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Hydra vulgaris ω10-lipoxygenase is used in vivo to synthesize new α-linolenic acid metabolites

Dedicated to the memory of Dr. Gianpaolo Nitti.

C. Gianfrani, V. Di Marzo*, L. De Petrocellis[a] and G. Cimino

Istituto per la Chimica di Molecole di Interesse Biologico and [a]Istituto di Cibernetica, C.N.R., Via Toiano 6, I-80072 Arco Felice, Naples (Italy)
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Abstract. Previous studies conducted in cytosolic extracts of the freshwater hydrozoan *Hydra vulgaris* led to the finding of an abundant 11(*R*)-lipoxygenase catalyzing the peroxidation of polyunsaturated fatty acid (PUFAs) on the tenth carbon atom from the aliphatic end (ω10 peroxidation). Here we describe experiments aimed at identifying the actual metabolites generated in vivo by such enzymic activity. Homogenates of *H. vulgaris* polyps were analyzed by HPLC. This showed the presence of three major components chromatographically identical to three metabolites obtained when incubating the homogenates with exogenous α-linolenic acid (α-LA). The presence, in extracts of polyps prelabelled with [^{14}C]-α-linolenic acid, of radioactive metabolites displaying the same chromatographic properties, substantiated the hypothesis that the natural products isolated in vivo are derived from α-LA. Gas chromatographic analyses revealed that this was the most abundant PUFA in both free and phosphoglyceride-bound fatty acid pools. [^{1}H]-NMR analysis of the endogenous substances, carried out in comparison with products obtained from exogenously incubated α-LA, indicated that their structures were those of 9-hydroxy-, 9-hydroperoxy- and 9-keto-octadeca-10E-12Z-15Z-trienoic acids (9-α-HOTrE, -HPOTrE and -KOTrE). *Hydra* homogenates transformed 9-α-HPOTrE partly into 9-α-HOTrE and partly into 9-α-KOTrE. Chiral phase HPLC conducted on 9-α-HOTrE established that this metabolite was composed mostly of the *R* enantiomer. These observations, and the finding that the presence of exogenous arachidonic acid in incubated homogenates significantly reduces the production of α-LA metabolites, provide strong evidence that these compounds are produced by an enzymic activity identical to the previously-described *H. vulgaris* (*R*)-ω10-lipoxygenase. Further experiments suggested that α-LA, acting as the native substrate for this enzyme, is mainly esterified on the 2 position of *Hydra* phosphoglycerides, and that the production of the α-LA metabolites described here for the first time from natural sources, can be potentially enhanced in vivo by stimuli activating phospholipase A_2.
Key words. Polyunsaturated fatty acids; invertebrate oxylipins; phospholipase A_2; *Hydra*; α-linolenic acid; lipoxygenase(s).

Lipoxygenases (LOs) are widespread enzymes. They catalyze the peroxidation of polyunsaturated (essential) fatty acids (PUFAs), such as arachidonic acid (AA), with formation of hydroperoxy-fatty acids[1–3]. LO-mediated reactions proceed through the enantiospecific abstraction of hydrogen from one of the doubly allylic methylene groups present in PUFAs, immediately followed by enantioselective addition of O_2 to the olefinic carbon atom β to the methylene group, a shift of the corresponding double bond, and the formation of a *cis*, *trans* conjugated diene α to the new hydroperoxy function[1–3]. While mammalian LOs lead to the biosynthesis of (*S*)-hydroperoxy-fatty acids, which may be transformed either into the corresponding hydroxy- and keto- fatty acids, or into the more complex leukotrienes and lipoxins[4,5], analogous enzymes from invertebrate sources often display the opposite enantioselectivity, thus catalyzing the production of (*R*)-hydroperoxy-fatty acids (for a review see ref. 6). The latter may undergo a wide series of biochemical transformations producing keto- and (*R*)-hydroxy-acids, hepoxilins and trioxilins[7], unstable allene oxides[8–10] and, in soft corals, even prostaglandins[9,10]. LOs are usually quite regioselective enzymes; the criterion by which the peroxidation is directed preferentially to one double bond instead of another varies according to the enzyme. Some LOs, like the enzymes from potato tubers and polymorphonuclear leukocytes[11,12], 'look' only for the double bond nearest to the carboxyl group, irrespective of the number of carbon atoms intervening between the two functions; other LOs 'count' the site of peroxidation starting from the aliphatic end of the PUFA, like the L-1 lipoxygenase from soybean which, at pH9, attacks specifically the ω6 unsaturated carbon atom, irrespective of the number of other double bonds separating this atom from the terminal methyl group[13].

Lately, we have described the partial characterization of a novel and abundant lipoxygenase-like enzyme in cell

* Corresponding author.

membrane-free extracts of the freshwater hydrozoan *Hydra vulgaris*[14,15]. Apart from considerations of a comparative and evolutionary nature, the importance of this finding comes also from the recently acquired evidence that, in species belonging to the genus *Hydra*, the enzyme responsible for PUFA release, i.e. phospholipase A_2 (PLA_2), as well as AA and LO-derived AA metabolites, may participate in the control of body pattern, tentacle regeneration and bud formation[16–18]. When AA was used as substrate, *H. vulgaris* LO catalyzed the enantioselective formation of high amounts of 11-(*R*)-hydroperoxy- and, subsequently, 11-(*R*)-hydroxy-eicosatetraenoic acid. This reaction was not inhibited by cycloxygenase or cytochrome p450 inhibitors, nor by the inhibitor of mammalian 5- and 12-LOs nordihydroguaiaretic acid (NDGA)[14]. When other PUFAs, with 18, 20 and 22 carbon atoms, were used as substrates, regioselective peroxidation of the tenth carbon atom counting from the aliphatic end, and, therefore, of the ω10 double bond, was always observed, with subsequent formation of hydroperoxy-, hydroxy- and, when starting from C_{18} PUFAs, keto-fatty acids[15]. The amount of *R*-enantiomer in the hydroxy-acids produced increased up to 95% when the substrate concentration was decreased and/or the degree of unsaturation of the substrate was increased as in eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA)[15]. On the basis of these findings, *H. vulgaris* LO can be classified as an (*R*)-ω10-lipoxygenase. The possible physiological role of such an unusual LO was studied by testing the effect on tentacle regeneration of commercially available hydroxy- and keto-derivatives which could originate from the action of the enzyme on some of the most abundant PUFAs in *Hydra* fatty acid pools[15]. However, the actual PUFA serving as the native substrate for this enzyme in vivo, as well as the nature of the metabolites thereby produced, was not investigated in these experiments.

