Epoxidation of plasmalogens: source for long-chain α -hydroxyaldehydes in subcellular fractions of bovine liver

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1. Masked long-chain α -hydroxyaldehydes were trapped in all subcellular fractions of bovine liver by application of pentafluorbenzyloxime derivatization [van Kuijk, Thomas, Stephens and Dratz (1986) *Biochem. Biophys. Res. Commun.* **139**, 144–149] and quantified via GLC/MS using characteristic ion traces. 2. The chain-length profile of long-chain 2-hydroxy-alkanals clearly indicates their relationship to plasmalogens as precursor molecules. 3. The previously postulated existence of α -acyloxyplasmalogens as precursor molecules of masked long-chain α -hydroxyaldehydes in bovine tissue lipids [Lutz and

Spiteller (1991) *Liebigs Ann. Chem.* **1991**, 563–567] was excluded. 4. The constant oxidation rate of plasmalogens in all subcellular fractions provides conclusive evidence for a non-enzymic plasmalogen epoxidation process (probably via hydroperoxy radicals). 5. The high reactivity of α -hydroxyaldehydes sheds some doubt on the postulation that plasmalogens protect mammalian cells against oxidative stress as postulated previously [Morand, Zoeller and Raetz (1988) *J. Biol. Chem.* **263**, 11590–11596; Morand, Zoeller and Raetz (1988) *J. Biol. Chem.* **263**, 11597–11606].

INTRODUCTION

Alk-1-enyl ether lipids (plasmalogens) are present in nearly all mammalian tissues [1,2], but obviously not in plants [3]. Their biological function is still unknown. The special surface charge and geometry of these lipids reduces membrane fluidity [4] and exerts other physicochemical effects concerning, for example, temperature of phase transition [5] and phospholipid structure [6].

Plasmalogens consist mainly of species containing polyunsaturated fatty acids at the sn-2 position. Thus alk-1-enyl ether lipids serve as a metabolic pool of arachidonic acid [7,8] being the precursors of leukotrienes, thromboxanes and prostaglandins.

Morand et al. [9,9a] found plasmalogens protect animal cells against photosensitized killing; Rüstow et al. showed a high plasmalogen content in the alveolar surfactant of type-II pneumocytes [10]. Thus plasmalogens are thought to function as biological antioxidants.

Recently our laboratory [11,12] reported the detection of long-chain α -hydroxyaldehydes in mammalian tissue samples. First, it was assumed that they are generated from α -acyloxyplasmalogens. Later we were able to demonstrate that oxidation of plasmalogens by fatty acid hydroperoxy radicals results in

highly reactive enol ether epoxides [13]. Traces of them are detectable as mixed acetals (see Scheme 1) after lipid extraction according to Folch et al. [14].

Plasmalogen epoxides and long-chain α -hydroxyaldehydes, their corresponding hydrolysis products, can be characterized efficiently by transformation to 2-(1-hydroxy)-1,3-dithiolanes (Scheme 2). These represent stable derivatives allowing their unambiguous detection by MS [12]. However, dithiolane formation requires a complex sequence of reactions: lipid extraction [14] is followed by high vacuum drying to remove water, since even traces of water prevent quantitative acetalization. The transformation to 1,3-dithiolane derivatives is catalysed by BF_3 and acetic acid (pH 1) and needs 48 h for completion. Several chromatographic steps and trimethylsilylation are necessary for the final detection of the derivatized long-chain α -hydroxyaldehydes by GLC/MS.

In the present study we show that plasmalogen epoxides can be detected in a much faster and less vigorous way after pentafluorbenzyloxime (PFBO) derivatization [15] in aqueous medium.

Analysis of gas-chromatograms and chain-length profile of plasmalogens and long-chain α -hydroxyaldehydes respectively provides conclusive evidence for their metabolic relationship. The subcellular distribution of plasmalogens and long-chain α -hydroxyaldehydes indicates an enzyme-independent plasmalogen epoxidation process.

