect may be implicated in the compensatory role of hepoxilin formation under oxidative stress conditions.

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