Positional- and stereo-selectivity of fatty acid oxygenation catalysed by mouse (12S)-lipoxygenase isoenzymes

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A quantitative stereochemical analysis of the products generated by recombinant mouse (12S)-lipoxygenase isoenzymes was performed with arachidonic acid and linoleic acid as substrates. The leucocyte-type (12S)-lipoxygenase generated, in addition to 12-hydroxyeicosatetraenoic acid (12-HETE) as the main product, 15- and 8-HETE from arachidonic acid and 13- and 9-hydroxyoctadecadienoic acid (13- and 9-HODE) from linoleic acid. The platelet-type enzyme oxygenated arachidonic acid to 12- and 8-HETE and linoleic acid to 13- and 9-HODE, whereas the epidermis-type (12S)-lipoxygenase reaction was essentially mono-specific with arachidonic acid but oxygenated linoleic acid to both 13- and 9-HODE. 12-HETE and 13-HODE were almost exclusively the *S* enantiomers. 8-HETE was the *R* enantiomer as a side-product of the platelet-type (12S)-lipoxygenase reaction but the *S* enantiomer as a side-product of the leucocyte-type reaction. 9-HODE was generated as the *R* enantiomer by the platelet-type and the epidermis-type isoenzymes and as the *S* enantiomer by the leucocyte-type (12S)-lipoxygenase. On the

basis of published models of lipoxygenase–substrate interaction, the stereochemistry of the products generated by the platelet- and epidermis-type (12S)-lipoxygenases is in agreement with a fixed ‘tail-to-head’ orientation of the substrate fatty acid in the binding pocket of these enzymes, whereas that of the reaction products of the leucocyte-type (12S)-lipoxygenase can be explained only when the inverse orientation of the substrate or a rotational isomerism along the longitudinal axis of the substrate is allowed. Both the product spectra generated and the sensitivity towards the 12-lipoxygenase selective inhibitors *N*-benzyl-*N*-hydroxy-4-phenylpentanamide and cinnamyl-3,4-dihydroxy- α -cyanocinnamate indicated the platelet-type and the epidermis-type isoenzymes to be biochemically more related to each other than to the leucocyte-type (12S)-lipoxygenase.

Key words: arachidonic acid, dioxygenation, epidermis, linoleic acid.

INTRODUCTION

Lipoxygenases are a family of structurally related non-haem iron-containing enzymes that insert molecular oxygen into *cis,cis*-1,4-pentadiene-containing polyunsaturated fatty acids [1,2]. Depending on the position of oxygen insertion into arachidonic acid, mammalian lipoxygenases are classified as 5-, 8-, 12- and 15-lipoxygenases. Primary reaction products are 5-, 8-, 12- and 15-hydroperoxyeicosatetraenoic acids (HpETEs) which are reduced to the corresponding hydroxyeicosatetraenoic acids (HETEs) or converted into various other types of eicosanoids including leukotrienes, lipoxins and hepoxilins. Oxygen insertion occurs with high enantioselectivity in that the main reaction products possess *S*-chirality. Only recently has a (12R)-lipoxygenase generating enantiomeric (12R)-HpETE been identified in mammalian skin [3–5].

The multiplicity of lipoxygenases is further increased by the identification of individual isoenzymes with similar positional specificities: two (15S)-lipoxygenase isoenzymes [6,7] and three (12S)-lipoxygenase isoenzymes [8] have been described. We and others have cloned three cDNA species from mouse tissues, encoding the leucocyte-type, the platelet-type and the epidermis-type 12-lipoxygenases, and expressed them as recombinant proteins [9–13]. After their cloning and expression there is a need for a detailed comparison of the three recombinant mouse (12S)-lipoxygenase isoenzymes regarding the differences and/or similarities in catalytic activity, regioselectivity and stereoselectivity and effects of inhibitors so as to be able to distinguish between these isoenzymes *in vivo*.

Here we describe a qualitative and quantitative analysis of the reaction products obtained by the three isoenzymes with free arachidonic acid and linoleic acid as substrates.

