Biosynthesis, structure and biological activity of hydroxyeicosatetraenoic acids in *Hydra vulgaris*

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Recent reports have suggested the involvement of arachidonic acid (AA) and its metabolites in the control of body pattern, head and tentacle regeneration and bud formation in *Hydra* spp. Here we describe for the first time the biosynthesis of hydroxyeicosatetraenoic acids (HETEs) *in vitro* by hydroid cytosolic extracts. Incubation of both unlabelled and tritiated AA with homogenates of *Hydra vulgaris* led to the conversion of up to 11 % of the exogenous fatty acid into mainly two metabolites. These were characterized as 11-hydroperoxyeicosatetraenoic acid (11-HPETE) and 11-HETE by means of a combination of chromatographic, chemical, ¹H-n.m.r. and electron-impact m.s. techniques. Trace amounts of 9-HETE and 12-HETE were also found. Analysis of 11-HETE by chiral-phase h.p.l.c. revealed that this metabolite was composed mainly of the *R* enantiomer. The production of 11-HPETE and 11-HETE was found to be: (1) associated with the cytosolic fraction of *Hydra* homogenates;

(2) dependent on AA concentration, incubation time and protein amount in the homogenates; (3) unaffected by co-incubation with the 5- and 12-lipoxygenase inhibitors, 5,8,11-eicosatriynoic acid and nordihydroguaiaretic acid, the cyclo-oxygenase inhibitor, indomethacin, or the cytochrome *P*-450 inhibitors, proadifen and methoxalen. These results strongly suggest the presence of a very active (*R*)-11-lipoxygenase in *H. vulgaris*. The activity of both *R* and *S* enantiomers of synthetic 9-, 11- and 12-HETE and of 'endogenous' 11-HETE was studied on tentacle regeneration and bud formation in decapitated *Hydra*. Although almost all compounds tested inhibited budding, only endogenous 11-HETE and synthetic (*R*)-11-HETE significantly enhanced the average number of tentacles, thus suggesting that this eicosanoid might be one of the cellular regulators of regeneration in *H. vulgaris*.

INTRODUCTION

Arachidonic acid [AA; 20:4 (*n*–6)], like other polyunsaturated fatty acids, is an abundant component of the animal cell membrane and a well-recognized precursor for the production, through the catalytic action of enzymes such as cyclo-oxygenase, cytochrome *P*-450 mono-oxygenase and various lipoxygenases, of a wide range of metabolites, known as eicosanoids, which exert a broad spectrum of biological activities in mammals. Eicosanoids, such as prostaglandins and hydroxyeicosatetraenoic acids (HETEs), have been suggested to participate also in many aspects of marine invertebrate physiology [for reviews see Stanley-Samuelson (1987) and (1991)], but only in a few cases has their biosynthesis been demonstrated and elucidated. Thus 12-lipoxygenase metabolites, used as second messengers for the action of 5'-hydroxytryptamine and histamine, have been characterized in the nervous system of the opisthobranch mollusc *Aplysia californica* (Piomelli et al., 1989; Buttner et al., 1989). A novel pathway leading to the production of high levels of prostaglandins and prostaglandin-1,15-lactones with multiple function has been reported for another opisthobranch, *Tethys fimbria* (Cimino et al., 1991; Di Marzo et al., 1991, 1992). (*R*)-8-HETE has been shown to be biosynthesized, probably via a specific 8-lipoxygenase, in the reproductive system of starfish where it controls oocyte maturation (Meijer et al., 1986). More recently the biosynthesis of prostaglandins and HETEs by blood cells of the crab, *Carcinus maenas*, has been reported (Hampson et al., 1992). Both mammalian-like and unusual eicosanoids have been isolated and characterized in many other marine organisms

[for a comprehensive review see Gerwick et al. (1993)], in which their function has not yet been fully clarified.

In coelenterates, eicosanoid biosynthesis has been investigated only in the class *Anthozoa* to which soft corals such as *Plexaura homomalla*, in which the first example of a marine prostaglandin was reported (Weinheimer and Spraggins, 1969), belong. These investigations have led to the suggestion that prostaglandin biosynthesis in these organisms follows a pathway different from that existing in mammals, with allene oxide instead of endoperoxides as intermediates (Corey et al., 1988; Brash, 1989; Baertschi et al., 1989; Gerwick et al., 1993). The other two coelenterate classes, *Hydrozoa*, to which the freshwater hydroid *Hydra vulgaris* belongs, and *Scifozoa* have been hardly studied.

