Polyunsaturated-fatty-acid oxidation in *Hydra*: regioselectivity, substrate-dependent enantioselectivity and possible biological role§

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A novel and abundant lipoxygenase-like activity converting *cis*-eicosa-5,8,11,14-tetraenoic acid (arachidonic acid) into (11*R*)-hydroxyeicosatetraenoic acid has been recently described in homogenates of the freshwater hydrozoan *Hydra vulgaris*. In this study, other substrates for this enzyme were selected from the polyunsaturated fatty acids (PUFAs) present in *H. vulgaris*, and the chemical natures of the hydroperoxy and hydroxy derivatives produced, as well as the activity of some of the latter on hydroid tentacle regeneration, were investigated. The highest conversion among C₂₀ fatty acids was observed for arachidonic acid, and among C₁₈ fatty acids for *cis*-octadeca-9,12,15- and *cis*-octadeca-6,9,12-trienoic (α - and γ -linolenic) acids. *Cis* double bonds on the 10th carbon atom from the aliphatic end of the substrate (e.g. C-9, C-11 and C-13 respectively in C₁₈, C₂₀ and C₂₂ PUFAs) were regiospecifically peroxidized. Conversely, *trans*-octadeca-9,12-

dienoic (linoelaidic) acid was not a substrate for lipoxygenase activity. Enantioselectivity of lipoxygenation depended on the degree of unsaturation of the substrate, with the amount of the *R* enantiomer increasing when passing, for example, from *cis*-eicosa-11,14-dienoic to *cis*-eicosa-5,8,11,14,17-pentaenoic acid. Regiospecific formation of keto acids was observed only when incubating C₁₈ PUFAs. Commercially available hydroxyacids corresponding to the reaction products of some of the most abundant *H. vulgaris* PUFAs were tested for effects on *Hydra* tentacle regeneration. An enhancement of average tentacle number, in a fashion depending on the stereochemistry and on the number of double bonds, was found for two compounds, thus suggesting for the 11-lipoxygenase-like enzyme a role in the production of metabolites potentially active in the control of hydroid regenerative processes.

INTRODUCTION

Polyunsaturated fatty acids (PUFAs) are abundant components of the animal cell membrane (Fisher, 1989; Cook, 1991). Once released by the action of specific phospholipases from membrane lipids, where they are usually found esterified at the 2-position of glycerophospholipids (Axelrod, 1990), PUFAs might serve as precursors for a wide range of bioactive metabolites, known as oxygenated fatty acids, through the catalytic action of several oxidizing enzymes including cyclo-oxygenase, cytochrome *P*-450 mono-oxygenases and various lipoxygenases (O'Brien, 1987; Fisher, 1989; Kromhout, 1992; Gerwick et al., 1993). In particular, the activation of enantiospecific lipoxygenases has been described as leading to the formation, via the corresponding hydroperoxides, of hydroxy acids and keto acids with several biological functions in many aspects of both mammalian and invertebrate physiology (Stanley-Samuelson, 1987; Spector et al., 1988). In some marine organisms, namely soft corals, lipoxygenase-catalysed production of (*R*)-hydroperoxy-derivatives of C₂₀ PUFAs has also been proposed as the first step in the biosynthesis of special oxygenated fatty acids, the prostaglandins, whose formation in mammalian tissues follows an altogether different pathway (Brash, 1989; Samuelsson, 1970). This emphasizes the importance of studying lipoxygenases from invertebrate as well as mammalian sources. Thus, for example, a (12*S*)-lipoxygenase has been shown to produce, starting from *cis*-5,8,11,14-eicosatetraenoic acid (arachidonic acid, AA), metabolites acting as second messengers for neural transmission

in ganglia of the mollusc *Aplysia californica* (Buttner et al., 1989; Piomelli et al., 1989). An (8*R*)-lipoxygenase has been described as catalysing the synthesis, in the reproductive system of starfish, of 8-*R*-hydroxyeicosatetraenoic acid [(8*R*)-HETE] involved in the control of oocyte maturation (Meijer et al., 1986). Enantioselective (11*R*)- and (12*R*)-lipoxygenase activities have been reported also in sea urchins (Hawkins and Brash, 1987), and lipoxygenase products have been detected in blood cells of the crab *Carcinus maenas* (Hampson et al., 1992).

More recently, several investigations have suggested the participation of phospholipase A₂, the enzyme mostly responsible for the liberation of AA and other PUFAs from cell membranes (Axelrod, 1990), and of enzymes of the 'AA cascade', in the control of hydroid body pattern, tentacle regeneration and bud formation (Di Marzo et al., 1993a; De Petrocellis et al., 1993a,b; Muller et al., 1993). HETEs and/or hydroxyoctadecadienoic acids (HODEs) have been detected in *Hydra magnipapillata* (Muller et al., 1993) and in other marine and freshwater hydroids (Di Marzo et al., 1993c). This work led to the finding of an abundant enantioselective enzymic activity leading to the formation of (11*R*)-hydroperoxyeicosatetraenoic acid [(11*R*)-HPETE] and (11*R*)-HETE in extracts of *Hydra vulgaris* incubated with AA (Di Marzo et al., 1993b). This activity was not decreased by either cyclo-oxygenase or cytochrome *P*-450 mono-oxygenase inhibitors, or by the antioxidant and 12- and 5-lipoxygenase inhibitor nordihydroguaiaretic acid, thus suggesting the presence of an (11*R*)-lipoxygenase-like enzyme in *H. vulgaris* (Di Marzo et al., 1993b).

Abbreviations used: AA, arachidonic acid; ATN, average tentacle number; DGLA, dihomogamma-linolenic acid; DHA, docosahexaenoic acid; EDA, eicosadienoic acid; e.i., electron impact; EPA, eicosapentaenoic acid; HDHE, hydroxydocosahexaenoic acid; HEPE, hydroxyeicosapentaenoic acid; HETE, hydroxyeicosatetraenoic acid; HETRe, hydroxyeicosatrienoic acid; HODE, hydroxyoctadecadienoic acid; HOTRe, hydroxyoctadecatrienoic acid; HPEPE, hydroperoxyeicosapentaenoic acid; HPETE, hydroperoxyeicosatetraenoic acid; HPODE, hydroperoxyoctadecadienoic acid; KODE, keto-octadecadienoic acid; LA, linoleic acid; α -LA, α -linolenic acid; γ -LA, γ -linolenic acid; PUFA, polyunsaturated fatty acid.