EXPERIMENTAL

Materials

(8S)- and (8R)-hydroxyeicosa-(5Z,9E,11Z,15Z)-tetraenoic acid [(8S)- and (8R)-HETE], (12S)- and (12R)-hydroxyeicosa-(5Z,8Z,10E,14Z)-tetraenoic acid [(12S)- and (12R)-HETE], (9S)- and (9R)-hydroxyoctadeca-(10E,12Z)-dienoic acid [(9S)- and (9R)-HODE] and (13S)- and (13R)-hydroxyoctadeca-(9Z,11E)-dienoic acid [(13S)- and (13R)-HODE] standards were from Reatec (Weiterstadt, Germany). *N*-Benzyl-*N*-hydroxy-4-phenylpentanamide (BHPP) was a gift from Dr K. V. Honn (Wayne State University, Detroit, MI, U.S.A.); cinnamyl-3,4-dihydroxy- α -cyanocinnamate (CDC) was purchased from Biomol (Hamburg, Germany). All other chemicals and solvents were of analytical grade. SEP-PAK C₁₈ cartridges were from ICT (Bad Homburg, Germany).

Expression of 12-lipoxygenase cDNA

For transient expression, plasmid DNA (10 μ g) and vector DNA as control were introduced into HEK-293 cells (seeded at 1.5×10^6 cells per 100 mm plate) by a modified calcium phosphate transfection procedure with a mammalian transfection kit, in accordance with the manufacturer’s protocol (Stratagene, La Jolla, CA, U.S.A.). Cells were harvested 48 h after transfection.

Abbreviations used: BHPP, *N*-benzyl-*N*-hydroxy-4-phenylpentanamide; CDC, cinnamyl-3,4-dihydroxy- α -cyanocinnamate; 8-, 12- and 15-HpETE, 8-, 12- and 15-hydroperoxyeicosatetraenoic acids; 8-, 12- and 15-HETE, 8-, 12- and 15-hydroxyeicosatetraenoic acids; 9- and 13-HpODE, 9- and 13-hydroperoxyoctadecadienoic acids; 9- and 13-HODE, 9- and 13-hydroxyoctadecadienoic acids.

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Preparation of 10000 g supernatants

Transfected cells were homogenized by sonication (Branson Sonifier, 10 pulses of 6 s on ice) in TEE buffer [50 mM Tris/HCl (pH 7.4)/1 mM EDTA/1 mM EGTA/10 µg/ml leupeptin]. The supernatant received after centrifugation (10000 g for 15 min at 4 °C) was used for the lipoxigenase assay.

Lipoxigenase assay

Aliquots of 250 µl of 10000 g supernatants were incubated with 100 µM arachidonic acid or linoleic acid for 12 min at 37 °C in the presence or absence of various concentrations of inhibitors dissolved in ethanol. The incubations were terminated by the addition of 40 µl of 1 M sodium formate buffer, pH 3.1. To reduce the hydroperoxy acids to hydroxy acids, 20 µl of trimethylphosphite was added and incubated at room temperature for 5 min. Samples were extracted twice by ethyl acetate. The combined organic extracts were evaporated to dryness, redissolved in 0.75 ml of ethanol and diluted to a final ethanol concentration of 15% (v/v) by the addition of 0.1 M sodium formate buffer. An enriched HETE or HODE fraction was obtained by solid-phase extraction by the method of Powell [14].

Analysis of HETEs and HODEs

For HPLC, a Zorbax Sil column (250 mm × 4.6 mm, 5 µm particle size; Bischoff, Stuttgart, Germany) was used. HETEs and HODEs were eluted at 1 ml/min with a solvent system of n-hexane/propan-2-ol/acetic acid/water (98.5:1.5:0.1:0.025, by vol.). The eluate was monitored at 236 nm. Individual HETEs and HODEs separated by straight-phase analysis were analysed by chiral-phase chromatography with a Chiracel column (250 mm × 4.6 mm, 5 µm particle size; Baker Instruments Corp., Deerfield, IL, U.S.A.) with the solvent system n-hexane/propan-2-ol/acetic acid (99:1:0.05, by vol.) for elution of the *R* and *S* enantiomers of 15- and 12-HETE and 13-HODE, and the solvent system n-hexane/propan-2-ol/acetic acid (97.5:2.5:0.05, by vol.) for elution of the *R* and *S* enantiomers of 8-HETE and 9-HODE. The flow rate of the solvent systems was 0.5 ml/min; the eluate was monitored at 236 nm. The recovery was 57 ± 7% for HETEs and 61 ± 11% for HODEs (means ± S.E.M.).