EXPERIMENTAL

Materials

The chemicals used were all of analytical grade. Kynuramine was supplied by Sigma, cytochrome *c* was obtained from Boehringer and *N*-methyltrimethylsilyltrifluoroacetamide (MSTFA) was ordered from Macherey and Nagel. All other chemicals were purchased from Fluka. 2-Hydroxyundecanal dipentylacetal [16],

Scheme 1 Detection of plasmalogen epoxidation

1-hydroxy-2-dodecanone [17] and the *O*-PFBO derivative of 2-hydroxyundecanal [18] were prepared as described previously. The subcellular fractions were stored at -60°C under argon, $\text{BF}_3\text{-Et}_2\text{O}$ (48 %) was stored at 4°C .

Preparation of subcellular fractions

Bovine liver (from the slaughterhouse) was homogenized (in a Waring Blender) in hypertonic buffer (0.25 M sucrose, 10 mM Hepes/KOH, pH 7.8, 0.2 mM EDTA, 1 mM 2-mercaptoethanol) and fractionated by differential centrifugation (4°C) as described previously [18,19]. Resuspension of the pellets was performed carefully with a Dounce homogenizator. The cell debris fraction was obtained after centrifugation at 3000 *g* (GSA rotor, $2\times$, 15 min). Mitochondria were separated by centrifugation at 26000 *g* (GSA rotor, $2\times$, 20 min). Ultracentrifugation (rotor A641, $1\times$, 120 min, 105000 *g*) yielded the microsome fraction (pellet) and the S100 sample (supernatant).

Assessment of membrane purity

The purity of the isolated membrane fractions was determined using marker enzymes as described previously (protein [20]; succinate dehydrogenase [21]; monoamine oxidase [22]; NADPH-cytochrome *c* reductase [23]; glucose 6-phosphatase [24,25]).

Lipid extraction

Lipid extraction was performed according to Folch et al. [14]. The solvent was supplied with 50 $\mu\text{g}/\text{ml}$ 2,6-di-*t*-butyl-4-methylphenol (BHT) in order to prevent artificial lipid peroxidation [15] (EDTA was included in the homogenization buffer, see above).

Plasmalogen analysis

The chain-length profile of plasmalogen aldehydes was determined after lipid extraction and derivatization to 2-alkyl-1,3-dithiolanes [12,26] by GLC/MS as described previously.

Analysis of long-chain α -hydroxyaldehydes

Analysis of masked long-chain α -hydroxyaldehydes in tissue lipids was performed after transformation to 2-(1-hydroxyalkyl)-1,3-dithiolanes according to Lutz and Spiteller [12]. Control experiments (see below) and detailed analysis of the complex mixture of reaction products by TLC and GLC/MS [18] revealed problems and disadvantages of this derivatization procedure (e.g. loss of volatile α -hydroxyaldehydes, risk of artificial lipid peroxidation, artefact production).

Therefore PFBO derivatization [15] was applied to detect long-chain α -hydroxyaldehydes in subcellular fractions of bovine liver.

An aliquot (20 ml) of 0.1 M pentafluorobenzyl hydroxylamine hydrochloride (PFBHA/HCl) was added to 50 ml of a subcellular fraction. 1-Hydroxy-2-dodecanone (13 μg) and 2-hydroxyundecanal pentafluorobenzyloxime (13.6 μg) were added (internal standards). After stirring at room temperature for 3 h the reaction was stopped by extraction (cyclohexane/diethyl ether; 4:1, v/v); the extracts (up to 300 mg) were separated by TLC (cyclohexane/ethyl acetate; 3:1, v/v). The relevant TLC areas ($R_f = 0.51\text{--}0.65$), marked with an external standard, were scraped off and eluted with diethyl ether.

After evaporation of the solvent the residue was split. An

aliquot (< 0.5 mg) was retained for trimethylsilylation performed as described previously [27].