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Starting from this evidence and from the knowledge that PUFAs are very abundant in *Hydra* (De Petrocellis et al., 1993a; Muller et al., 1993), the present study was aimed at investigating whether PUFAs other than AA could be substrates for lipoxygenase action in *Hydra* homogenates and how the chemical structure of the substrate would influence the yield, regio-specificity and enantioselectivity of its oxidation. Moreover, data on the potential regenerative activity of oxidation products of some of the PUFAs present in *H. vulgaris* were also sought, in order to assess the possible involvement of lipoxygenases in the control of hydroid tentacle regeneration.

EXPERIMENTAL

Materials

H. vulgaris were originally obtained from Prof. P. Tardent, University of Zurich, and were grown according to the procedure described by Loomis and Lenhoff (1956) with slight modifications. PUFAs were obtained from Sigma. (9*S*)-HODE, (9*R*)-HODE, (9*S*)-hydroperoxyoctadecadienoic acid (9-*S*-HPODE), 9-keto-octadecadienoic acid (9-KODE), (11*S*)-HETE, (11*R*)-HETE, 11-HPETE, (11*S*)-, (11*R*)-hydroxyeicosapentaenoic acid [(11*R*)-HEPE] and (11*R*)-hydroperoxyeicosapentaenoic acid [(11*R*)-HPEPE] standards were purchased either from Biomol (Plymouth Meeting, U.S.A.) or from Cayman Chemicals (Ann Arbor, MI, U.S.A.). Protein determination assay was carried out by means of Folin phenol reagent (Merck). H.p.l.c. and deuterated solvents were purchased respectively from Farmitalia Carlo Erba (Milan, Italy) and Merck.

Incubation of PUFAs with *Hydra* homogenate and purification and quantitative determination of metabolites

Usually 1000 polyps were homogenized in 4 ml 0.05 M Tris/HCl, pH 8.0, at 0 °C and sonicated for 30 s. The homogenate was then centrifuged for 30 min at 10000 *g* and 0 °C, and the supernatant treated with the PUFA, as free acid, at a concentration of 0.5 mg/ml. An aliquot was always taken for protein determination assay. In some experiments with AA and α -linolenic acid ($C_{18:3,n-3}$; α -LA) concentrations of fatty acid of 0.01 and 0.1 mg/ml were also used. The incubation was carried out for 1 h at 20 °C, and then stopped by lowering the temperature to 0 °C and the pH to 2 by addition of HCl. Incubations with each PUFA were repeated at least three times. Control incubations were carried out in either acidified or boiled homogenates, and did not lead to any detectable peroxidation of PUFAs. In a separate set of experiments, homogenates were incubated with [14 C]9-HPODE, -HODE or -KODE prepared from a previous incubation carried out with linoleic acid ($C_{18:2,n-6}$; LA) plus [14 C]LA. In all cases, incubates were pre-purified by means of extraction on Sep-paks (Waters Associates), according to the procedure described previously (Di Marzo et al., 1993b), and the eluates were dried down under flow of nitrogen and then loaded onto h.p.l.c. This was carried out using a Spherisorb ODS-2 semi-preparative column (5 μ m diameter bead; 25 cm $\times$ 1 cm) 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 up to 70% acetonitrile in water plus trifluoroacetic acid (0.1%). The flow rate was 2 ml/min. U.v. absorbance was monitored simultaneously at 205, 235, and 280 nm. H.p.l.c. fractions containing u.v.-visible peaks were collected and lyophilized to be submitted for structural elucidation. In some experiments, LA or α -LA was co-incubated with 2 μ Ci [14 C]LA

or [14 C] α -LA respectively (Du Pont; 53 and 52 mCi/mmol respectively), and treated as described above, and the radioactivity of 10% of each 1 min h.p.l.c. fraction counted.

H.p.l.c. analyses, carried out under the same conditions described above with monitoring of the u.v. absorbance at 235 nm, of known amounts of 9-HODE, 9-HPODE, 9-KODE, 11-HETE, 11-HPETE, 11-HEPE and 11-HPEPE standards allowed quantitative measurement of PUFA hydroperoxy-, hydroxy- and keto-derivatives produced by each incubation. Synthetic standards were used to quantitate also those compounds that are not commercially available, on the assumption that the molar absorption coefficients at 235 nm of, for example, 9-HODE and 9- α -hydroxyoctadecatrienoic acid (α -HOTrE) or 11-HETE and 11-hydroxyeicosatrienoic acid (11-HETrE), are the same ($\epsilon \approx 23000 \text{ l} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$).

^1H -n.m.r. electron-impact m.s. analyses, chemical reactions and chiral phase h.p.l.c.

Proton n.m.r. spectra were recorded, for both endogenous and synthetic compounds, in [^2H]methanol on a 500 MHz Bruker apparatus. Application of the n.m.r. technique was often limited by scarcity of material. Electron-impact m.s. (e.i.-m.s.) spectra were obtained by means of a TRI-O-2 g.c.-m.s. apparatus equipped with a VG quadrupole mass spectrometer and a FS-SE-30 capillary column (25 m $\times$ 0.32 mm) using a programmed temperature gradient (80–280 °C at 10 °C/min). Application of the e.i.-m.s. technique to the hydroperoxy derivatives, which would have exhibited spectra identical with those of the corresponding hydroxy acids, was not possible because of the extreme instability of these compounds during the chemical derivatization procedures used in this study.