GLC/MS

Derivatization of the HETEs and HODEs and qualitative GLC/MS were performed as described previously [15].

RESULTS

Determination of enzymic activity

HEK-293 cells were transiently transfected with the pcDNA3 expression vector carrying the cDNA encoding the leucocyte-type, platelet-type or epidermis-type (12*S*)-lipoxigenase. At 48 h after transfection, the cells were homogenized by ultrasonication and the enzymic activity was determined in 10000 g supernatants of the homogenates. When incubated with saturating concentrations of arachidonic acid for 12 min, i.e. during the linear time course of product formation, a linear relationship between the formation of 12-HETE and the sample's protein concentration of up to 0.2 mg/ml supernatant for both the leucocyte-type and the platelet-type enzyme and of 0.5–5.0 mg/ml supernatant for the epidermis-type isoenzyme was observed. The specific activities of leucocyte-type and platelet-type (12*S*)-lipoxigenases were approx. 600-fold and 300-fold that of the epidermis-type (12*S*)-lipoxigenase respectively (Table 1). With linoleic acid as a

Table 1 Specific enzymic activities and reaction products of recombinant mouse (12*S*)-lipoxigenase isoenzymes with arachidonic acid as substrate

Supernatants (10000 g) from sonicated HEK-293 cells transfected with leucocyte-type (0.1 mg/ml protein), platelet-type (0.2 mg/ml protein) or epidermis-type (5 mg/ml protein) (12*S*)-lipoxigenase cDNA, or pcDNA3 vector alone (5 mg/ml protein) were incubated in TEE buffer with 100 µM arachidonic acid for 12 min at 37 °C. The hydroperoxy derivatives of the fatty acids were reduced and extracted by the method of Powell [14]; the ethyl acetate fraction was analysed by straight-phase HPLC as described in the legend to Figure 1. Activities were measured by comparison of the areas of the 8-, 12- and 15-HETE peaks with corresponding standards. The amounts of 8-, 12- and 15-HETE generated by vector-transfected cells were subtracted. Results are means ± S.D. for 3 ≤ *n* ≤ 5 experiments. Abbreviation: n.d., not detectable.

(12 <i>S</i>)-Lipoxigenase type	Reaction products (pmol/min per mg of protein)		
	12-HETE	15-HETE	8-HETE
Leucocyte	13580 ± 2250	1020 ± 105	185 ± 25
Platelet	7655 ± 950	n.d.	22 ± 9
Epidermis	23 ± 5	n.d.	n.d.

Table 2 Specific enzymic activities and reaction products of recombinant mouse (12*S*)-lipoxigenase isoenzymes with linoleic acid as substrate

Supernatants (10000 g) from sonicated HEK-293 cells transfected with leucocyte-type (0.15 mg/ml protein), platelet-type (1.0 mg/ml protein) or epidermis-type (5 mg/ml protein) (12*S*)-lipoxigenase cDNA, or pcDNA3 vector alone (5 mg/ml protein) were incubated in TEE buffer with 100 µM linoleic acid for 12 min at 37 °C. The hydroperoxy derivatives of the fatty acids were reduced and extracted by the method of Powell [14]; the ethyl acetate fraction was analysed by straight-phase HPLC as described in the legend to Figure 2. Activities were measured by comparison of the areas of the 9- and 13-HODE peaks with corresponding standards. The amounts of 9- and 13-HODE generated by vector-transfected cells were subtracted. Results are means ± S.D. for 3 ≤ *n* ≤ 5 experiments.

(12 <i>S</i>)-Lipoxigenase type	Reaction products (pmol/min per mg of protein)	
	13-HODE	9-HODE
Leucocyte	1420 ± 55	40 ± 5
Platelet	185 ± 60	4.4 ± 0.8
Epidermis	15 ± 5	4.2 ± 2.5

substrate, 13-HODE formation was linear up to a protein concentration of 0.2 mg/ml for the leucocyte-type lipoxigenase, up to 5 mg/ml for the platelet-type enzyme and between 4 and 5 mg/ml protein for the epidermis-type enzyme. Again, leucocyte-type 12-lipoxigenase exhibited the highest enzymic activity, being approx. 8-fold and 80-fold more efficient than the platelet-type and epidermis-type enzymes respectively (Table 2).