The aim of the present study was to gain further evidence for the physiological significance of *H. vulgaris* ω10-LO by attempting:

1) To find its natural substrate;
2) to characterize the chemical structure and, where possible, the bioactivity of the putative metabolites derived in vivo by its action on this substrate;
3) to obtain preliminary information on the possible stimuli leading to the production of these metabolites.

Materials and methods

H. vulgaris were originally obtained from Prof. P. Tardent, University of Zurich, and were grown as previously described[14–17]. PUFAs, as free acids, were obtained from Sigma, UK. 9-hydroxy-, 9-hydroperoxy- and 9-keto-octadeca-10*E*-12*Z*-dienoic acids were purchased from Cascade, UK or from Cayman Chemicals, USA. Protein determination was carried out by means of Folin phenol reagent (Merck). HPLC and deuterated solvents were obtained, from Farmitalia Carlo Erba, Milan, Italy, and Merck, respectively. [^{14}C]-α-LA was purchased from NEN (USA, 52 mCi/mmol).

Both for the extraction of naturally occurring metabolites, and for incubation experiments, polyps were homogenized in 0.05 M/HCl, pH = 8.0, at 0 °C and sonicated for 30 s. The homogenate was then centrifuged for 30 min at 10,000 *g* and 0 °C. According to the type of experiment, homogenates were obtained from unlabelled polyps or from polyps pre-labelled overnight with [^{14}C]-α-LA (0.5 µCi/ml culture medium). The supernatant was either directly purified or incubated at 20 °C. Aliquots were always kept for protein content determination. The type of incubation varied according to the type of experiment. Homogenates from unlabelled hydra were used for 1 h incubations in the presenece of: 1) increasing concentrations of exogenously added α-LA(0.085, 0.17, 0.35, 1.00, 1.75 mM); 2) [^{14}C]-α-LA (0.1 µCi/ml homogenate) with or without AA or eicosanoic acid (0.5 mg/ml); 3) [^{14}C]-α-LA (1 µCi/ml homogenate), in order to produce radiolabelled α-LA metabolites; and 4) previously purified [^{14}C]-α-LA metabolites, in order to investigate their interconversion. Homogenates from pre-labelled hydra were used: 1) for incubations carried out for different intervals of time (0, 5, 20 and 60 min); and 2) to study the effect of PLA_2 (50 U) or phospholipase B (10 U) on the production of radioactive α-LA and of its metabolites; in this case whole homogenates, obtained from non-centrifuged extracts, were used. Control homogenates were inactivated by addition of HCl 10 N up to a pH = 2.5. *Artemia salina* nauplii were also extracted as described above.

In all the above cases, supernatants, either immediately after the centrifugation or following the incubation, were fractioned by means of Sep-pak C-18 (Waters Ass., USA). This was washed and eluted as described previously[14,15], and the eluates were lyophilized and analyzed by reverse phase HPLC. This chromatographic step was conducted as previously described[14,15]. Briefly, a Spherisorb ODS-2 semi-preparative column (5 µm diameter bead, 25 cm × 1 cm) was eluted with a 10 min isocratic step of 55% acetonitrile in water plus trifluoroacetic acid (0.1% v/v) followed by a 70 min linear gradient of up to 70% acetonitrile in water plus trifluoroacetic acid (0.1% v/v). The flow rate was 2 ml/min. In preparative analyses, fractions corresponding to peaks of UV absorbance, monitored at 235 and 280 nm, were pooled, lyophilized and redissolved in CD_3OD to be submitted to [^{1}H]-NMR spectroscopy, conducted on a Brucker 500 MHz instrument equipped with a reverse probe. In analyses of radioactive incubates, 5 ml scintillation liquid were added to 1 min (2 ml) fractions, and radioactivity counted. In quantitative analyses, the

amounts of metabolites were measured by comparison of the areas of their HPLC UV peaks with those of known amounts of standards of 9-hydroxy-, 9-hydroperoxy-and 9-keto-octadeca-10*E*-12*Z*-dienoic acids.

Methyl ester derivatives of samples of 9-α-HOTrE, prepared by reacting the compound with excess diazomethane, were analyzed for their enantiomeric composition by means of chiral phase HPLC carried out as previously described[14,15,20]. Briefly, a Chiralcel OB column (Daicel Chem. Ind., 25 cm × 4.6 mm) was eluted at 1.5 ml/min with *n*-hexane/propan-2-ol (98/2 v/v). UV absorbance was monitored at 235 nm. 9-(*S*) and 9-(*R*)-hydroxy-octadeca-10*E*-12*Z*-dienoic acids were used as standards.

Determination of PUFA content was carried out by means of gas-chromatographic (GC) analyses of the methyl ester derivatives. PUFAs present as free acids in cytosolic extracts were obtained by extraction of the latter with chloroform/methanol 2/1 (v/v). The extracts were then lyophilized and derivatized by reacting them with excess diazomethane. Phospholipid-bound fatty acids were obtained directly as methyl esters by reacting *H. vulgaris* phosphoglycerides, extracted as previously described[19], with Na_2CO_3 in anhydrous methanol overnight at 20 °C. GC of the fatty acid methyl esters was carried out as described previously[19], but an isotherm elution at 190 °C was used instead of a programmed temperature gradient in order to resolve α-LA-methyl ester from linoleic acid- and γ-LA-methyl esters.