The rest was transferred into a vial and mixed with 100–150 μl of dimethylformamide and 100–150 μl of *N*-methyl-*N*-*t*-butyldimethylsilyltrifluoroacetamide (M-*t*-BSTFA). In contrast to MSTFA, M-*t*-BSTFA allows further purification via TLC, avoiding hydrolysis. Incubation for 12 h at 50°C transformed hydroxy functions completely to *t*-butyldimethylsilyl (*t*-BDMS)-ether derivatives. The *O*-PFBO *t*-BDMS-ether derivatives of long-chain α -hydroxyaldehydes were separated by TLC using cyclohexane/ethylacetate (98:2, v/v) as solvent. The corresponding derivatives of 2-hydroxyheptanal, 2-hydroxyoctanal and 1-hydroxy-2-dodecanone were co-chromatographed as external standards. The R_f area 0.51–0.78 was scraped off, eluted and analysed by GLC/MS.

Quantification was achieved by comparing the peak areas of the common marker ion of *O*-PFBO *t*-BDMS-ether derivatives of α -hydroxyaldehydes (see later) via an ion trace at $m/z = 368$.

Control experiment

2-Hydroxyundecanal dipentylacetal was used to prepare the 2-(1-hydroxyalkyl)-1,3-dithiolane derivative of 2-hydroxyundecanal as described previously [12]. After substitution of the catalysing acetic acid against propionic acid this derivatization procedure was repeated to check the possibility of acylation reactions during 1,3-dithiolane derivatization. Further processing of the sample was carried out as described above.

GLC/MS analysis

GLC was carried out with a Carlo Erba HRGC 5160 Mega Series chromatograph equipped with a flame ionization detector (FID), using a DB-1 fused-silica glass capillary column (30 m $\times$ 0.32 mm i.d.), temperature programmed from 80 to 280°C at $3^{\circ}\text{C}/\text{min}$. The temperatures of the injector and detector were kept at 270 and 290°C respectively. The carrier gas was hydrogen and the splitting ratio was 1:30 (80 %). Peak area integration was achieved with a Merck D-2500 integrator.

GLC/MS was performed on a Finnigan MAT 312 system with a MAT-SS-300 data system. EI mass spectra were recorded at an ionization energy of 70 eV. A Varian 3700 gas chromatograph with a 30 m $\times$ 0.3 mm i.d. DB-1 fused-silica column was used for sample separation. The carrier gas was hydrogen and the temperature programme was the same as used for GLC.

A mixture of straight-chain hydrocarbons ($\text{C}_{12}\text{--}\text{C}_{30}$) was added to each sample to ensure determination of retention indices.

RESULTS

Subcellular fractionation yielded membrane fractions of sufficient purity (Table 1). Subsequent plasmalogen analysis showed a unique characteristic chain-length profile with a great portion of branched aldehydes [11]. Surprisingly the plasmalogen content was found to be highest in the lipids of mitochondria and not of microsomes where their biosynthesis occurs (Table 2).

Long-chain masked α -hydroxyaldehydes can be simultaneously detected by GLC/MS as 2-(1-trimethylsilyloxyalkyl)-1,3-dithiolane derivatives [12]. However, their preparation is connected with some disadvantages: the transformation to 1,3-dithiolane derivatives requires the complete removal of water from the tissue lipids. During extraction [14,15] and vacuum drying volatile α -hydroxyaldehydes are lost and artificial lipid peroxidation might occur. In addition use of a proton acid (to enhance catalytic activity) results in partial acylation of free

Table 1 Assessment of membrane purity using marker enzymes

Values are given as means $\pm$ S.D. values. Results are from at least three independent experiments.

Subcellular fraction	Specific activity (nmol/mg of protein per min)			
	Succinate dehydrogenase	Monoamine oxidase	NADPH-cytochrome <i>c</i> reductase	Glucose 6-phosphatase
Homogenate	6.13 $\pm$ 0.80	1.66 $\pm$ 0.16	6.82 $\pm$ 0.83	11.93 $\pm$ 1.37
Cell debris	8.27 $\pm$ 0.72	2.13 $\pm$ 0.31	5.66 $\pm$ 0.74	14.54 $\pm$ 1.89
Mitochondria	21.6 $\pm$ 1.73	5.97 $\pm$ 0.27	7.70 $\pm$ 0.43	19.25 $\pm$ 2.28
Microsomes	6.52 $\pm$ 0.31	1.26 $\pm$ 0.18	22.69 $\pm$ 1.55	30.06 $\pm$ 0.54
S100	0.16	0.053 $\pm$ 0.01	2.89 $\pm$ 0.34	3.04 $\pm$ 0.30
Recovery (%)	89.33	90.5	101.34	93.77

Table 2 Content of plasmalogen-derived aldehydes and 2-hydroxyaldehydes in subcellular fractions of bovine liver

Values for plasmalogen-derived aldehydes are given as means $\pm$ S.D. values. Results are from two or three independent experiments (different liver samples). Abbreviations: PlsAld, plasmalogen-derived aldehyde; α -HyAld, 2-hydroxyaldehyde.