Aliquots of both synthetic and endogenous hydroxy and keto acids were methylated by reacting them with excess diazomethane for 15 min. Methyl ester derivatives of hydroxy acids were either: (a) acetylated in 200 μ l of pyridine plus 50 μ l of acetic anhydride for 40 min at 60 °C for e.i.-m.s. analysis, or (b) analysed by chiral phase h.p.l.c. carried out using the method described by Brash and Hawkins (1990) and a Chiralcel OB column (Daicel Chemical Industries; 25 cm $\times$ 4.6 mm). This column was eluted at 1.5 ml/min with *n*-hexane and various percentages (v/v) of propan-2-ol, depending on the hydroxy acid (0.3% for 11-HEDE, 0.5% for 11-HETrE, 1% for 9-HODE and 11-HETE, 2% for 9 α - and 9 γ -HOTrE, 4% for 11-HEPE and 13-hydroxydocosahexaenoic acid (13-HDHE). U.v. absorbance was monitored at 235 nm. Where commercial availability of standards made it possible, for example for LA-, AA-, and eicosapentaenoic acid (EPA)-derived monohydroxy acids, (*R/S*) composition was determined by comparison with optically pure standards and analysed under the same conditions. In the case of the hydroxy derivatives of α -LA, γ -linolenic acid, eicosadienoic acid (EDA), dihomogamma-LA ($C_{20:3,n-6}$; DGLA) and docosahexaenoic acid ($C_{22:6,n-6}$; DHA), on the contrary, it was assumed that, like for the other compounds, the (*S*)-enantiomer is eluted before the (*R*)-enantiomer.

Hydra tentacle regeneration assay

The assay was conducted as described previously (De Petrocellis et al., 1993a; Di Marzo et al., 1993b). At least 20 excised polyps, incubated for 24 h with the hydroxy fatty acid under study and then washed with culture medium, were used for each test. After the incubation period, each polyp was kept in 2 ml of medium in a separate 35 mm $\times$ 10 mm plastic Petri dish and individually observed daily. Drugs were dissolved in methanol, and control tests were run using the same amount of solvent (which was

never higher than 0.1 %, v/v). The effect on regeneration was studied by measuring the average tentacle number (ATN) 10 days after the incubation. Statistical analyses were carried out by using the unpaired Student's *t* test. The assay of doses higher than those described in Table 4 (below) was not possible because of the occurrence of toxic effects of the compounds tested.

RESULTS

Incubation of *Hydra* homogenates with C_{18} polyunsaturated fatty acids

Incubation of homogenates with either LA, α -LA or γ -LA, followed by Sep-pak extraction and reverse-phase h.p.l.c. purification always led to four u.v.-visible h.p.l.c. components, two of which showed maximal u.v. absorption at 235 nm, whereas the other two were only visible, at higher retention times, when u.v. absorbance was monitored at 280 nm (e.g. for LA, see Figure 1). Moreover, when *Hydra* homogenates were incubated with [14 C]LA or with [14 C] α -LA together with, respectively, unlabelled LA or α -LA, and aliquots of the h.p.l.c. fractions were counted, intense radioactivity peaks co-eluting with both 235 nm and 280 nm u.v. peaks were observed (results not shown), thus confirming that the latter actually correspond to metabolites of linoleic or linolenic acids. Conversely, incubation of homogenates with *trans*-octadeca-9,12-dienoic acid (linoelaidic acid) did not yield any u.v.-visible h.p.l.c. peak (Figure 1). ^1H -n.m.r. and/or e.i.-m.s. analyses were successfully employed to elucidate the structure of three of the four h.p.l.c. components for all C_{18} PUFAs except γ -LA, for which only the more polar 235 nm-visible metabolite was present in amounts sufficient to allow chemical characterization.

^1H -n.m.r. spectra of the more polar 235 nm peaks derived from the three PUFAs showed similar features (Table 1). The presence of a *trans*,*cis*-conjugated diene structure β to a secondary alcohol function was indicated by the olefinic signals at δ 5.64–5.72 (*J* 6.7, 15.2 Hz), 6.51–6.60 (*J* 11.1, 15.2 Hz) and 5.98–6.05 (*J* 11.1, 11.1 Hz), and by the CH-OH signal at δ 4.10–4.14 (*J* 6.7 Hz). The main characteristic of the spectrum of α -LA-derived metabolite was also a signal of δ 2.98 typical of methylene groups between an olefinic and a diene group. These data suggested the formation of a monohydroxy derivative for each PUFA indicated (see Di Marzo et al., 1993b, and references cited therein). The site of the hydroxylation on C-9 was established by the e.i.-m.s. fragmentation pattern of the methyl ester acetoxy derivatives of these metabolites, and namely by the presence of fragments at *m/z* corresponding to the cleavage of the C–C bond adjacent to the CH–OH group (Table 2; see also Hampson et al., 1992). These data indicate that the more polar h.p.l.c. components produced by the incubation of LA, α -LA and γ -LA with *Hydra* homogenates were 9-HODE, 9- α -HOTrE and 9- γ -HOTrE respectively.

The enantiomeric composition of these three metabolites was then analysed by means of chiral phase h.p.l.c. analyses of the methyl ester derivatives; the results of these analyses are summarized in Table 3. None of the three compounds was found to be optically pure; however, two observations could be made: (a) there was a greater amount of the (*R*)-enantiomer present in 9- α -HOTrE and 9- γ -HOTrE than in 9-HODE; and (b) in incubations conducted with concentrations of α -LA lower than 0.5 mg/ml (i.e. 0.01 and 0.1 mg/ml), the highest optical purity was observed at the lowest concentration tested (Table 3).

The less polar 235 nm-visible peaks derived from LA and α -LA, although quite abundant immediately after the purification, were found to be extremely unstable, their height visibly decreas-

ing in following h.p.l.c. runs. ^1H -n.m.r. spectra were, therefore, run immediately after purification in n.m.r. tubes sealed under nitrogen. The spectra were superimposable on those of the corresponding more polar 235 nm-visible metabolites except for a few diagnostic differences (Table 1): a slight upfield shift of the proton on C-10 from δ 5.64–5.72 to δ 5.55–5.63 and, more importantly, a significant downfield shift of the signal corresponding to the proton on C-9 from δ 4.10–4.13 to δ 4.26–4.32.

Figure 1 Selected reverse-phase h.p.l.c. traces of *Hydra* homogenates incubated with PUFAs, after Sep-pak extraction

U.v. absorbances at 235 nm (and, for LA, 280 nm) are shown. U.v. traces for α -LA and γ -LA were similar to that of LA (except for the fact that the second 235 nm peak and the two 280 nm peaks were much smaller for γ -LA) and are not shown for the sake of clarity. The less polar 235 nm peaks yielded by eicosadienoic acid and DHLA were not characterized because they were highly unstable and present only in little amounts, but are likely to be the hydroperoxy derivatives, according to what has been observed with other PUFAs.