The intriguing phenomenon known as 'morphallactic regeneration', in which missing parts of an animal can be restored by the reorganization of some cells belonging to the remainder of the organism (Morgan, 1901), has been known in freshwater hydroids since 1744. Nevertheless, there is still very little known about the chemical signals involved in the regenerative processes, for example, of *Hydra* species. Although the existence of many factors has been postulated, their chemical structures and actual mechanisms of action have been seldom investigated, the only exception being the peptide 'head activator' (Schaller and Bodenmuller, 1981). Recently, several studies have suggested that two classes of cell membrane-derived lipids, diacylglycerols, through the activation of the enzyme protein kinase C, and AA, directly or via the production of its metabolites, might regulate body pattern, regeneration and bud formation in *Hydra* species (Muller, 1989, 1990; De Petrocellis et al., 1991, 1993a,b; Di

Abbreviations used: AA, arachidonic acid; e.i.m.s., electron impact mass spectrometry; TFA, trifluoroacetic acid; HETE, hydroxyeicosatetraenoic acid; HPETE, hydroperoxyeicosatetraenoic acid; NDGA, nordihydroguaiaretic acid; ETI, 5,8,11-eicosatriynoic acid; ATN, average tentacle number; ABN, average bud number.

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Marzo et al., 1993; Muller et al., 1993). It has also been hypothesized that diacylglycerols and AA, apart from acting *per se* on pattern formation and tentacle renewal, may also effect a reciprocal control on each other's action. Diacylglycerols might induce a protein kinase C-mediated activation of phospholipase A₂, the enzyme mainly responsible for AA liberation from membrane phosphoglycerides, and conversely, AA may act in part through the activation of protein kinase C (De Petrocellis et al., 1993a, Muller et al., 1993). *Hydra* has been shown to contain high levels of phospholipid-bound AA (De Petrocellis et al., 1993a), and a phospholipase A₂-like activity capable of releasing AA from phosphatidylcholine (V. Di Marzo and L. De Petrocellis, unpublished work) as well as measurable amounts of some eicosanoids such as 5-, 9-, 12- and 15-HETE (Muller et al., 1993). However, the chemical nature of the metabolites potentially arising from AA oxidation in *Hydra* and, at the same time, playing a role in these aspects of hydroid biology has not yet been characterized.

Starting from the experimental background described above, the present study investigates: (a) the biosynthesis, in *H. vulgaris* homogenates, of AA-derived metabolites; (b) their structure; (c) the effect of inhibitors of cyclo-oxygenase, lipoxygenase and cytochrome P-450 mono-oxygenase on their production; (d) their activity on hydroid tentacle regeneration and bud formation.

MATERIALS AND METHODS

Materials

H. vulgaris were originally obtained from Professor P. Tardent, University of Zurich, and were grown following the procedure described by Loomis and Lenhoff (1956) with minor modifications. *Hydra* were kept at $18 \pm 1^\circ\text{C}$ in a medium consisting of 1 mM NaHCO₃ and 1 mM CaCl₂ in water, and were fed on alternate days with an excess of living *Artemia salina* nauplii. *Hydra* starved for at least 2 days were used for the preparation of homogenates, after several washes with the medium. AA, indomethacin, 9-(±)-HETE, (R)-9-HETE, (S)-11-HETE, (R)-11-HETE, (S)-12-HETE and (R)-12-HETE were purchased from Sigma. [³H]AA (60 mCi/mol) was purchased from NEN, Dupont De Nemours GmbH. (S)-12-Hydroperoxyeicosatetraenoic acid (HPETE), proadifen, methoxalen, nordihydroguaiaretic acid (NDGA) and 5,8,11-eicosatriynoic acid (ETI) were obtained from Biomol. H.p.l.c. solvents were purchased from Farmitalia Carlo Erba. Protein determination was carried out using Folin phenol reagent purchased from Merck.

Biosynthesis and purification of AA metabolites from *Hydra* homogenates

In preliminary experiments, the whole homogenate of *H. vulgaris*, obtained by homogenization of 1000 polyps in 10 ml of 0.025 M Tris/HCl, pH 8.0, followed by sonication, was treated with 0.5 mg/ml AA and 0.1 µCi of [³H]AA and incubated at room temperature for 2 h. After the incubation had been stopped by lowering the temperature to 0 °C and the pH to 2.5 by addition of HCl, the incubation medium was centrifuged for 30 min at 25800 g (13000 rev./min) and the supernatant loaded on to Sep-pak C₁₈ cartridges (Waters Associates, U.K.). These were primed with methanol, equilibrated with water and eluted with methanol after a washing step with water. The eluates were dried under nitrogen flow and purified by means of reversed-phase h.p.l.c. carried out on a Spherisorb ODS 2 column (5 µm,