Product analysis

Although the positional specificity of oxygen insertion is thought to be a characteristic property of individual lipoxigenases, it is known from previous studies that the pig and mouse leucocyte-type (12*S*)-lipoxigenases can direct oxygen into the 12- and the 15-positions of arachidonic acid [9,10,16]. A more detailed product analysis of the mouse isoenzyme showed that, in addition to 12-HETE as the main product, 15-HETE was the predominant and 8-HETE a minor side-product (Figure 1 and Table 1). In contrast, platelet-type (12*S*)-lipoxigenase generated almost exclusively 12-HETE with very small amounts of 8-HETE as a side product; 12-HETE was the only reaction product generated by

Figure 1 Straight-phase HPLC analysis of lipid extracts obtained after incubation with arachidonic acid of 10000 *g* supernatants from sonicated HEK-293 cells transiently transfected with leucocyte-type (A), platelet-type (B) or epidermis-type (C) (12*S*)-lipoxygenase expression constructs or with pcDNA vector alone (D)

Supernatants (10000 *g*) from sonicated HEK-293 cells transfected with pcDNA3 expression constructs containing epidermis-type, leucocyte-type or platelet-type (12*S*)-lipoxygenase cDNA or with pcDNA3 vector alone were incubated in TEE buffer with 100 μ M arachidonic acid for 12 min at 37 °C. The protein concentration in each assay was 0.1 mg/ml protein [leucocyte-type (12*S*)-lipoxygenase], 0.2 mg/ml protein [platelet-type (12*S*)-lipoxygenase], 5 mg/ml protein [epidermis-type (12*S*)-lipoxygenase] and 5 mg/ml protein (vector). HpETEs were reduced and extracted by the method of Powell [14]. The ethyl acetate fraction was dried under vacuum, redissolved in *n*-hexane/propan-2-ol/acetic acid/water (98.5:1.5:0.1:0.025, by vol.), injected on a Zorbax Sil column and eluted at 1 ml/min. The eluate was monitored at 236 nm. Authentic 8-, 12- and 15-HETE and 9- and 13-HODE were used as standards. The identities of HETEs were confirmed by GC/MS.

epidermis-type (12*S*)-lipoxygenase (Figure 1 and Table 1). When linoleic acid was used as a substrate in addition to the main product 13-HODE, the isomer 9-HODE was generated as a by-product by all three isoenzymes with a ratio of 13-HODE to 9-HODE of approx. 50:1 for leucocyte- and platelet-type (12*S*)-lipoxygenase and approx. 3:1 for epidermis-type (12*S*)-lipoxygenase (Table 2 and Figure 2).

Chirality of the reaction products

The chiral analysis of the reaction products obtained after the incubation of arachidonic acid with preparations of the three

Figure 2 Straight-phase HPLC analysis of lipid extracts obtained after incubation with linoleic acid of 10000 *g* supernatants from sonicated HEK-293 cells transiently transfected with leucocyte-type (A), platelet-type (B) or epidermis-type (C) (12*S*)-lipoxygenase expression constructs or with pcDNA vector alone (D)

Supernatants (10000 *g*) were incubated with linoleic acid instead of arachidonic acid. The protein concentration in each assay was 0.15 mg/ml protein [leucocyte-type (12*S*)-lipoxygenase], 1.0 mg/ml protein [platelet-type (12*S*)-lipoxygenase], 5 mg/ml protein [epidermis-type (12*S*)-lipoxygenase] and 5 mg/ml protein (vector). For details see the legend to Figure 1.

isoenzymes showed a strict enantiomeric purity of the main product (12*S*)-HETE. In addition, the by-products 15-HETE and 8-HETE generated by leucocyte-type (12*S*)-lipoxygenase were predominantly the *S* enantiomers. However, 8-HETE generated by platelet-type (12*S*)-lipoxygenase was found to be the *R* enantiomer (Figure 3 and Table 3). All three isoenzymes oxygenated linoleic acid to (13*S*)-HODE, whereas the chirality of the by-product 9-HODE was predominantly the *S* enantiomer with leucocyte-type (12*S*)-lipoxygenase but *R* with the two other isoenzymes (Figure 4 and Table 4). HETEs and HODEs generated in 10000 *g* supernatants of vector-transfected cells were found to be racemic.