Table 1 Selected ^1H -n.m.r. data for some PUFA oxidation products in *Hydra*

Spectra were run on a 500 MHz Bruker instrument using $[\text{D}_2]\text{methanol}$ as solvent. The low amount (10–30 μg) of material did not allow n.m.r. analysis for all compounds described in this study. Significant coupling constants are reported in the text (see the Results section) for the sake of clarity. HPDHE, hydroperoxydocosahexaenoic acid; HPOTrE, hydroperoxyoctadecatrienoic acid; KOTrE, keto-octadecatrienoic acid.

Compound	Chemical shift (δ) (p.p.m.)
9-HPODE	4.26 [1H, dt, C-9], 5.55 [1H, dd, C-10], 6.51 [1H, dd, C-11], 5.98 [1H, dd, C-12], 5.43 [1H, dt, C-13], 0.93 [3H, t, C-18]
9-HODE	4.10 [1H, dt, C-9], 5.64 [1H, dd, C-10], 6.52 [1H, dd, C-11], 6.00 [1H, dd, C-12], 5.45 [1H, dt, C-13], 0.94 [3H, t, C-18]
9-KODE	2.65 [2H, t, C-8], 6.23 [1H, d, C-10], 7.60 [1H, dd, C-11], 6.21 [1H, dd, C-12], 6.00 [1H, dt, C-13], 0.94 [3H, t, C-18]
9 α -HPOTrE	4.32 [1H, dt, C-9], 5.63 [1H, dd, C-10], 6.60 [1H, dd, C-11], 6.03 [1H, dd, C-12], 5.44 [3H, m, C-13, C-15, C-16], 2.98 [2H, dd, C-14], 1.02 [3H, t, C-18]
9 α -HOTrE	4.13 [1H, dt, C-9], 5.72 [1H, dd, C-10], 6.60 [1H, dd, C-11], 6.05 [1H, dd, C-12], 5.44 [3H, m, C-13, C-15, C-16], 2.98 [2H, dd, C-14], 1.02 [3H, t, C-18]
9 α -KOTrE	2.65 [2H, t, C-8], 6.26 [1H, d, C-10], 7.65 [1H, dd, C-11], 6.24 [1H, dd, C-12], 5.96 [1H, dt, C-13], 3.12 [2H, dd, C-14], 5.43 and 5.48 [2H, m, C-15 and C-16], 1.02 [3H, t, C-18]
9 γ -HOTrE	4.14 [1H, dt, C-9], 5.69 [1H, dd, C-10], 6.55 [1H, dd, C-11], 6.00 [1H, dd, C-12], 5.45–5.50 [3H, m, C-13, C-6, C-7], 0.95 [3H, t, C-18]
11-HPEPE	4.35 [2H, dt, C-11], 5.63 [1H, dd, C-12], 6.58 [1H, dd, C-13], 6.01 [1H, dd, C-14], 2.96 [2H, dd, C-16], 2.81 [2H, dd, C-7]
11-HEPE	4.15 [2H, dt, C-11], 5.72 [1H, dd, C-12], 6.58 [1H, dd, C-13], 6.00 [1H, dd, C-14], 2.96 [2H, dd, C-16], 2.81 [2H, dd, C-7]
13-HPHDE	4.37 [2H, dt, C-13], 5.65 [1H, dd, C-14], 6.59 [1H, dd, C-15], 6.02 [1H, dd, C-16], 5.48 [1H, dt, C-17], 2.98 [2H, dd, C-18], 2.88 + 2.86 [4H, m, C-6, C-9]
13-HDHE	4.18 [2H, dt, C-13], 5.71 [1H, dd, C-14], 6.58 [1H, dd, C-15], 6.01 [1H, dd, C-16], 5.48 [1H, dt, C-17], 2.98 [2H, dd, C-18], 2.88 + 2.86 [4H, m, C-6, C-9]

In agreement with previously published data (see Di Marzo et al., 1993b, and references cited therein), this suggested for these metabolites a structure as hydroperoxy derivatives corresponding to the monohydroxy acids described above, e.g. 9-HPODE and 9 α -HPOTrE.

The 280 nm u.v. absorbance of the third h.p.l.c. peak from LA, α -LA and γ -LA incubation with *Hydra* homogenates suggested the presence of a carbonyl-conjugated diene. This was confirmed for LA and α -LA derivatives by means of e.i.-m.s. analyses of the methyl esters (Table 2). Molecular ions respectively at $m/z = 308$ and 306, accompanied by fragment ions at -31 atomic mass corresponding to the loss of methoxy groups, and by the fragmentation pattern subsequent to the cleavage of the C–C bond adjacent to the carbonyl group, placed the latter on C-9. A typical McLafferty rearrangement at $m/z = 166$ was also observed for the metabolite derived from LA. The structures of the two compounds were thus suggested to be those of 9-KODE and 9 α -keto-octadecatrienoic acid. ^1H -n.m.r. (Table 1), with diagnostic signals at δ 2.65 (J 7.4 Hz), assigned to the proton on C-8, and at δ 6.23–6.26 (J 15.1 Hz), 7.60–7.65 (J 11.4, 15.1 Hz), 6.21–6.24 (J 10.4, 11.4 Hz) and 5.95–6.00 (J 10.4, 14.5 Hz), assigned to the olefinic protons on C-10, C-11, C-12 and C-13 respectively, conclusively confirmed e.i.-m.s. data.

Chemical identification of C_{18} PUFA metabolites allowed their quantification by means of h.p.l.c. analyses run in comparison with commercially available standards. Synthetic 9-HODE, 9-HPODE and 9-KODE were used to quantitate respectively the hydroxy, hydroperoxy and keto acids described above. Total conversion of PUFAs into their metabolites (e.g.

Table 2 Selected e.i.-m.s. data for PUFA oxidation products in *Hydra* homogenates

Only the most significant fragments are shown for the sake of clarity. For each compound, the yield of each e.i.-m.s. fragment is given as a percentage of the most abundant fragment. Hydroperoxides underwent degradation during the derivatization procedures. HEDE, hydroxy-eicosadienoic acid; KOTrE, keto-octadecatrienoic acid.