25 cm × 4.6 mm) eluted with an isocratic step of 5% acetonitrile/0.1% trifluoroacetic acid (TFA) (solvent B) in water/0.1% TFA (solvent A), followed by a linear gradient from 5 to 70% solvent B in 65 min (flow rate 1 ml/min). In preparative experiments, samples from Sep-pak extractions were eluted with an isocratic program of 55% solvent B in order to allow resolution between HETEs. AA was eluted by means of a washing step with 99% solvent B. In another set of experiments, the homogenization was carried out as described above, and the incubation was conducted by varying AA concentration, the incubation time or the total amount of proteins (see below). The h.p.l.c. system was equipped with Waters pumps 510, and u.v. absorbance was monitored at 205, 235 and 280 nm using a Waters Multiwavelength 490E detector. Fractions of volume 1 ml were collected and samples counted on a Tricarb (Packard) liquid-scintillation counter after the addition of 5 ml of Picofluor 15 (Packard) scintillation liquid. H.p.l.c. analyses, carried out by monitoring u.v. absorbance at 235 nm, of known amounts of 11-HETE synthetic standard allowed quantitative measurement of 11-HETE and 11-HPETE in *Hydra* incubation mixtures from the various experiments.

¹H-n.m.r., g.c.-electron-impact m.s. (e.i.m.s.) analyses and chemical reactions

N.m.r. spectra were recorded in C²HCl₃ and C²H₅O²H on a 500 MHz Bruker apparatus. E.i.m.s. spectra were obtained by means of a TriO-2000 g.c.-m.s. apparatus equipped with a VG quadrupole m.s. and an FS-SE-30 capillary column (25 m × 0.32 mm), using a programmed temperature gradient from 80 to 280 °C at 10 °C/min. Under these conditions, methyl ester trimethylsilyl ether derivatives of HETE are eluted after 18 min. Synthetic and native HETEs were methylated by allowing them to react for 15 min with excess diazomethane. Methyl esters were then treated with bis(trimethylsilyl)trifluoroacetamide in pyridine for 15 min at 60 °C, to prepare the trimethylsilyl ethers.

Reduction with NaBH₄ was carried out at room temperature in ethanol by adding excess reducing agent, and the reaction was then stopped after 10 min by adding excess methanol.

Chiral-phase h.p.l.c.

Peak I from h.p.l.c. runs carried out using the isocratic elution method was methylated with excess diazomethane and analysed on a Chiralcel OB column (Daicel Chemical Industry; 25 cm × 4.6 mm) eluted with *n*-hexane/propan-2-ol (99:1, v/v) (flow rate 1.5 ml/min) (Brash and Hawkins, 1990). U.v. absorbance was monitored at 235 nm. Under these conditions (S)-11-HETE methyl ester is eluted after 17 min and (R)-11-HETE methyl ester after 25.5 min, thus allowing conclusive determination of the enantiomeric composition of 11-HETE in *Hydra* incubation mixtures.

Effect of varying incubation time, protein and substrate concentration and of AA cascade inhibitors on AA metabolism in *Hydra* homogenates

In order to study the effects of varying the total protein concentration in the homogenates on AA conversion into 11-HETE and 11-HPETE, 1000 polyps were homogenized and centrifuged as described above, and the supernatants were either diluted with the extraction/incubation Tris/HCl buffer or used as such. AA (0.5 mg/ml) was then added and 1 h incubation, extraction and purification of incubation mixtures was carried out as described. Total protein was then determined using the

Folin reagent. In a separate set of experiments, the centrifuged homogenate, containing 1.5 mg of protein/ml, was incubated for 1 h with increasing concentrations of AA, and the extraction and purification were conducted as usual. Finally, in yet another series of experiments, the centrifuged homogenate, again containing 1.5 mg of protein/ml, was incubated with 0.5 mg/ml AA, and samples were taken at different intervals of time, extracted and purified as usual.