Inhibitor experiments

Two selective inhibitors of the human platelet-type (12*S*)-lipoxygenase, BHPP [17] and CDC [18], were tested for (12*S*)-lipoxygenase isoenzyme selectivity. BHPP was more efficient in

Figure 3 Chiral-phase HPLC of the regioisomeric HETEs formed by (12*S*)-lipoxygenase isoenzymes expressed in HEK-293 cells

The 8-, 12- and 15-HETE eluates were subjected to chiral-phase chromatography with a Chiracel column (250 mm × 4.6 mm, 5 µm particle size) with *n*-hexane/propan-2-ol/acetic acid (99:1:0.05, by vol.) for the elution of the *R* and *S* enantiomers of 15- and 12-HETE and with *n*-hexane/propan-2-ol/acetic acid (97.5:2.5:0.05, by vol.) for the elution of the *R* and *S* enantiomers of 8-HETE. Authentic (15*S*)-, (12*S*)- and (8*S*)-HETE and the corresponding *R* enantiomers were used as external standards and were eluted at a flow rate of 0.5 ml/min. (A) Leucocyte-type (12*S*)-lipoxygenase, (B) platelet-type 12(*S*)-lipoxygenase and (C) epidermis-type 12(*S*)-lipoxygenase.

Table 3 Chiral-phase analysis of the reaction products of (12*S*)-lipoxygenase isoenzymes with arachidonic acid as substrate

Chiral-phase analysis was done with HETE fractions obtained by straight-phase HPLC (see Figure 1) with a Chiracel column (for details see the legend to Figure 3). Authentic (12*R*)-, (12*S*)-, (15*R*)-, (15*S*)-, (8*R*)- and (8*S*)-HETE (100 ng of each) were used as external standards. The concentrations of *S* and *R* enantiomers were determined from the peak areas and used to determine the ratios, the sum of which was set at 100%.

(12 <i>S</i>)-Lipoxygenase type	Chirality of HETE (%)		
	12 <i>S</i> :12 <i>R</i>	15 <i>S</i> :15 <i>R</i>	8 <i>S</i> :8 <i>R</i>
Leucocyte	99:1	84:16	81:19
Platelet	99:1	—	30:70
Epidermis	92:8	—	—

suppressing platelet-type (12*S*)-lipoxygenase activity than epidermis-type and leucocyte-type (12*S*)-lipoxygenases, both with arachidonic acid and with linoleic acid as a substrate (Table 5). CDC almost exclusively inhibited the platelet-type enzyme with arachidonic acid as a substrate. The high selectivity was not observed with linoleic acid as substrate (Table 6).

DISCUSSION

Phylogenetically, the three mouse (12*S*)-lipoxygenase isoenzymes are found in different subgroups; sequence comparisons yield only moderate amino acid identities of between 57.0 and 60.7% [5,19]. This structural diversity is reflected by differences in catalytic activity, positional selectivity and stereoselectivity, and sensitivity to inhibitors. Although the isoenzymes oxygenated arachidonic acid predominantly to (12*S*)-HpETE and linoleic acid to (13*S*)-hydroperoxyoctadecadienoic acid [(13*S*)-HpODE], the yields varied widely. In particular, the specific activity of the epidermis-type isoenzyme was 1–2.5 orders of magnitude lower than that of leucocyte-type or platelet-type (12*S*)-lipoxygenase. Epidermis-type (12*S*)-lipoxygenase shares low catalytic efficiency with other lipoxygenases preferentially expressed in epidermis, i.e. human (15*S*)-lipoxygenase 2, human and mouse (12*R*)-lipoxygenase and mouse 8-lipoxygenase [4,5,7,12,20]. This indicates that either (1) free arachidonic acid or linoleic acid are not the appropriate substrates or (2) that epidermis-specific cofactors or post-translational modifications [21–23] are not provided on expression of the recombinant protein in HEK-293 cells. The leucocyte-type (12*S*)-lipoxygenase oxygenated linoleic acid with approx. one-tenth the efficiency of arachidonic acid. In this respect, the mouse enzyme resembled the bovine analogue, whereas the pig analogue was reported to oxygenate linoleic acid

Figure 4 Chiral-phase HPLC of the regioisomeric HODEs formed by (12*S*)-lipoxygenase isoenzymes expressed in HEK-293 cells

