Effects of reduced glutathione on the 12-lipoxygenase pathways in rat platelets

Wen-Chang CHANG,*† Junko NAKAO,‡ Hajime ORIMO‡ and Sei-itsu MUROTA*

*Department of Pharmacology, Tokyo Metropolitan Institute of Gerontology, and ‡Department of Internal Medicine, Tokyo Metropolitan Geriatric Hospital, Itabashi-ku, Tokyo-173, Japan

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Arachidonic acid is converted into several more polar products in addition to 12-L-hydroperoxyeicosa-5,8,10,14-tetraenoic acid (12-HPETE) and 12-L-hydroxyeicosa-5,8,10,14-tetraenoic acid (12-HETE) by the cytosol fractions of rat platelets. The more polar products are formed via the lipoxygenase pathways in the same way as are 12-HPETE and 12-HETE, since their formation is not inhibited by indomethacin but by eicosa-5,8,11,14-tetraenoic acid (ETYA). The presence of 0.5–1.5 mM-reduced glutathione (GSH) in the reaction mixture prevents the formation of the more polar products and produces 12-HETE as the only metabolite from arachidonic acid by the 12-lipoxygenase pathway. L-Cysteine has the same effect as GSH. However, oxidized glutathione (GSSG) and L-cystine are not able to prevent the formation of the more polar products. The results indicate that 12-HPETE peroxidase in the 12-lipoxygenase pathway is a GSH-dependent peroxidase and the more polar products might be formed from the non-enzymic breakdown of the primary 12-lipoxygenase product of 12-HPETE, owing to insufficient capability of the subsequent peroxidase system to completely reduce 12-HPETE to 12-HETE. Thus the presence of GSH in the reaction mixture offers a convenient and precise cell-free assay system for 12-lipoxygenase in rat platelets. Routine assays of 12-lipoxygenase are carried out in the presence of 1 mM-GSH in the reaction mixture. The synthesis of 12-HETE by 12-lipoxygenase is linear during the first 4 min of incubation at 37°C, and has a pH optimum of 7.7. The 12-lipoxygenase reaches half-maximal activity at an arachidonate concentration of 20 μ M. Fractionation of cell homogenates indicates that the cytosol fraction possesses almost all the 12-lipoxygenase activity, whereas the microsomal fraction exhibits little enzyme activity.

In many tissues and cells, two distinct enzymic pathways exist for the oxygenation of arachidonic acid. Prostaglandins, prostacyclin and thromboxanes are formed by the cyclo-oxygenase pathways, whereas the various polyunsaturated hydroxy

Abbreviations used: 12-HPETE, 12-L-hydroperoxyeicosa-5,8,10,14-tetraenoic acid; 12-HETE, 12-L-hydroxyeicosa-5,8,10,14-tetraenoic acid; ETYA, eicosa-5,8,11,14-tetraenoic acid; HHT, 12-L-hydroxyheptadeca-5,8,10-trienoic acid; 8,9,12-THETA, 8,9,12-trihydroxyeicosa-5,10,14-trienoic acid; 8,11,12-THETA, 8,11,12-trihydroxyeicosa-5,9,14-trienoic acid; EHPA, 11,12-epoxy-10-hydroxyeicosa-5,8,14-trienoic acid; GSH, reduced glutathione; GSSG, oxidized glutathione.

† Present address and address for correspondence and reprint requests: Division of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, University of Kentucky, Lexington, KY 40536-0053, U.S.A.

fatty acids are formed by the lipoxygenase pathways. In washed platelets, arachidonic acid is converted into HHT and thromboxane B₂ by the cyclo-oxygenase pathways and into 12-HETE by the lipoxygenase pathways (Hamberg & Samuelsson, 1974). In addition to the formation of 12-HETE, arachidonic acid is also converted by washed platelets from man, horse and dog into a mixture of 8,9,12-THETA and 8,11,12-THETA (Jones *et al.*, 1978; Bryant & Bailey, 1979) and EHPA by washed human platelets (Bryant & Bailey, 1980a).

In this investigation, we have characterized the lipoxygenase pathways in a cell-free system of rat platelets and developed a simple precise radiometric assay for 12-lipoxygenase in rat platelets. Evidence is presented showing the direct involvement of GSH in 12-lipoxygenase pathways in platelets.