The effect of inhibitors was studied in three separate experiments. In one ([AA] = 0.5 mg/ml, [total protein] = 1.5 mg/ml, incubation time = 2 h), the effects of either NDGA or ETI (25 μ M) or proadifen (90 μ M) were examined and compared with controls. In another experiment ([AA] = 0.5 mg/ml, [total protein] = 2.2 mg/ml, incubation time = 1 h), the action of indomethacin (50 μ M) versus control was studied. Finally the other cytochrome *P*-450 inhibitor, methoxalen (140 μ M), was studied ([AA] = 0.5 mg/ml, [total protein] = 1.8 mg/ml, incubation time = 1 h). The concentrations used for proadifen and methoxalen were chosen bearing in mind the aspecific effects reported for cytochrome *P*-450 inhibitors on lipoxygenase activity when used at high doses (Fitzpatrick and Murphy, 1989). Indomethacin was used at a concentration slightly higher than that reported to exert a maximum inhibition of 11-HETE production in *Carcinus maenas* (Hampson et al., 1992), and the NDGA dose was higher than that normally used *in vitro* in order to compensate for the reduction in its efficiency observed in alkaline solutions (Van der Zee et al., 1989). In all cases, incubations were carried out in triplicate, and the amounts of 11-HETE and 11-HPETE were determined by comparison of h.p.l.c. peaks with those of known amounts of standards. Data are expressed as means $\pm$ S.D. and were compared by Student's *t* test.

Assay of *Hydra* regeneration and bud formation

To study the effect of HETEs on regeneration, *Hydra* were decapitated immediately below the tentacle whorl. In all experiments *Hydra*, originated from buds collected on the same day and cultured for a week, were of the same size, with six tentacles each. *Hydra* with no buds were used. Incubations with HETEs were carried out for 24 h. *Hydra* were then washed and cultured individually in 35 mm $\times$ 10 mm plastic Petri dishes with 2 ml of medium. The drugs were dissolved in methanol immediately before use and then diluted with culture medium to the desired concentration, always maintaining the level of methanol at 1 μ l/ml. Controls were treated with the same amount of methanol without any compound. At least 20 *Hydra* were used for each drug. The effect on regeneration was studied by measuring the average tentacle number (ATN) 10 days after the incubation. The effect on bud formation was studied by measuring the average bud number (ABN) 10 days after the incubation. ATN and ABN are expressed as means $\pm$ S.E.M. and compared using Student's *t* test.

RESULTS

Biosynthesis and structure determination of AA metabolites

In pilot experiments, 2 h incubations of 5 mg of AA and 0.1 μ Ci of [3 H]AA with a 10 ml whole homogenate of 1000 *Hydra*, followed by Sep-pak extraction and h.p.l.c. analysis using the linear solvent gradient, yielded two minor radioactivity peaks, eluted at 48 and 62 min, and a major one eluted at 68 min. Neither peak was present in control incubations conducted by incubating [3 H]AA with either heat-inactivated or trichloroacetic acid-treated homogenate. The most abundant peak corresponded

to a major u.v. (235 nm)-absorbance peak, which was absent from control incubations, and was further purified by reversed-phase h.p.l.c. using isocratic conditions (Figure 1). This yielded two radioactive and u.v. (235 nm)-visible peaks, the first of which, peak 1, was co-eluted, at 19.8 min, with synthetic 11-HETE, and the second, peak 2, was eluted at an elution time, 21.8 min, identical with that of synthetic 12- and 9-HETE, but was found to undergo rapid degradation, its height diminishing visibly in the following h.p.l.c. analyses. Therefore, in the next experiments, the incubation medium was extracted on Sep-pak and immediately purified using h.p.l.c. isocratic conditions, the two peaks were collected, dried under nitrogen and, the same day, either submitted to 1 H-n.m.r. analysis or methylated and trimethylsilylated for examination by e.i.m.s. The 1 H-n.m.r. spectrum of peak 1, obtained in both 2 HCl $_3$ and 2 H $_3$ O $_2$ H, was compared with those of synthetic HETEs and did not allow 11- and 9-HETE to be distinguished between. The presence of a double doublet at δ 2.82 instead of 2.98 (in 2 H $_3$ O $_2$ H), assigned to the protons of the methylene group between either two isolated double bonds or an isolated double bond and a diene, however, ruled out the possibility of this metabolite being either 12- or 8-HETE [see also Just and Wang (1986)], in agreement with its chromatographic behaviour. E.i.m.s. of the methyl ester trimethylsilyl ether derivative yielded a fragmentation pattern typical of 11-HETE (Figure 2a), with a base peak at m/z 225 (100%) corresponding to the cleavage of the C-10–C-11 bond adjacent to the derivatized hydroxy group. Other diagnostic peaks were found at m/z 283 (1.5%), corresponding to the cleavage of the C-11–C-12 bond, and at m/z 305 (0.3%), corresponding to the loss of (CH $_2$) $_3$ -COOMe, whereas the molecular ion at m/z 406 was only a minor fragment (0.2%). The 1 H-n.m.r. spectrum of peak 2 differed from that of 11-HETE and of peak 1 only in the presence of: (1) a double doublet at δ 4.41 (in 2 HCl $_3$) or δ 4.34 (in 2 H $_3$ O $_2$ H) instead of 4.24 or 4.17 respectively, assigned to the methynic proton adjacent to the hydroxy group on C-11; (2) a double doublet at δ 5.58 instead of 5.68, assigned to the olefinic proton on C-12; (3) a multiplet at