The 9- and 13-HODE eluates were subjected to chiral-phase chromatography by using a Chiracel column (250 mm × 4.6 mm, 5 μ m particle size) with *n*-hexane/propan-2-ol/acetic acid (99:1:0.05, by vol.) for the elution of the *R* and *S* enantiomers of 13-HODE and with *n*-hexane/propan-2-ol/acetic acid (97.5:2.5:0.05, by vol.) for the elution of the *R* and *S* enantiomers of 9-HODE. Authentic (13*S*)- and (9*S*)-HODE and the corresponding *R* enantiomers were used as external standards and were eluted at a flow rate of 0.5 ml/min. For platelet-type and epidermis-type (12*S*)-lipoxygenases, the 9-HODE chromatograms were performed by pooling several 9-HODE fractions obtained by straight-phase HPLC. (A) Leucocyte-type (12*S*)-lipoxygenase, (B) platelet-type (12*S*)-lipoxygenase and (C) epidermis-type (12*S*)-lipoxygenase.

Table 4 Chiral-phase analysis of the reaction products of (12*S*)-lipoxygenase isoenzymes with linoleic acid as substrate

Chiral-phase analysis was done with HODE fractions obtained by straight-phase HPLC (see Figure 4) with a Chiracel column (for details see the legend to Figure 4). (13*R*)-, (13*S*)-, (9*R*)- and (9*S*)-HODE (100 ng of each) were used as external standards. The concentrations of *S* and *R* enantiomers were determined from the peak areas and used to determine the ratios, the sum of which was set at 100%.

(12 <i>S</i>)-Lipoxygenase type	Chirality of HODE (%)	
	13 <i>S</i> :13 <i>R</i>	9 <i>S</i> :9 <i>R</i>
Leucocyte	97:3	65:35
Platelet	95:5	25:75
Epidermis	93:7	24:76

Table 5 Inhibition by BHPP of (12*S*)-lipoxygenase isoenzymes with arachidonic acid or linoleic acid as substrate

(12 <i>S</i>)-Lipoxygenase type	IC ₅₀ (μ M)	
	Arachidonic acid	Linoleic acid
Leucocyte	20.8	2.3
Platelet	0.8	0.3
Epidermis	3.7	0.7

Table 6 Inhibition by CDC of (12*S*)-lipoxygenase isoenzymes with arachidonic acid or linoleic acid as substrate

(12 <i>S</i>)-Lipoxygenase type	IC ₅₀ (μ M)	
	Arachidonic acid	Linoleic acid
Leucocyte	> 50	2.3
Platelet	0.3	6.2
Epidermis	36	6.7

at least as efficiently as arachidonic acid [8]. The mouse platelet-type (12*S*)-lipoxygenase also showed considerable enzymic activity with linoleic acid as substrate, whereas the bovine and human analogues were reported to exhibit low and undetectable activity respectively [8].

The leucocyte-type enzyme was unique among the three (12*S*)-lipoxygenase isoenzymes in that it exhibited a promiscuous positional specificity, generating considerable quantities of 15-HETE and 8-HETE as by-products. Dual positional specificity has been previously shown for the pig and the mouse leucocyte (12*S*)-lipoxygenases [8–10,16] as well as for the rabbit and human reticulocyte-type (15*S*)-lipoxygenases [24,25], which are considered to be the corresponding orthologues of the mouse leucocyte-type (12*S*)-lipoxygenase [8]. The platelet-type (12*S*)-lipoxygenase generated only small quantities of 8-HETE as a side product. In previous studies the human platelet-type (12*S*)-lipoxygenase was shown to generate small quantities of 15-HETE, whereas the mouse analogue was reported to be mono-specific [8–10]. The epidermis-type isoenzyme inserted molecular oxygen exclusively into the 12-position of arachidonic acid. With linoleic acid as substrate the leucocyte-type and platelet-type isoenzymes generated 13-HODE and 9-HODE in the ratio of approx. 50:1 and the epidermis-type (12*S*)-lipoxygenase in the ratio of approx. 3:1.

