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Mechanism of biosynthesis of 11*R*- and 12*R*-hydroxyeicosatetraenoic acids by eggs of the sea urchin *Strongylocentrotus purpuratus*

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11(*R*)-Hydroxyeicosatetraenoic acid [11(*R*)-HETE] and 12(*R*)-HETE are biosynthesized by eggs of the sea urchin *S. purpuratus*. We report here the isolation of the 11(*R*)- and 12(*R*)-hydroperoxy-eicosanoids from incubations of the desalted 30–50% $(NH_4)_2SO_4$ fraction of the egg homogenate; biosynthesis required the addition of calcium but not NADPH. Egg 11- and 12-HETE were formed from octadeuterated arachidonic acid without loss of geminal ^2H from C11 or C12, thus revealing that 11- or 12-keto intermediates are not involved in the biosynthesis. The results support the conclusion that egg 11(*R*)- and 12(*R*)-HETE are synthesized by a lipoxygenase and not by an NADPH-dependent cytochrome P450 monooxygenase mechanism.

Hydroperoxide; Lipoxygenase; Arachidonic acid; (Sea urchin egg)

1. INTRODUCTION

12(*R*)-Hydroxyeicosatetraenoic acid (12(*R*)-HETE) is a major eicosanoid product of human psoriatic scales, but its mechanism of biosynthesis in skin has not been elucidated [1]. Synthesis of 12(*R*)-HETE has also recently been reported to occur in microsomal preparations of liver [2] and corneal epithelium [3]; an NADPH-dependent cytochrome P450 monooxygenase is believed to be responsible for biosynthesis in these two systems [2,3]. It has been hypothesized that 12(*R*)-HETE could originate from 12(*S*)-hydroperoxy-eicosatetraenoic acid [12(*S*)-HPETE] by forming a 12-keto-eicosatetraenoic acid intermediate which might then undergo stereospecific reduction to 12(*R*)-HETE [4]; moreover, rat liver microsomes have now been reported to catalyze the NADPH-dependent reduction of 12-keto-eicosatetraenoic acid to 12(*R*)- and 12(*S*)-HETE [5].

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We recently described the biosynthesis of 11(*R*)-HETE and 12(*R*)-HETE by eggs of the sea urchin *S. purpuratus* [6]. The results of stereochemical analyses and hydrogen abstraction experiments were consistent with formation of these products by a lipoxygenase mechanism, but other pathways cannot be excluded. Here, we have established that the biosynthesis of egg HETEs does not proceed through the formation of 11- or 12-keto intermediates, and that there is no requirement for NADPH. We have detected the formation of 11(*R*)- and 12(*R*)-hydroperoxyeicosatetraenoic acids, providing further evidence that 11*R*- and 12*R*-lipoxygenase activities are responsible for the synthesis of 11*R*- and 12*R*-HETE in eggs of *S. purpuratus*.

2. MATERIALS AND METHODS

2.1. *Chemicals*

Solvents and reagents were obtained from previously described sources [6]. Octadeuterated arachidonic acid ([5,6,8,9,11,12,14,15-$^2H_8$]arachidonate) was prepared from eicosatetraynoic acid (ETYA) [7] and was a gift from Dr Ian

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Blair. Racemic HETE standards were prepared by the autoxidation of methyl arachidonate and purified as in [8,9].

2.2. *Egg $(NH_4)_2SO_4$ fractionation*

Strongylocentrotus purpuratus (Marinus, Long Beach, CA) eggs were isolated and washed in ice-cold calcium-free artificial seawater containing 2 mM EGTA as described [5]. A 30–50% $(NH_4)_2SO_4$ fraction (10000 × *g* pellet) was prepared from the 10000 × *g* supernatant of the egg homogenate [eggs:buffer, 1:10 (v/v) in 40 mM Tris, pH 7.9, plus 2 mM EGTA] and desalted on a Sephadex G-25M PD-10 gel filtration column (Pharmacia, Piscataway, NJ) equilibrated with 40 mM Tris, pH 7.9. Aliquots of the crude enzyme preparation were incubated with 100 μM arachidonate and 10 mM $CaCl_2$ for 5 min at 22–24°C, acidified and extracted into an equal volume of ice-cold dichloromethane. Lipid extracts were analyzed by straight-phase HPLC and stereochemical analyses of the methylated HETEs were performed on a dinitrobenzoyl phenylglycine chiral stationary phase HPLC column (Prikle type 1-A; Regis, Morton Grove, IL) as in [6,11,12].

2.3. *Gas chromatography-mass spectrometry*

GC-MS analyses were performed in the negative ion, chemical ionization (NI-CI) mode after conversion of the HETEs to pentafluorobenzyl (PFB) ester trimethylsilyl (TMS) ether derivatives [6,13]. Spectra were obtained by repetitive scanning in the mass range *m/z* 375–425; ions at *m/z* 399 and 391 correspond to the carboxylate anion after loss of the PFB moiety (M − 181) from the [$^2H_8$]HETE and non-deuterated HETE, respectively.