Figure 1 Radioactivity (c.p.m.) ($\square$) and u.v.-absorption (235 nm) (—) profiles of h.p.l.c. analyses of *H. vulgaris* incubations

Homogenates were incubated with AA and [3 H]AA as described in the Materials and methods section. Incubation mixtures were extracted by Sep-pak chromatography, dried down and analysed by isocratic elution (25 min) of 55% solvent B followed by a 10 min wash with 99% solvent B. Elution times of authentic standards of 5-, 8-, 9-, 11-, 12- and 15-HETE are shown. 11-HPETE was co-eluted with 9- and 12-HETE.

Figure 2 E.I.m.s. spectra of methylated and trimethylsilylated (a) peak 1 (11-HETE) and (b) peak 2 (11-HPETE plus traces of 9- and 12-HETE) after reduction with NaBH_4 , from h.p.l.c. isocratic analyses of *H. vulgaris* incubations (see the Materials and methods section)

The structures of derivatized HETEs with their main fragmentation patterns are shown. Fragments other than those discussed in the Results section are also shown: m/z 391 (—15) loss of $-\text{CH}_3$; m/z 375 (—31) loss of $-\text{OCH}_3$; m/z = 333 (—73) loss of $-\text{Si}(\text{CH}_3)_3$; m/z = 316 (—90) loss of $\text{HOSi}(\text{CH}_3)_3$.

δ 2.48 instead of 2.37 assigned to the methylene protons on C-10. These data, although ruling out the possibility of peak 2 being either 9- or 12-HETE, indicated for this compound a chemical structure identical with that of peak 1 and, therefore, 11-HETE, but for the functional group present on C-11. Its exceptional

instability suggested that this metabolite might be the hydroperoxide usually needed as an intermediate for the biosynthesis of 11-HETE, i.e. 11-HPETE. This hypothesis was supported by the ^1H -n.m.r. data reported for methynic protons adjacent to hydroperoxy groups (Coxon et al., 1984) and found for the

Figure 3 Influence of (a) total protein content, (b) incubation time and (c) AA concentration on the percentage of AA converted into 11-HPETE (◆) and 11-HETE (□) by *H. vulgaris* homogenates

AA concentration was 0.5 mg/ml in (a) and (b). Incubation time was 1 h in (a) and (c). Total protein content in (b) and (c) was 1.5 mg/ml of homogenate. Data are representative of at least three separate experiments. Error bars are not shown for the sake of clarity, but were never higher than 15% and 25% of the means for 11-HETE and 11-HPETE respectively.

proton on C-12 in the spectrum of synthetic 12-HPETE at δ 4.36. Moreover, when peak 2 was reduced with NaBH_4 immediately after its purification, a compound was formed which was co-eluted with both peak 1 and 11-HETE standard. The ^1H -n.m.r. spectrum of this compound was identical with that of 11-HETE, thus confirming that peak 2 was indeed 11-HPETE. Interestingly, the e.i.m.s. spectrum of peak 2, after reduction with NaBH_4 , methylation and trimethylsilylation (Figure 2b), showed the presence, apart from the signal at m/z 225 typical of 11-HETE, of minor peaks at m/z 255 and 295, corresponding to the analogous fragmentation pattern occurring in 9- and 12-HETE respectively. This indicated the presence in peak 2 also of traces of these two HETEs (left unaffected by the reduction), as might have been expected by its elution time in h.p.l.c. analyses.

The enantiomeric composition of the most abundant stable AA metabolite found in *Hydra* homogenates incubated with

exogenous AA, i.e. 11-HETE, was determined by means of chiral-phase h.p.l.c. of its methyl ester, following a widely used procedure (Brash and Hawkins, 1990). This showed that the eicosanoid consisted mainly of the *R* enantiomer ($81.2 \pm 3.8\%$, $n = 3$). Owing to its rapid degradation, it was never possible to obtain a preparation of the methyl ester of 11-HPETE pure enough to determine its *R/S* composition by h.p.l.c. However, 11-HETE obtained by reduction of 11-HPETE showed the same enantiomeric composition as that of 11-HETE directly produced by *Hydra* homogenates.