Whereas the main products, 12-HETE and 13-HODE, generated by the three (12*S*)-lipoxygenase isoenzymes were almost exclusively *S* enantiomers, the chirality of the by-products differed with the isoenzyme. Thus (15*S*)-HETE and (8*S*)-HETE were generated by leucocyte-type (12*S*)-lipoxygenase and (8*R*)-HETE by platelet-type (12*S*)-lipoxygenase; linoleic acid was oxygenated to (9*R*)-HODE by both the epidermis-type and the platelet-type (12*S*)-lipoxygenases but to the *S* enantiomer by the leucocyte-type enzyme.

The positional and enantiomeric specificity of (12*S*)-lipoxygenases might cast light on mechanisms involved in the catalytic process. On the basis of biochemical [26,27], X-ray [28–30] and spectroscopic analyses [31], models have been proposed that predict the product spectrum of lipoxygenases [32–34]. The initial, rate-limiting step of the lipoxygenase reaction

Figure 5 Positional selectivity and stereoselectivity of the (12S)-lipoxygenase reaction with arachidonic acid as substrate

The 1,4-*cis*-pentadiene unit with the bis-allylic C-10 binds to the active centre of the enzyme. Abstraction of hydrogen 10 with the L configuration directs oxygen insertion into the 12 L_S or 8 L_R position and the hydrogen with the 10 D configuration into the 12 D_R or 8 D_S (not shown) position of arachidonic acid. Oxygen insertion into the 15 L_S position requires the abstraction of hydrogen 13 with the L configuration.

is the abstraction of one of the two bis-allylic methylene hydrogens from one (or two different) 1,4-pentadiene structure(s) followed by a rearrangement of the intermediary pentadienyl radical driven by a *Z,E*-diene conjugation, and the stereospecific (antarafacial) addition of molecular oxygen to one of the two possible radical carbons. The intermediate peroxide radical is then reduced by the addition of a hydrogen atom to the corresponding hydroperoxy fatty acid.

As far as (12S)-lipoxygenase is concerned, isotope tracer experiments have shown that the hydrogen with the L configuration at C-10 of arachidonic acid is removed and oxygen is directed into the 12 L_S position, yielding (12S)-HpETE as the major primary product [26]. The generation of regioisomeric by-

products can be due either to oxygen insertion into one of the mirror-image-specific carbons of the intermediary pentadienyl radical or to a double regioselectivity in that hydrogen abstraction occurs from two different bis-allylic methylene groups (Figure 5). Thus the generation of (8R)-HpETE by platelet-type (12S)-lipoxygenase might result from oxygen insertion into the 8 L_R position instead of the mirror-image-specific 12 L_S position, yielding a product with an inverse chirality (Figure 5) (see also [35]), whereas the formation of (8S)-HpETE by leucocyte-type (12S)-lipoxygenase is compatible with the removal of the 10 D hydrogen, directing oxygen to the 8 D_S position. In fact, the epidermal 8-lipoxygenase generating (8S)-HETE as the main product has been shown to abstract the 10 D rather than the

10 L hydrogen [36]. With linoleic acid as a substrate, leucocyte-type, platelet-type and epidermis-type (12S)-lipoxygenases produced (13S)-HpODE and (9R)-HpODE as by-products, most probably through abstraction of the 11 L hydrogen, which directs oxygen insertion into the 13 L_S or 9 L_R position. In contrast, the formation of (9S)-HpODE required the abstraction of hydrogen 11 D.

Provided that in all (12S)-lipoxygenase isoenzymes hydrogen abstraction is occurring at the same site of the substrate-binding cavity, the mutual removal of either the L or D hydrogen is possible only under two conditions: (1) the substrate can be twisted by 109°, corresponding to the tetrahedral angle, thus permitting the abstraction of either the L or D hydrogen, or (2) the orientation of the fatty acid in the substrate cavity can have two orientations, i.e. a terminal methyl group ('tail-first') or a carboxy group ('head-first') towards the centre of the enzyme, a concept previously put forward by Brash et al. [35]. Thus the product spectra of the three (12S)-lipoxygenase isoenzymes might be explained by a 'tail-first' positioning of arachidonic acid and a binding pocket of differing volumes, permitting the regiospecific positioning of the bis-allylic methylene groups with respect to the site of hydrogen abstraction. An exception is the generation of (8S)-HETE and (9S)-HODE by the leucocyte-type (12S)-lipoxygenase and (8S)-lipoxygenase because it requires an opposite orientation ('head-first') or a twisted fixation of the substrate in the binding cavity.

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