Subcellular fraction ...	Aldehyde content				
	Homogenate	Cell debris	Mitochondria	Microsomes	S100
PlsAld (mg/g of lipid)	6.74 $\pm$ 0.92	6.43 $\pm$ 1.14	7.92 $\pm$ 1.08	2.58 $\pm$ 0.61	1.28 $\pm$ 0.31
α -HyAld (μ g/g of lipid)	2.99	3.12	2.40	0.82	0.16

Scheme 2 Side reactions of 1,3-dithiolane derivatization

hydroxy functions. Thus the existence of α -acyloxyplasmalogens is mimicked (Scheme 2a).

Supporting evidence for this side reaction was obtained by a control experiment with undecanal dipentylacetal: derivatization of this compound with dithioethylenglycol, BF_3 and acetic acid [12] yielded 2-(1-acetoxydecyl)-1,3-dithiolane as a side product. After substitution of acetic acid with propionic acid the propionoxy derivative was detected. Thus α -acyloxyplasmalogens, the assumed precursor molecules of masked long-chain

Figure 1 Search for *O*-PFBO TMS-ether derivatives of long-chain α -hydroxyaldehydes in bovine liver homogenate [total ion current (lower panel) and ion trace at m/z 326 (upper panel)]

Bovine liver was homogenized in hypertonic buffer. α -Hydroxyaldehydes were analysed after transformation to *O*-PFBO TMS-ether derivatives (*) by GLC/MS. Abbreviation: HKS, α -hydroxyketone (internal standard).

α -hydroxyaldehydes in biological tissue [12,26], turned out to be artefacts.

In addition glycerol esters are also able to react with dithioethylenglycol (A. Dudda and G. Spiteller, unpublished work). The acidic reaction conditions (pH 1) favour the dehydration of the resulting 2-alkyl-2-hydroxy-1,3-dithiolanes (Scheme 2b). This was unambiguously proven by the isolation of 2-alkyldien-1,3-dithiolanes of variable chain-length in bovine tissue lipids [18].

Consequently there was a requirement for a new, gentler, faster and quantitative derivatization method to analyse masked

Figure 2 EI mass spectra of the PFBO t-BDMS-ether derivative of 2-hydroxyhexadecanal

Figure 3 Long-chain α -hydroxyaldehydes in cell debris of bovine liver: (a) gas chromatogram and (b) total ion current (lower panel) and ion trace at m/z 368 (upper panel)

Bovine liver was homogenized in hypertonic buffer. Centrifugation at 3000 g yielded the cell debris fraction. α -Hydroxyaldehydes were analysed after transformation to O -PFBO t-BDMS-ether derivatives by GLC/MS. Abbreviations: HAS and HKS, internal standards (α -hydroxyaldehyde, and α -hydroxyketone); A, artifact. (For quantification and peak identification see Table 3).

α -hydroxyaldehydes. Application of the PFBO-derivatization [15,28] to subcellular fractions of bovine liver provided optimal results.

Even 1.5 g of a biological tissue sample was sufficient to detect long-chain α -hydroxyaldehydes in all subcellular fractions of

bovine liver. All O -PFBO trimethylsilyl (TMS)-ether derivatives are characterized by their key fragment at m/z 326 produced by α -cleavage [27]. If the ion trace of this fragment was recorded instead of the total ion current very informative GLC were obtained which only indicated molecules of this class of compounds (Figure 1).