Factors affecting the biosynthesis of AA metabolites by *Hydra* homogenates

Centrifugation at 13000 rev./min produced cell membrane-free *Hydra* homogenates which were still fully capable of converting both unlabelled and labelled AA into peaks 1 and 2, whereas pellets produced by the centrifugation step, once resuspended in Tris/HCl buffer, did not exhibit AA-metabolizing activity (not shown). The pH of the homogenate was rigorously kept at 8.0 in order to minimize possible base-catalysed degradation of the hydroperoxide derivatives. Moreover, this pH value, although being far from the pH optima of most plant and mammalian lipoxygenases, was identical with that used in most successful *in vitro* eicosanoid-biosynthesis experiments conducted in invertebrates such as those leading to the formation of high levels of (*R*)-8-HPETE in soft corals (Corey et al., 1988; Baertschi et al., 1989). A single experiment was also carried out at pH 8.8, and no significant change in the total conversion of AA into 11-HETE and 11-HPETE was observed. Finally, lowering the pH to 2.5 by addition of HCl resulted in the complete loss of HETE-synthesizing activity.

As shown in Figure 3(a), the percentage of AA converted into 11-HPETE and 11-HETE was directly proportional to the total amount of protein present in the homogenate. Some 11.7% of exogenously added AA (0.5 mg/ml) was metabolized by 1 h of incubation with 2.4 mg of protein/ml of homogenate (obtained by extracting about 800 *Hydra* in 6 ml of Tris/HCl buffer). The 11-HPETE/11-HETE ratio, moreover, seemed to remain constant at around 2 (1.7–2.2).

Maximal conversion of AA (0.5 mg/ml) into 11-HPETE was obtained with an incubation time of 1 h (Figure 3b; 1.5 mg of total protein/ml). With longer incubation periods, 11-HPETE seemed to decrease and 11-HETE to increase correspondingly, although the total conversion remained approximately constant (from 6.8 to 5.7%), with the 11-HPETE/11-HETE ratio reaching 1 after a 5 h incubation.

The percentage AA conversion was found to depend also on AA concentration (Figure 3c), a maximum (10.4%) being observed (1 h incubation time and 1.5 mg of total protein/ml) with 0.05 mg/ml. At this and higher AA concentrations, the 11-HPETE/11-HETE ratio was again found to remain approximately constant (2.7–3.3).

Effect of AA cascade inhibitors on AA metabolism in *Hydra* homogenates

To gain some preliminary data on the type of enzyme responsible for biosynthesis of 11-HPETE and 11-HETE in *Hydra*, a series of incubations of *Hydra* homogenates with AA in the presence of AA cascade inhibitors was carried out (not shown). The amount of the fatty acid converted into the two eicosanoids was not significantly decreased by either the 5- and 12-lipoxygenase inhibitors, ETI and NDGA (25 μM), or the cyclo-oxygenase

Table 1 Effect of *R* and *S* enantiomers of HETEs on *H. vulgaris* ATN and ABN

The compounds were tested at different doses but, for the sake of simplicity, only the doses eliciting the maximal effect are shown. Data are expressed as means $\pm$ S.E.M. ($n > 18$) of percentage enhancement of control ATN and percentage decrease of control ABN. ATN and ABN in control experiments were respectively 6.0 ± 0.1 and 0.77 ± 0.1 ($n = 6$). * $P < 0.005$. n.d. = not determined.

Compound	Dose (μ M)	Percentage of control ATN (% enhancement)	100 — percentage of control ABN (% inhibition)
9-($\pm$)-HETE	15	7.0 ± 2.0	10.0 ± 2.0
(<i>R</i>)-9-HETE	15	0.4 ± 3.7	$43.0 \pm 7.0^*$
(<i>S</i>)-11-HETE	15	5.0 ± 2.0	$81.7 \pm 5.5^*$
(<i>R</i>)-11-HETE	8	$22.0 \pm 3.4^*$	$81.0 \pm 19.0^*$
(<i>R</i>)-11- + (<i>S</i>)-11-HETE	8	-7.8 ± 3.4	n.d.
Endogenous 11-HETE (81.2% <i>R</i>)	5	$23.1 \pm 5.0^*$	$61.0 \pm 9.0^*$
(<i>S</i>)-12-HETE	8	4.8 ± 3.0	$41.6 \pm 25.0^*$
(<i>R</i>)-12-HETE	15	8.5 ± 4.0	4.3 ± 7.6

inhibitor, indomethacin (50 μ M). More importantly, neither of the two inhibitors of cytochrome *P*-450 mono-oxygenase used, proadifen (90 μ M) and methoxalen (140 μ M), affected the synthesis of 11-HPETE and 11-HETE.