Compound	E.i.-m.s. fragments: m/z (% parts of the molecule lost upon fragmentation)
9-HODE	153 (100, M^+ -acetyl - $(\text{CH}_2)_7$ -COOMe), 185 (52, M^+ -acetyl - $\text{CH}_2=\text{CH}-\text{CH}=\text{CH}-\text{CH}_2$), 135 (45, M^+ -acetic acid, - $(\text{CH}_2)_7$ -COOMe), 310 (6, M^+ -acetyl), 292 (6, M^+ -acetic acid)
9-KODE	151 (100, M^+ - $(\text{CH}_2)_7$ -COOMe), 237 (93, M^+ - $(\text{CH}_2)_4$ - CH_3), 166 (62, M^+ - $(\text{CH}_2)_6$ -COOMe, + H, McLafferty rearrangement), 308 (45, M^+), 277 (50, M^+ -OMe)
9 α -HOTrE	290, 100, M^+ -acetic acid), 133 (50, M^+ -acetic acid, - $(\text{CH}_2)_7$ -COOMe) 147 (50, M^+ -acetic acid, - $(\text{CH}_2)_6$ -COOMe), 239 (18.7, M^+ -acetyl, - $\text{CH}_2-\text{CH}=\text{CH}-\text{CH}_2-\text{CH}_3$)
9 α -KOTrE	149 (100, M^+ - $(\text{CH}_2)_7$ -COOMe), 275 (3.3, M^+ -OMe), 306 (1, M^+)
9 γ -HOTrE	123 (100, M^+ -acetic acid, - $(\text{CH}_2)_2-\text{CH}=\text{CH}-\text{CH}_2$ -COOMe), 195 (15.5, - $\text{CH}_2-\text{CH}=\text{CH}-\text{CH}_2$ -COOMe), 153 (14.5, M^+ -acetyl, - $\text{CH}_2-\text{CH}=\text{CH}-\text{CH}_2$ -COOMe), 290 (8.1, M^+ -acetic acid), 308 (1, M^+ -acetyl)
11-HEDE	123 (100, M^+ -acetic acid, - $(\text{CH}_2)_{10}$ -COOMe), 135 (35.3, M^+ -acetic acid, - $(\text{CH}_2)_9$ -COOMe), 149 (34.8, M^+ -acetic acid, - $(\text{CH}_2)_8$ -COOMe), 153 (33.3, M^+ -acetyl, - $(\text{CH}_2)_9$ -COOMe), 320 (13.3, M^+ -acetic acid), 338 (6.5, M^+ -acetyl)
11-HETrE	153 (100, M^+ -acetyl, - $\text{CH}_2-\text{CH}=\text{CH}-\text{CH}_2$ -COOMe), 195 (20, - $\text{CH}_2-\text{CH}=\text{CH}-\text{CH}_2$ -COOMe), 135 (19, M^+ -acetic acid, - $\text{CH}_2-\text{CH}=\text{CH}-\text{CH}_2$ -COOMe), 318 (2.0, M^+ -acetic acid)
11-HEPE	173 (100, M^+ -acetic acid, - $\text{CH}_2-\text{CH}=\text{CH}-\text{CH}_2$ -COOMe), 151 (87.0, M^+ -acetyl, - $\text{CH}_2-\text{CH}=\text{CH}-\text{CH}_2$ -COOMe), 314 (76, M^+ -acetic acid), 213 (41.0, M^+ -acetic acid, - $(\text{CH}_2)_3$ -COOMe), 193 (28.0, M^+ - $\text{CH}_2-\text{CH}=\text{CH}-\text{CH}_2$ -COOMe), 245 (20, M^+ -acetic acid, - $\text{CH}_2-\text{CH}=\text{CH}-\text{CH}_2$ -COOMe)
13-HDHE	133 (100, M^+ -acetic acid, - $\text{CH}_2-\text{CH}=\text{CH}-\text{CH}_2$ -COOMe), 151 (10.0, M^+ -acetyl, - $\text{CH}_2-\text{CH}=\text{CH}-\text{CH}_2$ -COOMe), 173 (6.0, M^+ -acetic acid, - $\text{CH}_2-\text{CH}=\text{CH}-\text{CH}_2$ -COOMe), 340 (4.0, M^+ -acetic acid), 213 (3.0, M^+ -acetic acid, - $\text{CH}_2-\text{CH}=\text{CH}-\text{CH}_2$ -COOMe)

the two 235 nm and the more polar 280 nm u.v.-visible h.p.l.c. components) was found to be higher for γ -LA and α -LA (Table 3).

The coexistence of hydroperoxy, hydroxy and keto derivatives of C_{18} PUFAs in *Hydra* homogenate incubations raised the question of their biosynthetic relationships. Therefore, a set of experiments was run to assess whether incubation of homogenate with one of the three classes of derivatives would lead to the production of any others. These homogenates were incubated under the same conditions carried out in PUFA incubations. [^{14}C]9-HPODE (5840 c.p.m.), 9-HODE (9400 c.p.m.) and 9-KODE (4000 c.p.m.), purified from a previous incubation conducted with LA plus [^{14}C]LA, were incubated, in separate experiments, with homogenates from 1000 hydra (1.5 mg of total protein/mg of homogenate) for 1 h at room temperature. After the experiment, the homogenates were extracted on Sep-pak and analysed by h.p.l.c. as usual. The incubation with 9-HPODE yielded radioactive peaks with the retention times characteristic of 9-HODE (36 min, 587 c.p.m.), 9-HPODE (39 min, 528 c.p.m.) and 9-KODE (43 min, 175 c.p.m.). Other more polar peaks were also detected and their nature was not investigated. Incubation with 9-HODE yielded no metabolites other than itself (36 min, 1977 c.p.m.). The *Hydra* homogenates treated with [^{14}C]9-KODE contained both 9-KODE itself (43 min, 2027 c.p.m.) and an unidentified peak (46 min, 696 c.p.m.), which corresponded with

Table 3 PUFA oxidation by *Hydra* homogenates: yield, regiospecificity and enantioselectivity

The percentage conversion of each PUFA into its oxidation products, e.g. hydroxy-plus hydroperoxy-derivatives for C₂₀ PUFAs and hydroperoxy- plus hydroxy- plus keto-derivatives for C₁₈ PUFAs, was measured by means of quantitative h.p.l.c. analyses. Data are means $\pm$ S.E.M. for at least three separate incubations. Standards for the oxidation products of C_{18:3,*n*-3}, C_{18:3,*n*-6}, C_{20:2,*n*-6}, C_{20:3,*n*-6} and C_{22:6,*n*-3} are not commercially available and their stereochemistry was assumed to be (*R*) on the basis of their behaviour on chiral phase h.p.l.c. compared with that of hydroxy derivatives of the other PUFAs. The effect of varying fatty acid (FA) concentrations on optical purity was studied only for C_{18:3,*n*-3} and C_{20:4,*n*-6}. N.D., not determined because of scarcity of material (less than 1 μ g).