Quantification of long-chain α -hydroxyaldehydes in biological tissue required sample enrichment. Therefore the samples were derivatized with M-t-BSTFA instead of MSTFA to avoid hydrolysis in the subsequent TLC step. EI-mass spectra of the resulting O -PFBO t-BDMS-ether derivatives (Figure 2) are well suited for the detection of tiny amounts of α -hydroxyaldehydes. Characteristic ions (M-15, M-57, m/z 368) are produced by α -cleavage at the t-butyldimethylsilylated oxygen.

The fragment m/z 368 represents a unique marker ion for all derivatized α -hydroxyaldehydic compounds. Consequently an ion trace at m/z 368 can be used for quantification (Figure 3; Table 3). The O -PFBO t-BDMS-ether derivative of 2-hydroxyundecanal which does not occur in biological tissue served as an internal standard.

Comparison of the results after plasmalogen analysis (1,3-dithiolane derivatives) and analysis of long-chain α -hydroxyaldehydes (Table 3) clearly indicates the relationship of these two classes of compounds.

(1) Plasmalogen-derived aldehydes and long-chain α -hydroxyaldehydes of the same chain-length show a constant R_f -shift of $340 (\pm 17)$.

(2) The unusual and typical branching of the plasmalogen-derived aldehydes is found again in the very similar corresponding chain-length profile of O -PFBO t-BDMS-ether derivatives (Figure 4, Table 4).

(3) Analogous results (corresponding chain-length profiles) were obtained for all subcellular fractions of bovine liver (Table 4).

Thus the occurrence of long-chain α -hydroxyaldehydes in bovine tissue lipids is unambiguously proven to be a result of plasmalogen oxidation.

DISCUSSION

During oxidative stress hydroperoxy radicals were proposed to act as chain propagators [29,30]. Later it was shown that this reagent is able to co-oxidize even double bonds [31,32]. In the case of plasmalogens highly reactive plasmalogen epoxides are formed representing masked long-chain α -hydroxyaldehydes. This has been confirmed by a model experiment with ^{18}O -labelled hydroperoxide [33]. Supporting evidence for a non-enzymic plasmalogen-oxidation process in mammalian tissue is provided by constant plasmalogen epoxidation rates (Table 4) in subcellular fractions of bovine liver. These results are independent of the sequence of analysis (different subcellular fractions) and thus indicate plasmalogen epoxidation *in vivo*.

However, the reported figures represent only the amount of 2-hydroxyalkanales which could be detected under the chosen experimental conditions. Obviously many plasmalogen epoxide molecules reacted with biomolecules before addition to our trapping reagent. In addition the nucleophilic power of sulphur (dithioethylenglycol) exceeds that of nitrogen (PFBHA/HCl). Dithioethylenglycol also attacks, for instance, aldehydes bound in plasmalogens and transforms them to 2-alkyl-1,3-dithiolane derivatives. This explains the differences obtained by quantification after 1,3-dithiolane [12] and pentafluorobenzyl derivatization, respectively.

We assume that epoxidation is the only, or at least the most common way, that plasmalogens are oxidized in biological

Table 3 Quantification and peak identification (see Figure 3)

R_f values of 2-alkyl-1,3-dithiolanes (plasmalogen-derived aldehydes) and PFBO t-BDMS-ether derivatives of long-chain α -hydroxyaldehydes. Abbreviations: α -HyAld, α -hydroxyaldehyde; PlsAld, plasmalogen-derived aldehyde; i, iso; ai, anteiso; br, branched; HAS and HKS, internal standards (α -hydroxyaldehyde, α -hydroxyketone); tr, traces.

Peak number	Chain length	R_f GLC/FID α -HyAld	α -HyAld content	R_f GLC/FID PlsAld	ΔR_f GLC/FID
HAS	11:0	2390	—	—	—
HKS	12:0	2380/2383	—	—	—
1a	br 14:0	2546	412	2184	362
1b	n 14:0	2585	288	2224	361
2a	i 15:0	2645	406	2295	350
2b	ai 15:0	2656	665	2300	356
2c	n 15:0	2682	102	2333	349
3a	br 16:0	2742	166	2400	342
3b/3c	n 16:0	2771/2780	727	2441	330/339
4a	i 17:0	2835	39	2509	326
4b	ai 17:0	2843	83	2517	326
4c	n 17:0	2852	tr	2544	308
5	n 18:0	2977	196	2652	325