Materials and methods

Chemicals

[1-¹⁴C]Arachidonic acid (sp. radioactivity 58 Ci/mol) was purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Arachidonic acid and indomethacin were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. ETYA was generously provided by Nippon Roche K.K., Tokyo, Japan. GSH and GSSG were purchased from Boehringer-Mannheim G.m.b.H., Germany, and P-L Biochemicals, Milwaukee, WI, U.S.A., respectively. L-Cysteine, L-cystine, dithiothreitol, thioglycolic acid and bovine serum albumin (fraction V) were obtained from Wako Pure Chemical Industries, Tokyo, Japan. 2-Mercaptoethanol was purchased from Tokyo Kogyo Co., Tokyo, Japan. Prostaglandin and thromboxane standards were gifts from Ono Pharmaceutical Co., Osaka, Japan. T.l.c. plates of silica gel 60 F₂₅₄, 0.25 mm in thickness, were purchased from E. Merck, Darmstadt, Germany. All reagents not specified above were of analytical grade.

Preparation of platelets

Blood was withdrawn into a 3.8% solution of sodium citrate (9:1, v/v) from the abdominal aorta of male Wistar rats aged 9–10 weeks under diethyl ether anaesthesia. Platelet-rich plasma was prepared by centrifugation of citrated blood at 200g for 10 min at room temperature. Platelet pellets were obtained by centrifuging the platelet-rich plasma at 1000g for 10 min at room temperature.

Preparation of cytoplasmic supernatant fractions

Platelet pellets were resuspended in 25 mM-Tris/HCl buffer (pH 7.7) and freeze-thawed three times by using liquid N₂ and a water bath at 37°C respectively. These preparations were centrifuged at 105 000g for 60 min. The resulting supernatants were designated the cytoplasmic supernatant fractions (cytosols). For experiments of subcellular localization of 12-lipoxygenase, the preparations were centrifuged at 10 000g for 20 min. The supernatants were then ultracentrifuged as described above, and the resulting precipitates were designated the microsomal pellets. Unless otherwise stated, the cytoplasmic supernatant fractions were used as 12-lipoxygenase source throughout the study.

Enzyme assay

Platelet lipoxygenase activity was assayed with [1-¹⁴C]arachidonic acid as substrate. The assay mixture contained 12 µg of [1-¹⁴C]arachidonic acid (0.2 µCi/assay) and cytosol fraction in a final volume of 1 ml of 25 mM-Tris/HCl buffer (pH 7.7). Incubations were performed in air at 37°C for 2 min, unless otherwise stated, with shaking. The

incubations were stopped by quickly adding an appropriate amount of 1 M-HCl to bring the pH of the reaction mixture to 3.0. The reaction mixture was then extracted with 10 ml of ethyl acetate. The resulting organic phase was evaporated to dryness under reduced pressure. Residues were dissolved in a small amount of ethanol and applied to t.l.c. plates. The plates were developed in a solvent system of ligroin/diethyl ether/acetic acid (50:50:1, by vol.). The procedures of t.l.c. were performed at room temperature unless otherwise stated. The radioactive products were detected by a Dünnschicht scanner. In some cases, the developed plates were autoradiographed by using Fuji X-ray film no. Kx medical. Reference standards of prostaglandins and arachidonic acid were detected visually by using 10% phosphomolybdic acid in ethanol. Zones corresponding to the radioactive products were extracted with chloroform/methanol (1:1, v/v) for further analysis or scraped into a scintillation vial for determination of radioactivity. For the preparation of 12-HPETE from [1-¹⁴C]arachidonic acid transformed by platelet lipoxygenase, the incubation conditions and product purification were performed by the same methods described above, except that all procedures including extraction and separation after the incubation reaction were carried out at 4°C to avoid the breakdown of 12-HPETE. Details of the procedures for extraction, separation and identification of arachidonate metabolites have been described previously (Chang *et al.*, 1976a,b, 1977).