PUFA	Total % conversion/mg protein	Peroxidation site	[FA] (mg/ml) ...	% <i>R</i> enantiomer of the hydroxyacid		
				0.5	0.1	0.01
18:2, <i>n</i> -6	0.73 $\pm$ 0.21	C-9 (C-10')	48			
18:2, <i>n</i> -6	Not detectable					
all- <i>trans</i>						
18:3, <i>n</i> -3	2.68 $\pm$ 1.32	C-9 (C-10')	69	81	89	
18:3, <i>n</i> -6	2.50 $\pm$ 0.78	C-9 (C-10')	73			
20:2, <i>n</i> -6	0.38 $\pm$ 0.08	C-11 (C-10')	60			
20:3, <i>n</i> -6	1.40 $\pm$ 0.30	C-11 (C-10')	73			
20:4, <i>n</i> -6	4.80 $\pm$ 0.35	C-11 (C-10')	81*	90	N.D.	
20:5, <i>n</i> -3	1.10 $\pm$ 0.90	C-11 (C-10')	95			
22:6, <i>n</i> -3	0.63 $\pm$ 0.27	C-13 (C-10')	91			

* Data at 0.5 mg/ml were obtained from Di Marzo et al. (1993b).

the second 280 nm u.v.-visible peak shown in the upper trace of Figure 1. This suggests that the unknown compound, which was not characterized in this study because of the scarcity of material, might be a 9-KODE metabolite. These data, taken together, strongly suggest that: (1) 9-HPODE produced from incubation of LA with *H. vulgaris* homogenates is partly chemically or enzymically degraded into some as yet unknown metabolite, and partly both oxidized to 9-KODE and reduced to 9-HODE; and (2) the latter are produced independently from each other and directly from the corresponding hydroperoxide.

Incubation of *Hydra* homogenates with C₂₂ polyunsaturated fatty acids

The outcome of incubation of *Hydra* homogenates with AA has been described previously (Di Marzo et al., 1993b). In this study, homogenates were incubated with other C₂₀ PUFAs, e.g. EDA, DGLA and EPA under the same conditions previously described for AA and used for C₁₈ PUFAs. H.p.l.c. analysis of the Sep-pak extracts yielded in all cases two major peaks with maximal u.v. absorbance at 235 nm (Figure 1). The more polar components were chemically characterized by means of e.i.-m.s. analysis of their methyl ester acetoxy derivatives and, for the EPA-derived metabolite, by means of ¹H-n.m.r. spectroscopy. No h.p.l.c. peak absorbing at 280 nm was detected in measurable amounts.

E.i.-m.s. spectra of the more polar metabolites were diagnostic of methyl ester, acetyl derivatives of PUFA-derived mono-hydroxy acids (Table 2). Signals at *m/z* corresponding to the loss of either the acetyl function of acetic acid (−42 and −60 atomic masses respectively) were always observed, whereas strong fragment ions at *m/z* corresponding to the cleavage of the C–C bond adjacent to the CH–OH group placed in all cases the site of hydroxylation on C-11. This strongly suggested that the first eluted 235 nm u.v.-visible component of each incubation mixture had the structure of the 11-hydroxy-derivative of the PUFA incubated, e.g. of 11-hydroxyeicosadienoic acid, 11-HETrE and

11-HEPE respectively for the metabolites of EDA, DGLA and EPA. For the latter, the amount of the metabolite was sufficient for ¹H-n.m.r. analysis. The presence of a *trans,cis*-diene structure β to a hydroxy group was confirmed by the olefinic signals at δ 5.72 (*J* 6.6, 15.2 Hz), 6.58 (*J* 11.0, 15.2 Hz) and 6.00 (*J* 11.0 Hz), and by the CH–OH signal at δ 4.15 (*J* 6.6 Hz) (Table 1).

Enantiomeric composition of the hydroxy derivatives of C₂₀ PUFAs was again established by means of chiral phase h.p.l.c. of the methyl ester derivatives. The results are summarized in Table 3. Interestingly, the amount of the *R* enantiomer present, in the C₂₀ metabolites, increased significantly and continuously with the amount of conjugation present in the substrate. Moreover, when incubated at the lower concentration of 0.1 mg/ml, AA generated 11-HETE with a higher percentage composition of the (*R*)-enantiomer.

The less polar 235 nm peaks again were very unstable. They degraded quickly and the amounts present were insufficient for structural analysis except for the metabolite produced from EPA. Its ¹H-n.m.r. spectrum (Table 1) displayed signals present in either the 9-HPODE or the 11-HEPE spectrum, thus strongly corroborating the hypothesis that this compound, and possibly the analogous ones derived from the other C₂₀ PUFAs, is the 11-hydroperoxide-derivative of EPA, e.g. 11-HPEPE.

Also in this case, identification of the chemical structure of the metabolites present in incubates allowed their quantitation by h.p.l.c. Standards of 11-HETE or 11-HEPE and of 11-HPETE and 11-HPEPE were used. The total percentage conversion per mg of total protein of each PUFA into its derivatives is summarized in Table 3. The highest conversion observed was that previously described for AA (Di Marzo et al., 1993b).