Effect of HETEs on *Hydra* tentacle regeneration and bud formation

The effects of 11-HETE purified from *Hydra* homogenates incubated with AA and of either the (*R*) or (*S*) enantiomers of synthetic 9-, 11- and 12-HETE were studied on ATN and ABN in decapitated polyps. As shown in Table 1, almost all compounds tested inhibited the formation of buds in regenerating *Hydra* irrespective of their stereochemistry. However, only 'native' 11-HETE (maximal enhancement of ATN 23.1% of control at 5 μ M; $P < 0.005$) and synthetic (*R*)-11-HETE (maximal enhancement 22% of control at 8 μ M; $P < 0.005$) enhanced *Hydra* tentacle regeneration. Synthetic (*S*)-11-HETE did not exhibit a significant effect up to a 30 μ M dose (maximal enhancement 5% of control at 15 μ M), but counteracted the effect of (*R*)-11-HETE.

DISCUSSION

This study has demonstrated that cell-membrane-free extracts of a hydroid species, *H. vulgaris*, can operate a quantitative conversion of AA into two major metabolites possessing chromatographic, chemical and spectral characteristics identical with those of 11-HETE and of the corresponding hydroperoxy derivative, 11-HPETE. Two of the findings reported here seem to indicate that the enzyme responsible for this conversion is an 11-lipoxygenase and not a cytochrome *P*-450 mono-oxygenase. First, the enantioselectivity of the conversion leads mostly to the formation of the *R* enantiomer, whereas less selective syntheses of both enantiomers have been often, but not always, reported for cytochrome *P*-450-catalysed oxidations (Fitzpatrick and Murphy, 1989). More importantly, both cytochrome *P*-450 inhibitors used in this study, proadifen (also known as SKF525A) and methoxalen, failed to inhibit the formation of 11-HETE and

11-HPETE. (*R*)-11-HETE has been shown also to be a by-product of either other lipoxygenases or cyclo-oxygenase-induced oxidation of AA (Setty et al., 1985; Corey and Nagata, 1987). However, this does not appear to be the case in *H. vulgaris* homogenates, as both the cyclo-oxygenase inhibitor, indomethacin, and the 5- and 12-lipoxygenase inhibitors, NDGA and ETI, did not affect the synthesis of either AA metabolite in this study.

The occurrence of (*R*)-11-HETE has also been reported in echinoderms (Hawkins and Brash, 1987), for which the involvement of *R*-enantioselective lipoxygenase(s) was also suggested. However, the present study provides the first example of enantiospecific biosynthesis of (*R*)-11-HETE in a hydroid species. Another recent study (Muller et al., 1993) presented g.c.-m.s. evidence for the presence of 12-, 8-, 9- and 5-HETE, of which the enantiomeric composition was not determined, in extracts of *Hydra magnipapillata*, in which 11-HETE was detected only in traces. Conversely, we detected the formation of only trace amounts of 9- and 12-HETE by *H. vulgaris* homogenates. This discrepancy may be ascribed to the different procedures and species used in this study. *Hydra* species behave differently in response, for example, to diacylglycerol treatment in relation to ectopic head formation (L. De Petrocellis and V. Di Marzo, unpublished work), and, therefore, species-specific effects, although unlikely, cannot be ruled out. Alternatively, the *H. vulgaris* enzymic apparatus may behave differently *in vivo* from *in vitro* where disruption of subcellular compartments may activate some enzymes and inactivate others.

As previously shown in other organisms (Stanley-Samuelson, 1987; Pace-Asciak and Asotra, 1989; Yamamoto, 1991), (*R*)-11-HETE is very probably formed by *Hydra* homogenates via the corresponding short-lived hydroperoxide intermediate, which was also isolated and characterized in the present study. This is suggested by the outcome of experiments in which the incubation time is varied: after maximal 11-HPETE synthesis is reached within 1 h, the amount of this metabolite seems to decrease and the amount of 11-HETE to increase correspondingly. Conversely, with 1 h incubations, the 11-HPETE/11-HETE ratio does not appear to change when the concentration of either the total protein or the substrate AA is varied in the incubation medium, thus suggesting that the amount of 11-HETE formed under these conditions is directly proportional to the amount of 11-HPETE produced by the oxidation of AA. Unfortunately, neither unlabelled nor radiolabelled 11-HPETE are commercially available and therefore conclusive experiments on the conversion of this compound into 11-HETE could not be performed.