Reduction with NaBH₄

Samples were dissolved in 2 ml of methanol precooled to 0°C and 10 mg of NaBH₄ was added. Reactions were allowed to proceed at 0°C for 20 min and then at room temperature for 60 min. The reaction mixtures were diluted with 2 ml of water, acidified to pH 3.0 with 1 M-HCl and extracted three times with diethyl ether. The organic layer was evaporated to dryness under a stream of N₂.

Preparation of derivatives

Methylation. Diazomethane was freshly prepared from *N*-methyl-*N*-nitro-*N*-nitrosoguanidine by the method of Fales *et al.* (1973). Samples were dissolved in 0.1 ml of methanol, 0.9 ml of ethereal diazomethane was added and they were placed in the dark for 60 min. After this interval, the solvent was evaporated to dryness under a stream of N₂.

Trimethylsilylation. Trimethylsilyl imidazole/bis(trimethylsilyl) trifluoroacetamide (2:1, v/v; 50 µl) was added to each sample and the reaction was allowed to proceed for 2 h at room temperature.

Gas chromatography-mass spectrometry

A Shimatsu-LKB 9000B combined gas chromato-

graph-mass spectrometer equipped with a column (1m x 2mm) of 3% OV-101 on Celite 545 was employed. The temperature of column and ion source were maintained at 220°C and 310°C respectively. The electronic energy and accelerating voltage were kept at 70eV and 3.5kV respectively. The carrier gas was helium with a flow rate of 30ml/min.

Protein determinations

Protein contents were determined by the method of Lowry *et al.* (1951) with bovine serum albumin (fraction V) as standard.

Results

Transformation of [1^{14}C]arachidonic acid by platelet lipoxygenase

When platelet cytosol fraction was incubated with [1^{14}C]arachidonic acid, several radioactive metabolites in the polar region of the thin-layer chromatogram were observed as shown in Fig. 1. The least-polar two products, 12-HPETE and 12-HETE, migrated with an R_F value of 0.43 and 0.40 respectively. Peaks that were more polar than 12-HETE, including the one at the origin, were not identified. They were designated the 'more polar products'. The more polar products constituted 50–60% of total products transformed from arachidonic acid by platelet cytosols.

Fig. 1. Transformation of [1^{14}C]arachidonic acid by platelet cytosol fraction

Abbreviations used: D₂, prostaglandin D₂ standard; AA, arachidonic acid standard.

Identification of 12-HPETE and 12-HETE

Samples of 12-HETE and 12-HPETE peaks, extracted from the thin-layer plates, were treated with NaBH₄ and chromatographed on a thin layer of silica gel. The chromatograph showed that the 12-HETE peak is inert to this reduction, whereas the 12-HPETE peak co-chromatographs with the 12-HETE peak after reduction. The structure of the 12-HETE peak was then identified by gas chromatography-mass spectrometry. The mass spectrum of 12-HETE exhibited ions at 391 ($M-15$), 375 ($M-31$), 316 ($M-90$), 295 ($M-111$), 229 and 205 (295–90). The data are in agreement with the published spectrum of 12-HETE synthesized by washed human platelets (Hamberg & Samuelsson, 1974).

Effect of inhibitors on the formation of reaction products

Since the more polar unidentified products significantly constitute the reaction products transformed from arachidonic acid by platelet cytosol fractions, it is necessary to confirm whether they are formed via lipoxygenase pathways. Indomethacin, a fatty acid cyclo-oxygenase inhibitor (Vane, 1971), showed no significant effect on the formation of 12-HPETE, 12-HETE and the more polar products, when it was present at 20 μM during the reaction, whereas ETYA, a lipoxygenase and fatty acid cyclo-oxygenase inhibitor (Hammarström, 1977), showed a dose-dependent inhibition of the formation of reaction products. The formation of all reaction products was almost completely inhibited by 40 μM -ETYA. The results indicated that all of the reaction products transformed from arachidonic acid by platelet cytosol fractions are formed via lipoxygenase pathways. When the purified 12-HPETE was chromatographed at room temperature, formation of the more polar products was observed. The breakdown profile of 12-HPETE was almost the same as that shown in Fig. 1. Therefore, formation of the more polar products might be derived from the non-enzymic breakdown of 12-HPETE during reaction due to the insufficient capability of 12-HPETE peroxidase in the lipoxygenase pathways.