Incubation of *Hydra* homogenates with docosahexaenoic acid

In order to assess whether the lipoxygenase-like activity present in *Hydra* homogenates is capable of recognizing also C₂₂ PUFAs as a substrate, incubations were also conducted with DHA. These yielded two major u.v.-visible (235 nm) h.p.l.c. peaks (Figure 1) which were characterized by means of ¹H-n.m.r. and e.i.-m.s. The spectra (Tables 1 and 2) displayed signals analogous to those described above for C₁₈ and C₂₀ hydroperoxy and hydroxy acids. Briefly, an e.i.-m.s. fragmentation pattern placed the site of hydroxylation of the more polar compound on C-13, thus suggesting for this metabolite the structure of 13-hydroxydocosahexaenoic acid (13-HDHE). N.m.r. analysis established that the less polar metabolite was the hydroperoxy analogue of 13-HDHE.

Chiral phase h.p.l.c. established that 13-HDHE comprised 91 % of the *R* enantiomer. Quantitative data for DHA conversion into its metabolites, obtained by using 11-HEPE and 11-HPEPE as standards, are shown in Table 3.

Regenerative activity on *Hydra* of some polyunsaturated fatty acid metabolites

Both the (*R*)- and (*S*)-enantiomers of some of the hydroxy-fatty acids described above were tested for their ability to effect the hydroid ATN. The selection of the enantiomers was based on commercial availability, the presence of endogenous PUFA precursors, in free as well as phospholipid-bound fatty acid pools in *H. vulgaris*. The results of the bioassays are reported in Table 4, along with the previously reported data relative to 11- (*R*)- and (11*S*)-HETE (Di Marzo et al., 1993b). Among the compounds tested, only (9*S*)-HODE and, to a much lower extent, (9*S*)-HPODE were found to enhance ATN. (9*R*)-HODE

Table 4 *Hydra*-tentacle-regenerating activity of some PUFA oxidation products

Among PUFA-derivatives characterized in this study, only the commercially available ones have been tested. Doses higher than those shown were toxic to *hydra*. Increase in ATN is given as a percentage of ATN in the absence of metabolite. Data are means $\pm$ S.E.M. of the ATN of at least 20 treated excised *Hydra* 10 days after treatment. Data for (11*R*)- and (11*S*)-HETE have been partly reported previously (Di Marzo et al., 1993b). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.0001$.

Metabolite	Concentration (μ M)	ATN	Increase (%)
(9 <i>R</i>)-HODE	0	5.81 $\pm$ 0.19	
	8	5.75 $\pm$ 0.26	
	30	5.32 $\pm$ 0.17	
(9 <i>S</i>)-HODE	0	5.81 $\pm$ 0.19	
	3	5.73 $\pm$ 0.20	
	8	6.90 $\pm$ 0.27**	19.0
	30	8.00 $\pm$ 0.40***	37.9
(9 <i>S</i>)-HPODE	0	5.21 $\pm$ 0.17	
	8	5.78 $\pm$ 0.21*	10.9
	30	5.64 $\pm$ 0.18	
9-KODE	0	5.21 $\pm$ 0.17	
	8	5.43 $\pm$ 0.15	
	30	5.80 $\pm$ 0.19	
(11 <i>R</i>)-HETE	0	5.80 $\pm$ 0.19	
	8	7.00 $\pm$ 0.21**	20.7
	12	6.70 $\pm$ 0.17	
(11 <i>S</i>)-HETE	0	5.75 $\pm$ 0.26	
	8	5.10 $\pm$ 0.27	
	12	6.29 $\pm$ 0.29	
(11 <i>R</i>)-HEPE	0	5.33 $\pm$ 0.20	
	8	5.37 $\pm$ 0.19	
	30	5.48 $\pm$ 0.19	
(11 <i>S</i>)-HEPE	0	5.33 $\pm$ 0.20	
	8	4.95 $\pm$ 0.16	
	30	5.45 $\pm$ 0.17

Figure 2 The effect of (9*R*)H and (9*S*)-HODE (30 μ M) on excised *Hydra* ATN at different times after the initial 24 h incubation

This experiment is representative of two separate ones conducted with 20 *hydra* each. S.E.M. bars are not shown for the sake of clarity; S.E.M. was never higher than 5% (see also Table 4). Control, no (9*R*)- or (9*S*)-HODE added.

and 9-KODE were completely inactive. The (9*S*)-HODE effect was time- and dose-related and maximal at a 30 μ M concentration with a 37.9% increase of ATN. Tentacle regeneration in excised *Hydra* treated with this compound was first inhibited, probably because of its toxicity (higher doses were, in fact, lethal to *Hydra*), and then significantly enhanced with respect to control after 9 days of incubation (Figure 2). After 10 days, the time at

which the assay was interrupted, the effect had not yet reached a plateau and was higher than that reported for (11*R*)-HETE (Table 4). (11*R*)- and (11*S*)-HEPE were not found to be active up to a 30 μ M dose.

DISCUSSION

In the present study, we investigated whether the previously described (Di Marzo et al., 1993b) 11-lipoxygenase-like activity present in extracts of *H. vulgaris* could catalyse the peroxidation of PUFAs other than AA. The biochemical relevance of such investigation relies on two considerations. First, 11-lipoxygenase products are not widespread in animal cells, although their presence and biological function in many mammalian and invertebrate tissues has been recognized by several studies (cited by O'Brien, 1987; Spector et al., 1988; Gerwick et al., 1993). Moreover, PUFAs such as LA, α -LA, and EPA are very abundant in *Hydra* fatty acid pools (De Petrocellis et al., 1993a; Muller et al., 1993; C. Gianfrani, V. Di Marzo, L. De Petrocellis and G. Cimino, unpublished work), where their function is not yet clear. Therefore, experiments aimed at defining the substrate specificity, regiospecificity and enantioselectivity of an abundant 11-lipoxygenase-like enzymic activity such as that present in *H. vulgaris* are crucial to the understanding of its potential applications to studies on mammalian 11-lipoxygenases as well as of its possible biological role in hydroids.

The findings described herein can be summarized as follows: (a) formation of typical lipoxygenase products, e.g. hydroperoxy and hydroxy acids was observed for all PUFAs tested; keto acids were formed only when using C₁₈ PUFAs as substrates; (b) in all metabolites formed the site of oxidation was always and only the tenth carbon atom counting from the aliphatic end of the fatty acid; (c) the enantioselectivity of the reaction depended on the degree of unsaturation of the substrate; and (d) PUFA oxidation by the 11-lipoxygenase-like enzyme leads, when the substrates are LA or AA, e.g. two of the most abundant *H. vulgaris* fatty acids, to hydroxy acids enantiospecifically active on *Hydra* tentacle regeneration.