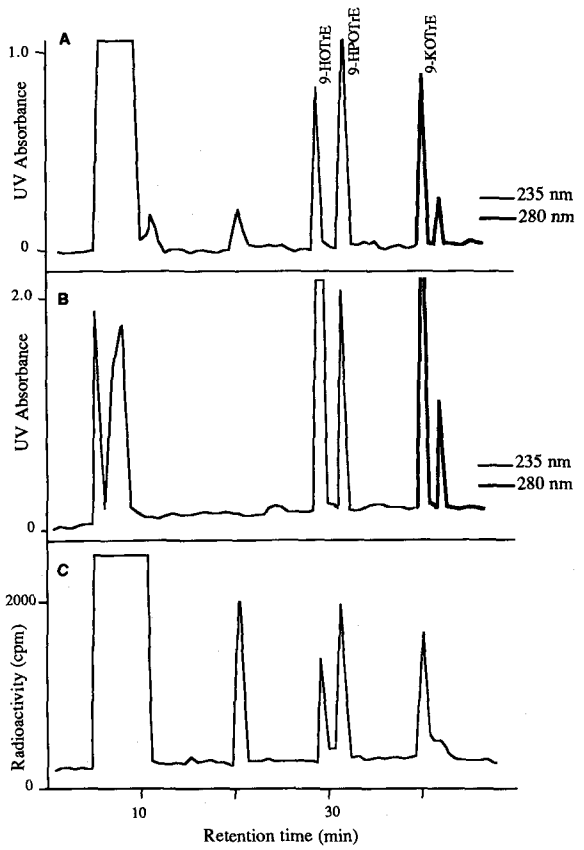

Figure 1. UV (*A* and *B*) and radioactivity (*C*) profiles of HPLC analyses of Sep-pak eluates from homogenates of *Hydra vulgaris* untreated (*A* and *C*) or incubated for 1 h at 20 °C with exogenous α-LA (0.5 mg/ml) (*B*). 5000 (*A*) and 1000 (*B*) unlabelled polyps and 1000 polyps pre-labelled overnight with [^{14}C]-α-LA (*C*) were used. HPLC conditions are described in 'Materials and methods'.

Results and discussion

The first aim of the present study was to assess whether substances that could in theory be derived from the catalytic activity of *H. vulgaris* ω10-LO on an available endogenous PUFA actually do occur in vivo. Therefore, aqueous extracts from polyps of the hydrozoan were fractionated by means of elution through Sep-paks C-18 and analysed by reverse phase HPLC. A typical HPLC chromatogram is shown in figure 1A. Two major components were detected when the UV absorbance was monitored at 235 nm, while two more hydrophobic peaks were observed when the UV detector was set at 280 nm. It was noticed that the retention times of these four peaks, using elution conditions under which most PUFA oxidation products are well resolved[14,15], were identical to those of four metabolites formed after 1 h incubation of *H. vulgaris* cytosolic extracts with exogenous α-linolenic acid (α-LA) [this study and ref. 15] (fig. 1B). In a previous study[15], three of the latter metabolites were identified, by means of gas chromatographic-mass spectrometric analyses of the acetoxy-methyl ester derivatives, and of [^{1}H]-NMR analyses, as 9-hydroxy-, 9-hydroperoxy- and 9-keto-octadeca-10*E*-12*Z*-15*Z*-trienoic acids (9-α-HOTrE, -HPOTrE and -KOTrE) which appeared in the more and the less polar 235 nm peaks and the more polar 280 nm peak, respectively. The structure of the less polar 280 nm peak could not be determined owing to its low amount.

Therefore, it was decided to examine the possibility that the components of *H. vulgaris* untreated extracts were, indeed, these three derivatives of α-LA previously produced in vitro from the exogenously added fatty acid precursor. Since extracts of nauplii of *Artemia salina*, used to feed the hydra, did not contain these substances (not shown), it was deduced that they had been produced by synthesis de novo. Evidence for the biogenetic origin of the endogenous compounds was then sought by analyzing extracts of polyps that had been pre-labelled overnight with [^{14}C]-α-LA. All four native metabolites were found to specifically incorporate radioactivity (fig. 1C). However, bearing in mind that [^{14}C]-α-LA could have been transformed into other n-3 PUFAs such as EPA or DHA, this finding did not indicate unequivocally that the endogenous compounds were derived from α-LA. For this reason it was necessary to obtain definitive confirmation of their chemical structure.

For this purpose, extracts from 5000 polyps were extracted and the extracts immediately purified as de-