Effect of thiol and disulphide compounds on the transformation of arachidonic acid by platelet lipoxygenase

When GSH in a range between 0.5 and 6 mM was included during the reaction, 12-HETE was the only metabolite of arachidonic acid found. 12-HPETE and the more polar products were not observed as shown in Fig. 2. GSH at 0.5, 1 and 1.5 mM showed no significant effect on the total lipoxygenase activity. However, a significant inhibition was observed at 3 and 6 mM, as shown in Fig. 3. GSH under 0.5 mM (at 0.1 and 0.05 mM) showed no

significant effects on the total lipoxygenase activity and the prevention of formation of the more polar products. Effects of other thiol and disulphide compounds are indicated in Table 1. L-Cysteine had the same effect as GSH. 2-Mercaptoethanol slightly

Fig. 2. Effect of GSH on the transformation of [1-¹⁴C]arachidonic acid by platelet cytosol fraction. GSH (3 mM) was present in the reaction mixture during reaction. Abbreviations are defined in the text and in the legend to Fig. 1.

Fig. 3. Effect of GSH concentration on 12-lipoxygenase activity.

Incubation was carried out in the absence or in the presence of various concentrations of GSH. The lipoxygenase activity is expressed by the percentage of 12-HETE formation in the presence of GSH as a proportion of the total transformation including 12-HPETE, 12-HETE and the more polar products from arachidonic acid in the absence of GSH (control). Each point is the mean of duplicate assays, and the deviation was less than 10%.

Table 1. Effect of thiol and disulphide compounds on the transformation of arachidonic acid by platelet lipoxygenase. Each value is the mean result for duplicate assays, and the deviation was less than 10%. Values in parentheses are percentages compared with those of the GSH-treated group. Total lipoxygenase activity represents the sum of 12-HETE, 12-HPETE and more polar products.

Compound	Concn. (mM)	Product formation (nmol/min per mg)			Total lipoxygenase activity (nmol/min per mg)
		12-HETE	12-HPETE	More polar products	
Control		1.1 (31%)	0.6	2.0	3.7 (100%)
Thiols					
GSH	1	3.6 (100%)	0	0	3.6 (100%)
L-Cysteine	1	3.6 (100%)	0	0	3.6 (100%)
2-Mercaptoethanol	1	2.9 (82%)	0	0	2.9 (82%)
Dithiothreitol	1	0.6 (17%)	0	28.1*	
Thioglycolic acid	1	0.4 (11%)	0	26.4*	
Disulphides					
GSSG	1	0.9 (25%)	0.6	2.5	4.0 (111%)
	0.5	1.0 (28%)	0.7	1.8	3.5 (97%)
L-Cystine	1	0.9 (25%)	0.4	2.3	3.6 (100%)
	0.5	1.2 (33%)	0.5	2.1	3.8 (105%)

* Over 90% of the products were non-enzymically formed. See the Results section for details.

inhibited the total lipoxygenase activity, though it was able to prevent the formation of the more polar products. Other thiol compounds tested (dithiothreitol and thioglycolic acid) failed to protect the formation of 12-HETE. They induced a tremendously non-enzymic breakdown of arachidonic acid into several more polar products, since the formation of more polar products was not inhibited by indomethacin nor by EYTA and could be observed under the conditions of incubation with boiled enzyme or buffer instead of viable enzyme source. In the case of disulphide compounds, GSSG and L-cystine failed to prevent the formation of more polar products. Routine assay of 12-lipoxygenase in the following experiments was then carried out in the presence of 1 mM-GSH in the reaction mixture, and the 12-lipoxygenase activity in platelets was expressed by the formation of 12-HETE.

Assay conditions for platelet lipoxygenase with [$1\text{-}^{14}\text{C}$]arachidonic acid as substrate

The amount of 12-HETE produced as a function of incubation time was linear during the first 4 min of incubation, and 2 min of incubation was therefore chosen. A linear relationship between cytosol protein and the amount of 12-HETE was observed up to 0.7 mg of protein content. All subsequent experiments were carried out within this range of protein concentrations. The effect of pH on the rate of 12-HETE biosynthesis was then studied. The rate of 12-HETE formation was optimal at pH 7.7, and a pH value of 7.7 was therefore chosen for routine assays. The dependence of the rate of the reaction on arachidonic acid concentrations was examined. An apparent K_m of $20\text{ }\mu\text{M}$ for arachidonic acid was

estimated from a Lineweaver–Burk double-reciprocal plot of the reaction rate versus the substrate concentration as shown in Fig. 4. The subcellular localization of 12-lipoxygenase was investigated under these assay conditions. About 94% of the 12-lipoxygenase appeared in the soluble cytoplasmic fractions, and only 6% appeared in the microsomal fractions.