The PUFA concentration used in all incubations (0.5 mg/ml) was about 50 times higher than that found *in vivo*. In 1000 specimens of *H. vulgaris*, the total amount of fatty acids in free and phospholipid-bound pools was 20 μ g for LA, 24 μ g for EPA, 35 μ g for AA and 92 μ g for α -LA (De Petrocellis et al., 1993a; C. Gianfrani, V. Di Marzo, L. De Petrocellis and G. Cimino, unpublished work), and similar amounts can be found in *Hydra oligactis* (Di Marzo et al., 1993c) and *Hydra magnipapillata* (Muller et al., 1993). Unfortunately, the use of more physiological concentrations of fatty acid would have prevented the production of oxidation products in amounts sufficient for structure characterization, namely for ¹H-n.m.r. spectroscopy, which is necessary to distinguish between hydroxy and hydroperoxy derivatives. In pilot experiments conducted with 0.01 and 0.1 mg/ml PUFAs, however, no difference in the h.p.l.c. patterns of incubation mixtures and, presumably, in the regioselectivity of the oxidation, was observed (results not shown), although a different enantiomeric composition was found (Table 3).

The nature of the enzymic activity was not investigated. However, if one assumes that PUFA oxidation is catalysed by one single enzyme, as suggested by the regiospecificity of PUFA hydroxylation, this is very likely to be the same as that described previously as acting on AA in a [substrate]-, [protein]-, pH- and time-dependent fashion (Di Marzo et al., 1993b). This enzyme was not inhibited by cyclo-oxygenase and cytochrome P-450 mono-oxygenase inhibitors nor by the 12- and 5-lipoxygenase inhibitor eicosa-5,8,11-triynoic acid and by the antioxidant and

mammalian lipoxygenase inhibitor nordihydroguaiaretic acid, and led to the formation of 11-HETE via the intermediate 11-HPETE (Di Marzo et al., 1993b). The regioselectivity of PUFA peroxidation suggests that the enzyme recognizes the aliphatic end of the molecule. The remaining part, and its degree of unsaturation, would be important in anchoring the substrate within the active site, exposing a specific side of the double bond to O₂ attack and thus determining the enantioselectivity of the reaction. This could explain why the percentage of the (*R*)-enantiomer present in the hydroxy acid metabolites produced increases when additional double bonds are available in the substrate (Table 3). The overall degree of unsaturation seems to influence the amount of PUFA converted per milligram of protein. Too few or too many double bonds in the substrate seem to reduce the yield of metabolite, as observed for EDA or docosahexaenoic acid (DHA). Thus, among C₂₀ PUFAs, *Hydra* 11-lipoxygenase displayed a higher affinity for AA (Table 3).

The presence of *trans* double bonds in the molecule, as in linoleic acid, does not allow the oxidation to occur, possibly by modifying its conformation, in agreement with the general observation that *trans*-unsaturated fatty acids compared with *cis* isomers are not very reactive to enzymic membrane adsorption, esterification and oxidation (Cook, 1991).

In all cases, hydroxy acids would be formed upon reduction of the corresponding hydroperoxy derivatives. The latter would be the first reaction products, and, in the case of C₁₈ PUFAs, would be also subject to oxidation to keto acids, a process which is not likely to occur non-enzymically because it cannot be observed with C₂₀ PUFAs or DHA. No interconversion between 9-hydroxy and 9-keto acids was found in contrast to the situation described in mammalian tissues (Agins et al., 1987; Earles et al., 1991).

The possible biological activity of PUFA oxidation products on *Hydra* tentacle regeneration was also investigated. The choice of the substances to be tested was directed by their commercial availability, which ensured a source of compounds richer and optically purer than that produced by *Hydra* homogenates. For monohydroxy derivatives, both (*R*)- and (*S*)-enantiomers were tested, because enantiospecific properties have been reported, e.g., for HETEs in mammals or echinoderms (Meijer et al., 1986; Wollard et al., 1989; for a review, see Spector et al., 1988). Among the substances tested, (9*S*)-HODE, which is shown here to be produced from LA and was previously found to be lipoxygenase product (Van Os et al., 1979), was found to be the most active, affording a striking enhancement of ATN at a dose of 30 µM (Table 4). (9*S*)-HPODE was less active, although in this case the rapid degradation of this compound and/or its rapid conversion, described here, into metabolites other than (9*S*)-HODE (including 9-KODE) must be considered as an activity-limiting factor. (9*R*)-HODE and 9-KODE were completely inactive, thus raising the question of the biological purpose of their production. Other possible physiological functions in *Hydra* cell biology must be examined for these two compounds, although it must be pointed out that the *in vitro* biosynthesis procedure used in this study may lead to the production of metabolites that are not necessarily synthesized *in vivo*.

Interestingly, (11*R*)-HEPE was found to be inactive, despite its close structural resemblance to (11*R*)-HETE. The decreasing potency in enhancing *Hydra* ATN, observed when passing from (9*S*)-HODE to (11*R*)-HETE and (11*R*)-HEPE (Table 4), seems to indicate that this activity is negatively influenced by the length and/or the degree of unsaturation of the monohydroxy acids. This, bearing in mind the enantiospecificity of the effect, is

somehow in contrast with the increasing enantioselectivity with which these metabolites are respectively synthesized *in vitro*. Indeed, one would expect those compounds that are biosynthesized with the highest enantioselectivity to be also the most biologically active. It must however be stressed that *in vivo* physiological concentrations of substrate may lead to more enantioselective PUFA oxidation, as suggested by the data shown in Table 3 (see also Hampson et al., 1992).

In conclusion, the present study has shown that the previously described 11-lipoxygenase-like enzymic activity present in *H. vulgaris* extracts is capable of catalysing the peroxidation of several PUFAs, including the very ones that are found as abundant components of hydroid FA fractions, thereby generating potentially bioactive metabolites. Further studies are now needed to characterize this enzymic activity as well as the mode of action of PUFA-derived metabolites as chemical mediators of hydroid regenerative processes.

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