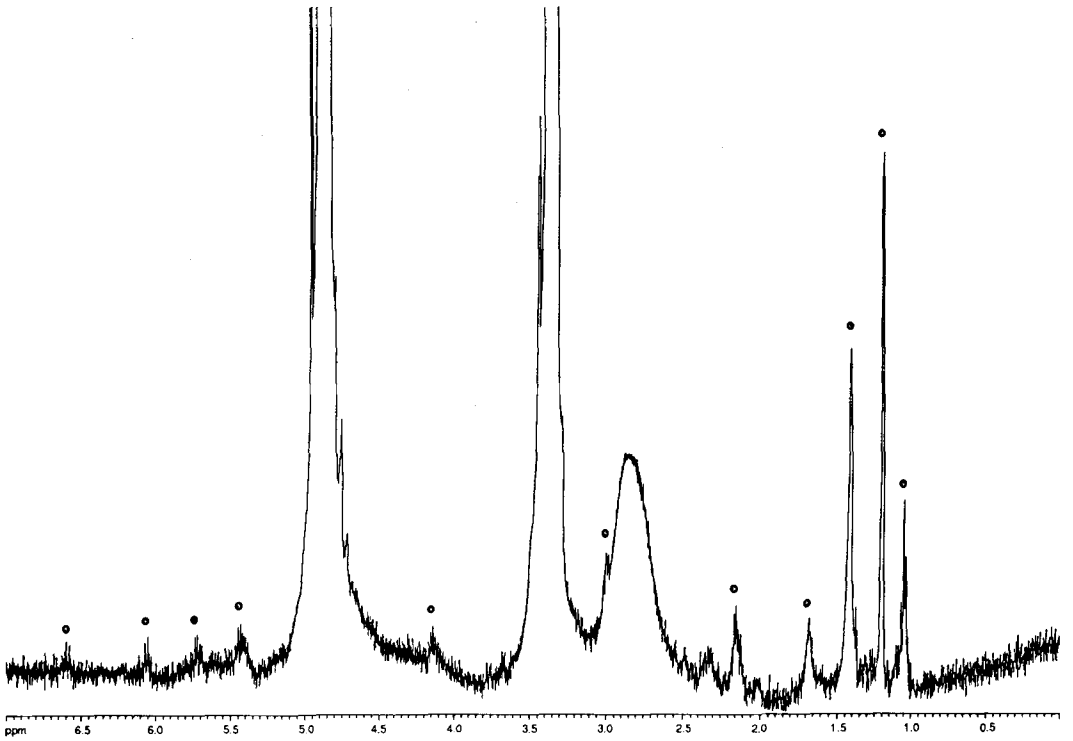

Figure 2. [^{1}H]-NMR spectrum (CD_3OD, 500 MHz, 1056 scans) of the more hydrophilic endogenous metabolite, i.e. 9-α-HOTrE (see fig. 1A), from *Hydra vulgaris* aqueous extracts. It was estimated that approximately 10 μg of sample were used to obtain this spectrum. Offscale peaks were due to solvent impurities visible because of the low sample concentration. Dots show signals present also in the spectrum of 9-α-HOTrE obtained in higher amounts from α-LA added exogenously to homogenates (see fig. 1B).

scribed. HPLC fractions corresponding to the three most abundant metabolites were lyophilized, dissolved in CD_3OD and analysed by [^{1}H]-NMR spectroscopy. The more hydrophilic compound displayed a [^{1}H]-NMR spectrum (fig. 2) perfectly superimposable on that of 9-α-HOTrE obtained from exogenous α-LA[15]. Signal multiplicities cited here refer to the latter spectrum since the signals from the endogenous compound (ca 10 μg) were only large enough to determine their chemical shifts and areas. Signals at $\delta = 5.71$ (dd, 1H), 6.59 (dd, 1H) and 6.04 (dd, 1H) suggested the presence of a conjugated diene; the signal at $\delta = 4.13$ (dt, 1H) was assigned to a CH-OH; the double doublet at $\delta = 2.98$ (2H) was typical of a methylene group between a conjugated diene group and a double bond, while the two protons of the latter resonated as a multiplet at $\delta = 5.42$; the triplet at $\delta = 1.01$ (3H) indicated univocally the presence of a terminal methyl group β to a double bond, as in all n-3 PUFAs; the multiplet at $\delta = 2.15$ (4H) was assigned to two methylene groups near either to a carboxylic function or to a double bond; the multiplet at $\delta = 1.66$ (2H) indicated the presence of a CH_2 α to a tertiary alcohol; finally, signals at $\delta = 1.38$ and 1.22 accounted for isolated methylene groups.

The [^{1}H]-NMR spectrum (not shown) of the less polar 235 nm peak was virtually identical to that previously reported for 9-α-HPOTrE (ref. 15) and, accordingly, almost identical to that of 9-α-HOTrE. The only differences from the latter were the signal at $\delta = 4.32$ instead of 4.13 (dt, 1H), assigned to CH-OOH, and the signal of the olefinic proton on C-10 at $\delta = 5.63$ instead of 5.71 (dd, 1H). Finally, [^{1}H]-NMR data for the more hydrophilic of the two 280 nm peaks coincided with those previously published for 9-α-KOTrE (ref. 15). Briefly, the presence of the carbonyl group conjugated with the *trans, cis* diene group was supported, apart from the strong UV absorbance at 280 nm, also by the signals at $\delta = 6.26$ (d, 1H), 7.65 (dd, 1H), 6.24 (dd 1H) and 5.85 (dt, 1H) assigned to the olefinic protons on C-10, C-11, C-12 and C-13, respectively; protons on the methylene group between the conjugated system and the isolated double bond displayed a signal at $\delta = 3.12$ (dd, 2H), while those on the methylene α to the carbonyl function were at $\delta = 2.65$ (t, 2H).

Thus, NMR analyses of the three most abundant metabolites found in *H. vulgaris* extracts confirmed their structures as those of the 9-hydroxy-, 9-hydroperoxy- and 9-keto-derivatives of α-LA. This allowed a quantitation of these compounds, carried out by means of HPLC in comparison with standards of 9-hydroxy-, 9-hydroperoxy- and 9-keto-octadeca-10*E*-12*Z*-dienoic acids, on the assumption that the presence of a further isolated double bond would not significantly influence the molar absorption coefficients at 235 and 280 nm. The amounts found in homogenates immediately after extraction and centrifugation were 2.1 ± 0.5, 2.2 ± 0.4

and 4.8 ± 0.8 µg/1000 specimens, respectively (means ± SEM, n = 3).

On the basis of the chemical characterization of the endogenous metabolites, it was justifiable, unless some very complicated endogenous pathway was envisaged, to hypothesize that these compounds were the products of an in vivo peroxidation of α-LA catalyzed by the previously described (*R*)-ω10-LO. In order to substantiate this hypothesis some questions had to be answered:

1) Are the endogenous substances produced by an enzymatic activity or by spontaneous lipid peroxidation?
2) Is their putative enzymatic production enantiospecific?
3) Is the putative substrate for this reaction, i.e. α-LA, actually available in vivo as a free acid?
4) Is AA, the preferential substrate for *H. vulgaris* ω10-LO (ref. 15), capable of competing with α-LA in enzymatic peroxidation?