Figure 4 Chain-length profile of plasmalogen-derived aldehydes (1,3-dithiolane derivatization) and long-chain α -hydroxyaldehydes (PFBO-derivatization) in cell debris of bovine liver

surroundings. In spite of addition of BHT prior to analysis and fast and careful handling artificial lipid peroxidation may not have been totally prevented. During cell homogenization and subcellular fractionation oxidative stress is enhanced. Lytic enzymes and other proteins involved in the inflammatory process are activated, e.g. lipoxygenases oxidizing polyunsaturated fatty acids. The resulting hydroperoxides in turn react with plasmalogens producing plasmalogen epoxides. Nevertheless long-chain α -hydroxyaldehydes can also be detected in low-density lipoprotein samples under conditions which seem to exclude artefact production (R. Felde and G. Spiteller, unpublished work).

The observation that α -hydroxyaldehydes were produced by plasmalogen epoxidation rises the question of the biological function of alk-1-enyl ether lipids. As pointed out in the Introduction plasmalogens were assumed to protect biological material against oxidative stress. In fact they are able to react with reactive oxygen species like hydroperoxy radicals of unsaturated fatty acids [33]. Taking into account the high proportion of polyunsaturated acids at the sn-2 position of plasmalogens, this seems to be a reaction of great relevance. Sim-

Table 4 2-Alkyl-1,3-dithiolanes (plasmalogen-derived aldehydes) and PFBO t-BDMS-ether derivatives of long-chain α -hydroxyaldehydes in subcellular fractions of bovine liver

Abbreviations: α -HyAld, α -hydroxyaldehyde; PlsAld, plasmalogen aldehyde; br, branched; n.d., not detectable. Values represent percentages of total PlsAld and α -HyAld, respectively. Coefficient of variation $\bar{s} = \sigma/\bar{x}$ for PlsAld: $10.49 \pm 9.45\%$; α -HyAld: $12.59 \pm 6.25\%$.

Subcellular fractions ...	Aldehydes (%)									
	Homogenate		Cell debris		Mitochondria		Microsomes		S100	
	PlsAld	α -HyAld	PlsAld	α -HyAld	PlsAld	α -HyAld	PlsAld	α -HyAld	PlsAld	α -HyAld
br 13:0	1.5	1.9	1.5	2.1	1.7	1.5	1.8	1.2	1.6	n.d.
n 13:0	0.7	0.5	0.6	n.d.	0.8	n.d.	0.8	n.d.	1.0	n.d.
br 14:0	15.9	14.6	12.1	13.2	14.0	13.6	14.3	14.1	13.3	16.1
n 14:0	9.5	8.2	9.3	9.2	10.6	12.2	10.4	10.6	9.0	11.0
br 15:0	27.9	30.9	28.6	34.3	30.4	32.6	30.5	36.6	30.4	38.1
n 15:0	4.0	3.6	4.3	3.3	4.3	2.9	4.4	n.d.	3.9	3.2
br 16:0	6.5	5.1	6.6	4.3	6.6	4.1	6.4	4.3	6.3	5.2
n 16:0	32.5	23.4	24.8	23.3	24.0	25.3	24.0	26.4	23.0	20.0
br 17:0	3.6	5.6	3.6	3.9	3.3	3.2	3.4	3.3	4.5	n.d.
n 17:0	0.5	0.6	0.7	n.d.	0.4	n.d.	0.4	n.d.	0.9	n.d.
n 18:0	6.2	5.6	8.0	6.3	3.9	4.5	3.6	3.5	6.1	6.5

ultaneously plasmalogen epoxides are produced representing masked long-chain α -hydroxyaldehydes. These very reactive non-radical secondary products of lipid peroxidation form adducts spontaneously with biomolecules [32] and are able to exert long-distance effects. Thus they should be discussed as possible 'second toxic messengers' like 4-hydroxy-2-alkenals [34,35]. It might be that either α -hydroxyaldehydes or their secondary products trigger special cellular responses to oxidative stress. Alternatively their detection could indicate a 'cell suicide' due to membrane lysis.

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