Discussion

In a cell-free system, 12-lipoxygenase, which transforms arachidonic acid into 12-HETE, was first found in the soluble fraction of bovine platelets by Nugteren (1975). Siegel *et al.* (1979) recently investigated the effects of aspirin-like drugs on 12-lipoxygenase and 12-HPETE peroxidase activities in cytosol fractions of human platelets, and they described 12-HPETE and 12-HETE as the only metabolites of arachidonic acid by 12-lipoxygenase. We performed the same experiments as the methods described by Siegel *et al.* (1979) in rat platelets and found that several more polar products in addition to 12-HPETE and 12-HETE are formed by rat platelet lipoxygenase (Fig. 1). All of the more polar products are formed by lipoxygenase since their formation is inhibited by EYTA and is inert to indomethacin. The structures of the more polar products were not identified in the present study, but we presume that they are composed of 8,9,12-THETA, 8,11,12-THETA, EHPA and other unknown products. They might be formed from the non-enzymic breakdown of the primary 12-lipoxygenase product of 12-HPETE due to the insufficient capability of the subsequent peroxidase system to completely reduce 12-HPETE to 12-HETE, since the breakdown products of purified 12-HPETE show the same pattern as these compounds. In agreement with our findings, Sun *et al.* (1981) recently reported the formation of 8,11,12-THETA from arachidonic acid by the cytosol fraction of human platelets. As shown in Fig. 1, the more polar products constitute a large part of the 12-lipoxygenase-derived products; it is thus impossible to neglect them during the assay of 12-lipoxygenase.

The enzymes in the lipoxygenase pathways in human platelet cytosol have been partially purified by Siegel *et al.* (1980), and they found aspirin-like anti-inflammatory drugs inhibit 12-HPETE peroxidase activity. They therefore concluded that glutathione peroxidase is not a likely candidate for the source of 12-HPETE peroxidase activity because human glutathione peroxidase is not inhibited by aspirin and indomethacin, though some reagents that modify sulphhydryl moieties show an inhibitory effect on lipoxygenase activity. In the present investigation, direct evidence shows the dependence of peroxidase activity in the lipoxygenase pathways on

Fig. 4. Effect of substrate concentration on 12-lipoxygenase activity

Each assay mixture contained 1 ml of enzyme source (0.66 mg of cytosol protein), 1 mM-GSH and various concentrations of [$1\text{-}^{14}\text{C}$]arachidonic acid, and the incubation was carried out at 37°C for 2 min. A Lineweaver–Burk plot is shown on the right.

GSH. GSH prevents the formation of the more polar products, and produces 12-HETE as the only metabolite from arachidonic acid (Fig. 2). L-Cysteine has the same effect as GSH. However, GSSG and L-cystine fail to prevent the formation of more polar products (Table 1). Therefore, our results indicate the direct involvement of glutathione-dependent peroxidase in the lipoxygenase pathways in rat platelets. In accordance with our results, Bryant & Bailey (1980b) recently reported that washed platelets from selenium-deficient rats show a 3–4-fold increase in synthesis of isomeric trihydroxy fatty acids converted from arachidonic acid and suggested that the result is due to a limitation of selenium-dependent glutathione peroxidase. Since GSH can be involved in the lipoxygenase pathways in platelets, the presence of GSH at a concentration that is able to prevent the formation of the more polar products (but at a concentration that does not inhibit the 12-lipoxygenase activity in platelet lipoxygenase assay mixtures) would be more convenient and elicit the precise information during the assays of 12-lipoxygenase in rat platelets. Our results in this investigation offer a good assay system for 12-lipoxygenase in the cytosol fraction of rat platelets.

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