In order to address the first of these questions, extracts of polyps pre-labelled with 1 µCi [^{14}C]-α-LA were incubated at 20 °C and aliquots were taken after 0, 5, 20 and 60 min, pre-purified and analyzed by HPLC as usual. A control incubation was carried out with an extract inactivated with HCl (pH = 2.5). The amounts of radioactivity incorporated at each interval of time into all three major components increased already after 5 min, except in the inactivated extract (not shown). For example, counts associated with 9-α-HPOTrE, expressed as percent of total radioactivity incorporated, went from 0.14%, at time 0 or in inactivated extracts, to 0.33% (5 min), 0.53% (20 min) and 0.57% after 1 h incubation. This finding suggested that these substances are produced by enzymatic oxidation of endogenous pools of α-LA. The presence and time-dependent formation of 9-α-HPOTrE, in extracts identical to those previously used to demonstrate the abundant ω10-LO-like activity, allows the further hypothesis to be proposed that the latter enzyme and the protein catalyzing the production of the hydroperoxide are identical.

Analysis by chiral phase HPLC of the methly ester derivative of native 9-α-HOTrE allowed the establishment of its stereochemistry at C-9 as *R* for 89% of the metabolite (fig. 3). 9-S- and 9-R-hydroxy-octadeca-10*E*-12*Z*-dienoic acids were utilized as standards, on the assumption that the presence of the third double bond at Δ15 does not modify the relative chromatographic behaviour of *R* and *S* enantiomers under the elution conditions used[15,20]. This represents the first report of 9(R)-α-HOTrE from natural sources, whereas the optical antipode of this compound, 9(*S*)-α-HOTrE, has already been isolated from plants such as rice, where it seems to play a defensive role against pests[21,22]. Moreover, the knowledge of the enantiomeric composition of one of the major products of endogenous enzymatic peroxidation in *H. vulgaris* substantiates further the hypothesis that the enzyme involved in its biosynthesis is

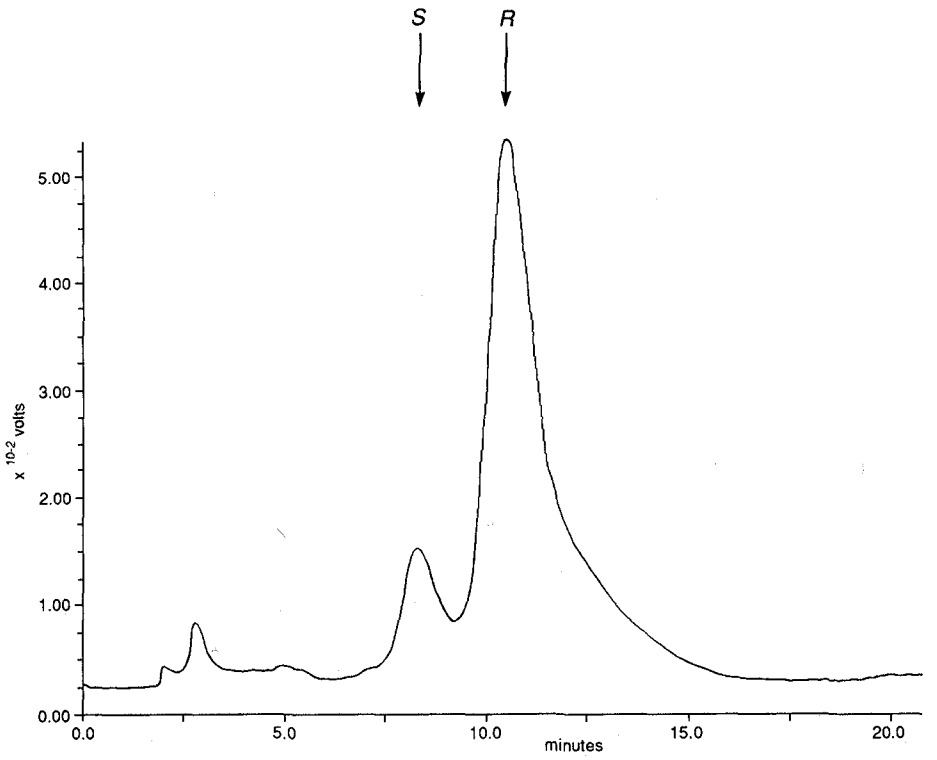

Figure 3. UV absorbance (235 nm) profile of chiral phase HPLC analysis of the methyl ester derivative of endogenous 9-α-HOTrE (c.a. 5 µg). HPLC conditions are described in 'Materials and methods'. Arrows show the relative elution sequence of synthetic 9-(*S*)-and 9-(*R*)-hydroxy-octadeca-10E-12Z-dienoic acid methyl esters in similar HPLC analyses.

Table 1. Amounts (μg/1000 polyps) of polyunsaturated fatty acids (PUFAs) as free acids and phosphoglyceride-bound esters in *Hydra vulgaris*.

PUFA	Free fatty acid (μg/1000 specimens)	Phosphoglyceride-bound (μg/1000 specimens)
$C_{18:2,\ \Delta 9,\ 12}$, (LA)	8	12
$C_{18:3,\ \Delta 9,\ 12,\ 15}$, (α-LA)	32	60
$C_{18:3,\ \Delta 6,\ 9,\ 12}$, (γ-LA)	0.5	3
$C_{20:4,\ \Delta 5,\ 8,\ 11,\ 14}$, (AA)	4	50
$C_{20:5,\ \Delta 5,\ 8,\ 11,\ 14,\ 17}$, (EPA)	6	29

LA = linoleic acid, α-LA = α-linolenic acid; γ-LA = γ-linolenic acid; AA = arachidonic acid; EPA = eicosapentaenoic acid.

the previously described *R*-lipoxygenase. Other enzymes possibly involved in simple lipid peroxidation, such as cytochrome p450 monoxygenases, have been reported to act mostly in a non-enantioselective fashion[23].