The possibility of 11-HPETE being transformed into other eicosanoids cannot be excluded by our findings. It is commonly accepted that a controlled free-radical pathway proceeding through (*R*)-11-HPETE or the 11-peroxy radical may lead to prostaglandin biosynthesis in mammals (Samuelsson, 1970; Porter et al., 1980). Autoxidative cyclization of lipid hydroperoxides to prostaglandin-like bicyclic endoperoxides has been shown to also occur non-enzymically (O'Connor et al., 1984). In coelenterates such as soft corals, it has been suggested that prostaglandin formation is not mediated by cyclo-oxygenase (Corey et al., 1988; Brash, 1989) and might also occur through the formation of (*R*)-11-HPETE (Brash et al., 1989). Thus, in hydroids, the finding described here of (*R*)-11-HPETE probably produced by the catalytic action of (*R*)-11-lipoxygenase might provide a potential pathway for prostaglandin formation.

HETEs possess a wide spectrum of biological activities, among which regulation of chemotaxis and chemokinesis has been shown in mammals [for a review see Spector et al. (1988)]. As cell motility has been suggested to play a major role in hydroid

pattern formation and in the early phases of regeneration and bud formation (Bode et al., 1986), these eicosanoids would be ideal candidates as cellular mediators for such processes. Therefore the effect of *R/S* enantiomers of 11-HETE and of the less abundant 9- and 12-HETE, detected in this study in *H. vulgaris* homogenates, was investigated here on excised *Hydra*. Almost all compounds tested inhibited to some extent bud formation during the head-regeneration period. This effect was not dependent on the stereochemistry of the C-OH in each HETE and seems to substantiate the previous suggestion (De Petrocellis et al., 1993b) that fatty acids structurally related to AA and other polyunsaturated fatty acids inhibit the process of budding non-specifically. Conversely, among all the compounds tested and produced by *Hydra* homogenates, only the most abundant, (*R*)-11-HETE (both synthetic and endogenous), significantly enhanced tentacle regeneration by increasing ATN up to 23.1 % of control experiments, whereas (*S*)-11-HETE was completely inactive. It is worthwhile mentioning that (*R*)-HETEs have been reported to be more potent chemotactic agents than the corresponding *S* enantiomers [for example see Wollard et al. (1989) and Spector et al. (1988)], and therefore there might indeed be a parallelism between regenerating activity in *Hydra* and chemotactic activity of HETEs. Interestingly, when added together with (*R*)-11-HETE at equimolar concentrations, (*S*)-11-HETE appeared to counteract its regenerating action on hydroid tentacle regeneration.

The effect observed with (*R*)-11-HETE on ATN, albeit enantio-specific, was not as strong as that described, for example, for activators of protein kinase C (De Petrocellis et al., 1993a), and was somewhat weaker than that expected from previous studies on the involvement of phospholipase A₂, AA and its metabolites in *Hydra* body pattern and regeneration (Di Marzo et al., 1993; Muller et al., 1993). Although the potency of the regenerating action may depend on the conditions of assay used here, it is possible that AA derivatives other than (*R*)-11-HETE and yet to be discovered would exert a more striking effect on ATN. As mentioned above, 11-HPETE, isolated in this study from *Hydra* homogenates, might produce *in vivo* other metabolites with a regenerating activity higher than that observed for (*R*)-11-HETE. Unfortunately, the extraordinary instability of endogenous 11-HPETE, as well as the commercial unavailability of this compound, prevented an accurate study of its effect on ATN. A further analysis of the minor AA metabolites produced by *Hydra* homogenates must be performed in order to ascertain whether eicosanoids play a physiologically relevant role in hydroid regenerative processes.

In conclusion, the present study has provided evidence for the presence of an enantiospecific, and possibly (*R*)-11-lipoxygenase-like, (*R*)-11-HETE-synthesizing activity in *H. vulgaris*. The physiological and biochemical relevance of this finding is clear if one bears in mind: (1) that AA is a major component of *H. vulgaris* cell membrane phospholipids (De Petrocellis et al., 1993a); (2) that an abundant and membrane-bound phospholipase A₂-like activity has been found in this hydrozoan (V. Di Marzo and L. De Petrocellis, unpublished work); (3) the potential functional role, suggested here, of (*R*)-11-HPETE and -HETE in the control of tentacle regeneration. Moreover, the extraordinarily good yield described here of these two AA metabolites suggests the use, in future investigations, of *H. vulgaris* as both a selective source and a model for the study of (*R*)-11-lipoxygenase.

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