A necessary condition for the LO-mediated production of α-LA derivatives in vivo is the availability of free α-LA in cytosolic extracts of *H. vulgaris*. Therefore, these were extracted with $CHCl_3/CH_3OH$, the organic phases lyophilized, the fatty acids esterified with diazomethane and the mixture analyzed by capillary-column gas chromatography in comparison with standards of fatty acid methyl esters. The isotherm elution gradient employed allowed the separation of α-LA-methyl ester from γ-LA- and linoleic acid-methyl esters. Starting from 1000 polyps, 32 μg of α-LA metabolites was found in free fatty acid pools. This value is perfectly compatible with the total amount of α-LA metabolites found in the extracts of 1000 polyps, i.e. ca 9.1 μg (see above). The fatty acid was also the most abundant PUFA among the phosphoglyceride-bound fatty acids (table 1), thus pointing to this pool as a further potential source of substrate for LO action.

Once the co-existence in cell membrane-free fractions of (*R*)-ω10-LO and α-LA had been ascertained, definitive evidence that the endogenous metabolites are derived from the action on the fatty acid of the former, for which no inhibitor has yet been found, was sought by studying the effect of AA, the PUFA displaying the best affinity for *H. vulgaris* ω10-LO[14,15], on the amount of [^{14}C]-α-LA incorporated into α-LA derivatives. As shown in table 2, AA (0.5 mg/ml), but not the saturated homologue eicosanoic acid – which is not a substrate for LO action – significantly inhibited the formation of radiolabelled 9-α-HOTrE, -HPOTrE and -KOTrE during 1 h incubations of extracts at 20 °C. Correspondingly: 1) the amount of free [^{14}C]-α-LA in the presence of the C_{20} PUFA was found to be increased; 2) strong UV peaks at the retention times of 11-hydroxy- and -hydroperoxy-eicosatetraenoic acid, the major products of ω10-LO action on AA [14], were found (not shown). This finding conclusively indicated that AA competes with endogenous α-LA as a substrate for the same enzyme, i.e. the previously reported (*R*)-ω10-lipoxygenase.

Table 2. The effect of arachidonic acid (0.5 mg/ml) and its saturated homologue, eicosanoic acid (0.5 mg/ml), on the metabolism of [^{14}C]-α-LA (0.1 μCi/ml) by homogenates of *Hydra vulgaris*.

	Control	+ Arachidonic acid ($C_{20:4,\ \Delta 5,\ 8,\ 11,\ 14}$)	+ Eicosanoic acid ($C_{20:0}$)
9-α-HOTrE + 9-α-HPOTrE (cpm)	12093 ± 422	9747 ± 241*	12067 ± 643
9-α-KOTrE (cpm)	16155 ± 897	11409 ± 677*	14895 ± 1129
α-LA (cpm)	21150 ± 127	31678 ± 4671*	21125 ± 2385

1000 polyps homogenised in 10 ml Tris buffer were used. Protein content in the supernatant after 10,000 × *g* centrifugation was 2.5 mg/ml. Radiolabelled α-LA and the exogenous PUFA were added at the same time before starting the 1 h incubation. Incubates, conducted in triplicate, were purified and analyzed as described in 'Materials and methods'. Data are expressed in cpm ± SEM, n = 3. Means were compared by Student's *t* test. * $p < 0.05$.

In order to gain some information on the affinity of this enzyme for α-LA, different concentrations of the fatty acid were incubated with *H. vulgaris* cytosolic extracts, and the amount of the metabolites formed measured by HPLC. As shown in figure 4, the percentage conversion of the PUFA into its LO-derived metabolites was maximal at a substrate concentration of 0.35 mM, and decreased at higher concentrations. This behaviour resembled that previously reported when using AA as substrate[14], in agreement with the above finding that the same enzyme is responsible for the oxidative metabolism of the two PUFAs in vitro and/or in vivo. Interestingly, the enantiomeric composition of 9-α-HOTrE produced also seemed to depend on α-LA concentration; a significantly lower amount of the *R* isomer was found at [α-LA] = 0.35 and 1.65 mM (81 and 69%, respectively), in agreement with that previously observed for AA[15].

Once the chemical and enzymatic aspects of α-LA peroxidation in *H. vulgaris* had been clarified, the possible biogenetic relationship between the identified metabolites was examined. [^{14}C]-labelled 9-α-HOTrE, -HPOTrE and -KOTrE were purified from previous incubations of *Hydra* homogenates with [^{14}C]-α-LA, and used to conduct separate 1 h incubations with other homogenates under the same conditions. Incubates were then fractionated and analysed by HPLC as usual. Due

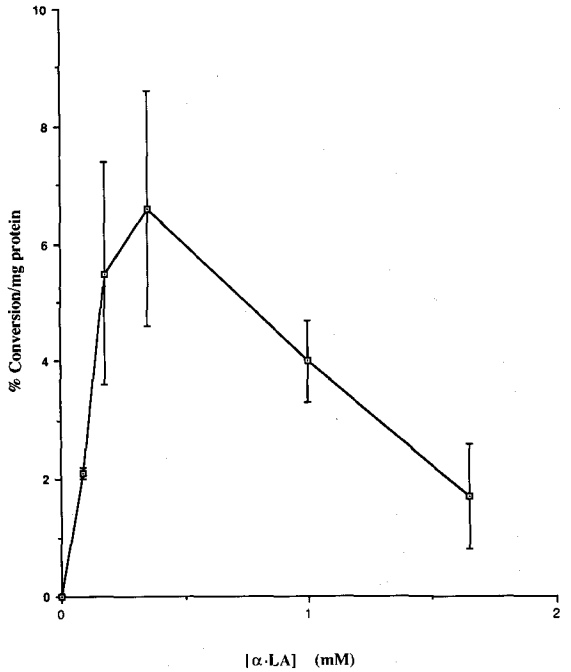

Figure 4. Dependence of α-LA oxidative metabolism on its concentration in *Hydra vulgaris* homogenates. The percent of the fatty acid converted into 9-α-HOTrE plus 9-α-HPOTrE plus 9-α-KOTrE per mg of total protein content in the homogenate is shown on the y axis. The amounts of endogenous metabolites present in homogenates without addition of exogenous α-LA (blanks) were subtracted. Data are means ± SD of three separate experiments.

to the long incubation time employed, small recoveries of radioactivity were observed in all three incubations. When starting from [^{14}C]-9-α-HPOTrE (11600 cpm) most of the radioactivity was found associated aspecifically with highly hydrophilic unidentified components, but specific incorporation was observed in HPLC fractions corresponding to 9-α-HOTrE and -KOTrE (400 cpm and 256 cpm, respectively, background 33 cpm). Homogenates incubated with [^{14}C]-9-α-HOTrE (14000 cpm) yielded a negligible amount of radiolabelled 9-α-KOTrE (135 cpm, background 30 cpm), possibly due to contamination of the HOTrE sample with some hydroperoxide, while 1712 cpm remained associated with 9-α-HOTrE. Finally, upon incubation with [^{14}C]-9-α-KOTrE (10900 cpm) no radiolabel was found associated with the hydroxy-derivative, and the major radioactivity peak corresponded with 9-α-KOTrE itself (1413 cpm, background 24 cpm); another peak (537 cpm) was found to co-elute with the second 280 nm UV-visible HPLC component, whose structure was not elucidated in this study due to scarcity of material, thus suggesting that this unknown compound may derive from 9-α-KOTrE. These findings, taken together, suggest that the hydroperoxide functions as precursor for the synthesis of both the hydroxy- and the keto-derivative, and that very little, if any, interconversion occurs between these two metabolites.

The lack of commercially available sources of the metabolites characterized in this study, and the incomplete enantiospecificity of their natural source, i.e. *H. vulgaris*, prevented a study of their biological activity. In a previous investigation[15], on the basis of earlier findings pointing to the involvement of the 'AA cascade' in the control of hydroid regeneration and body pattern[16–18], compounds derived from ω10-LO action on PUFAs different from α-LA were tested for their regenerating activity on *Hydra* head and tentacles. A correlation between stereochemistry of the hydroxylated carbon atom, unsaturation/length of the metabolite and regenerating activity was found[15]. While 11(*R*)-hydroxy-eicosapentaenoic acid, derived from EPA, was not active, 11(*R*)-hydroxy-eicosatetraenoic acid, derived from AA, and 9(*S*)-hydroxy-octadecadienoic acid (but not 9-keto-octadecadienoic acid), derived from linoleic acid, were capable of enhancing the average tentacle number. This might suggest an analogous role for 9-α-(*R*)-HOTrE which, unlike the above substances, occurs in vivo in fair amounts. However, any conclusion on the biological function of this compound, and of the other metabolites isolated in this study, must await their chemical synthesis in amounts sufficient to allow extensive bioactivity testing in *Hydra*.

Nevertheless, in order to gain some further understanding of the physiological relevance of the findings described here, the potential mechanism by which α-LA and, therefore, its metabolites, are produced in *H. vulgaris*, was preliminarily investigated in vitro. When deciding on the type of experiment to be performed for this purpose, three pieces of evidence obtained in the hydrozoan were taken into consideration: 1) the previously reported correlation between PLA_2 activation and tentacle regeneration[16,17]; 2) the recent finding of a membrane-bound PLA_2-like enzyme in *H. vulgaris* homogenates[24]; and 3) the high amounts of α-LA found in the present study to be esterified onto phosphoglyceride

Table 3. Effect of phospholipases A_2 (PLA_2) and B (PLB) on the release of α-LA and on its metabolism, in whole homogenates of *Hydra vulgaris* pre-labelled with [^{14}C]-α-LA.

Sample	Oxidation products (cpm)	α-LA (cpm)
Control	2103 ± 132	1689 ± 77
+ PLA_2	4743 ± 65**	3875 ± 224**
Control	695 ± 23	863 ± 67
+ PLB	768 ± 64	1166 ± 24*

In two sets of experiments, non-centrifuged homogenates were incubated for 1 h with either 50 U of PLA_2 or 10 U of PLB. The incubates were purified and analyzed as described in 'Materials and methods', and the radioactivity of HPLC fractions corresponding to either the three oxidation products or to α-LA was counted and compared with that of control incubations. Data for the three metabolites were pooled for the sake of simplicity, and are expressed in cpm as means ± SEM of triplicates. Means were compared by Student's *t* test. * $p < 0.05$, ** $p < 0.01$.

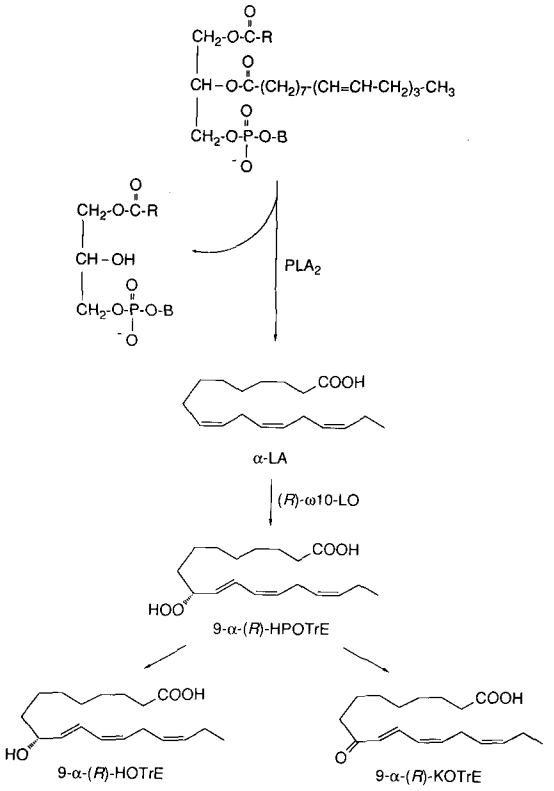

Figure 5. Schematic representation of α-LA release and metabolism in *Hydra vulgaris*, based on the findings described in the present paper. PLA_2 = phospholipase A_2; B = phospholipid base.

pools. Therefore the effect of exogenously added PLA_2 on the production of α-LA metabolites in whole extracts, containing cell membranes, of polyps prelabelled with [^{14}C]-α-LA, was examined. As shown in table 3, co-incubation of these extracts with porcine pancreatic PLA_2 (50 U) led to a considerable increase in the amounts both of free [^{14}C]-α-LA (129%) and of radiolabelled metabolites (125%), whereas co-incubation with PLB (10 U) produced only a little augmentation (35 and 11%, respectively). This finding has two implications. First, bearing in mind the low regioselectivity of PLB-catalyzed hydrolysis of phosphoglycerides, which, in the presence of this enzyme, are attacked on carboxylic groups bound to both C-1 and C-2 of the glycerol moiety[25], it can be deduced that, as in most animal tissues, α-LA is esterified mostly on the 2 position of membrane phospholipids; this ester bond is subjected to specific hydrolysis in the presence of PLA_2 (ref. 25) (fig. 5). More importantly, it can be hypothesized that stimuli, such as Ca^{2+} influx, GTP-binding protein and protein kinase C activation, leading to activation of PLA_2 in mammals (for a review see 26) and to tentacle regeneration in *H. vulgaris*[17], may also lead to α-LA release and peroxidation. Obviously, although *H. vulgaris* PLA_2 seems to belong to the same class as the porcine pancreatic enzyme used here, i.e. low molecular weight PLA_2s (ref. 24), the likely differences between the procedure described above and the actual situation occurring in vivo must be taken into due account. However, the effect observed above with the exogenous enzyme may be useful for future studies aimed at correlating α-LA metabolism with PLA_2 activation and regenerative phenomena in hydrozoans.

In conclusion, the present study has described the elucidation of the structure, origin and biogenetic relationship of unprecedented α-LA derivatives in *Hydra vulgaris* (fig. 5). A somewhat unusual strategy has led to the characterization of these secondary metabolites. While the discovery of new natural products usually precedes the finding of the enzymes catalyzing their formation, here the knowledge of the occurrence of an enantioselective and regiospecific enzymatic activity[14,15] led to the finding, at the same time, of the products derived from its action in vivo and of its actual substrate. Future efforts will have to be directed towards the design of a strategy for the chemical synthesis of these metabolites, in order to allow the testing of the hypothesis of their control of the cell differentiation, proliferation and motility process involved in the establishment of the hydroid body pattern. Moreover, further investigations are needed to clarify the molecular mechanisms regulating α-LA metabolism in *Hydra* and its correlation with biological phenomena typical of hydrozoans.

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1 Pace-Asciak, C. R., and Asotra, S., Free radic. Biol. Med., *7* (1989) 409.
2 Schewe, T., and Kuhn, H., Trends biochem. Sci. *16* (1991) 369.
3 Yamamoto, S., Free Radic. Biol. Med. *10* (1991) 149.
4 Keppler, D., Rev. Physiol. Biochem. Pharmac. *121* (1992) 1.
5 Serhan, C. N., Bioenergetics Biomembranes *23* (1991)105.
6 De Petrocellis, L., and Di Marzo, V., Prostaglandins Leukotrienes ess. fatty Acids (1994), in press.
7 Pace-Asciak, C. R., and Martin, J. M., Prostaglandins Leukotrienes Med. *16* (1984) 173.
8 Corey, E. J., Matsuda, S. P. T., Nagata, R., and Cleaver, M. B., Tetrahedron Lett. *29* (1988) 2555.
9 Brash, A. R., Baertschi, S. W., Ingram, C. D., and Harris, T. M., Adv. Prostaglandin Thromboxane Leukotriene Res. *19* (1989) 70.
10 Brash, A. R., Baertschi, S. W., and Harris, T. M., J. biol. Chem. *265* (1990) 6705.
11 Galliard, T., and Phillips, D. R., Biochem. J. *124* (1971) 431.
12 Borgeat, P., Hamberg, M., and Samuelsson, B., J. biol. Chem. *251* (1976) 7816.
13 Hamberg, M., and Samuelsson, B., J. biol. Chem. *242* (1967) 5329.
14 Di Marzo, V., De Petrocellis, L., Gianfrani, C., and Cimino G., Biochem. J. *295* (1993) 23.
15 Di Marzo, V., Gianfrani, C., De Petrocellis, L., Milone, A., and Cimino, G., Biochem. J., *300* (1994), 501.
16 De Petrocellis, L., Di Marzo, V., and Cimino, G., Experientia *49* (1993) 57.
17 De Petrocellis, L., Di Marzo, V., Gianfrani, C., and Minei, R., Comp. Biochem. Physiol. *105C* (1993) 219.

18 Muller, W. A., Leitz, T., Stephan, M., and Lehmann, W. D., Roux's Arch. devl. Biol. *202* (1993) 70.
19 Di Marzo, V., Cimino, G., Crispino, A., Minardi, C., Sodano, G., and Spinella, A., Biochem. J. *273* (1991) 593.
20 Brash, A. R., and Hawkins, D. J., Meth. Enzym. *187* (1990) 187.
21 Kato, T., Yamaguchi, Y., Hirano, T., Yokohama, T., Uyehara, T., Namai, T., Tamanaka, S., and Harada, N., Chem. Lett. (1984) 409.
22 Rama-Rao, A. V., Rajarathnam-Reddy, E., Purandare, A. V., and Varaprasad, C. V. N. S., Tetrahedron *43* (1987) 4385.
23 Fitzpatrick, F. A., and Murphy, R. C., Pharmac. Rev. *40* (1989) 229.
24 Borrelli, L., Carginale, V., Capasso, A., Schneider, T., Leitz, T., De Petrocellis, L., and Di Marzo, V., submitted.
25 Waite, M., in: Biochemistry of lipids, lipoproteins and membranes, p. 269. Eds D. L. Vance and J. Vance. Elsevier, Amsterdam 1991.
26 Axelrod, J., Burch, R. M., and Jelsema, C. L., Trends Neurosci. *11* (1988) 117.