3. RESULTS

3.1. *Isolation of 11R- and 12R-hydroperoxy-eicosanoids*

Whole homogenates of *S. purpuratus* eggs convert arachidonic acid to the hydroxy derivatives, 11*R*- and 12*R*-HETE [6]. Following incubation of the desalted egg 30–50% $(NH_4)_2SO_4$ fraction, two additional products with UV absorbance at 235 nm were detected by SP-HPLC (fig.1). These were identified as 11- and 12-hydroperoxy-eicosatetraenoic acids on the basis of their characteristic UV chromophore, cochromatography with authentic HPETE standards, and by their reduction with triphenylphosphine to the corresponding HETE. In contrast to the desalted egg $(NH_4)_2SO_4$ fraction, fresh unfractionated egg homogenates likely contain reducing co-factors such as glutathione which would facilitate (non)enzymatic reduction of the hydroperoxides to HETEs. H(P)ETE biosynthesis required the addition of calcium [6], but did not require and was not stimulated by the addition of NADPH. The desalted egg $(NH_4)_2SO_4$ fraction formed relatively little 11-HPETE in comparison with whole egg homogenate although the ratios of 11-HETE to 12-HETE do vary in homogenates [6].

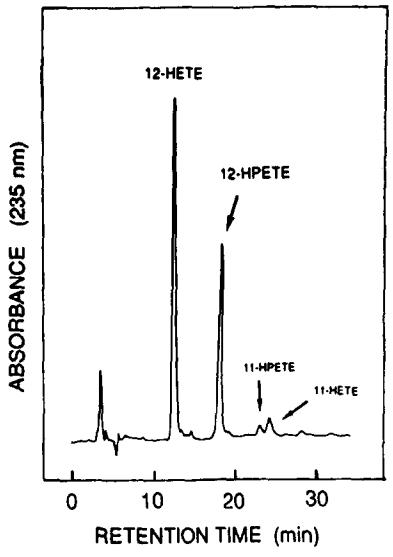

Fig.1. HPLC UV chromatogram (235 nm) of egg H(P)ETEs. Products from an incubation of arachidonic acid with egg $(NH_4)_2SO_4$ fraction were analyzed on an Alltech 5 μm silica HPLC column eluted at a flow rate of 1 ml/min with 1.0:100:0.1 *n*-hexane/isopropanol/acetic acid (by vol.).

3.2. *Product stereochemistry*

The hydroperoxides were reduced to the hydroxy fatty acids by treatment with triphenylphosphine, and the absolute stereochemical configurations of the HETE methyl esters were determined by chiral phase HPLC. Both of the reduced hydroperoxy-eicosanoids were of the *R* configuration, as shown by their appearance as a single peak which cochromatographed with the later eluting *R* enantiomers [6,12] of the corresponding racemic standards (fig.2). The two products recovered as the HETEs (fig.1) were also of the *R* configuration (not shown).

3.3. *Absence of keto intermediates in egg HETE biosynthesis*

Octadeuterated arachidonic acid was incubated with an egg homogenate; the 11- and 12-HETE products were recovered and the isotopic composition was measured by GC-MS (see section 2). A 15(*S*)-[$^2H_8$]HETE standard was prepared from the

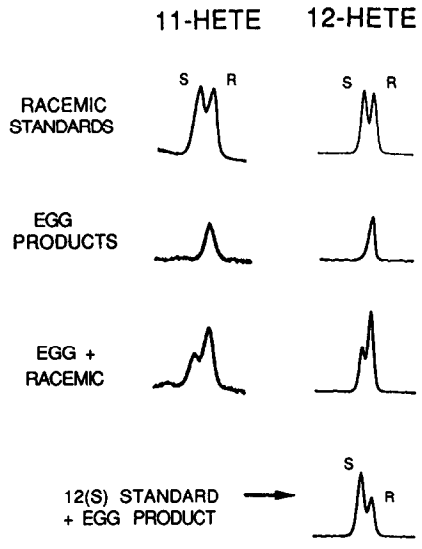

Fig.2. Chiral-phase HPLC partial UV chromatogram (235 nm) of the egg 11- and 12-hydroperoxide after reduction with triphenylphosphine. Egg 11- and 12-hydroperoxy products were isolated as shown in fig.1, reduced to hydroxy analogs, and the methyl esters chromatographed on the chiral phase HPLC column (see section 2). HETE methyl esters were eluted with 100:0.5 *n*-hexane/isopropanol (v/v) at a flow rate of 0.5 ml/min. UV absorbance was monitored at 235 nm. 12- and 11-HETE elution volumes were 23–25 and 25–28 ml.

same batch of [$^2H_8$]arachidonic acid using soybean lipoxygenase; this reaction is known to proceed with complete retention of the heavy atoms. The major ion in the spectrum of the labeled standard is found at *m/z* 399, representing the octadeuterated molecules (fig.3, lower panel). The ions at *m/z* 396, 397 and 398 correspond to molecules containing only 5, 6 and 7 deuterium atoms, and reflect incomplete labeling of the original batch of arachidonic acid.

Prominent ions in the egg products correspond to HETE derived from endogenous (non-deuterated) arachidonic acid at *m/z* 391, and from [$^2H_8$]arachidonic acid at *m/z* 399. HETE synthesis from [$^2H_8$]arachidonate via 11-keto and 12-keto intermediates would result in a loss of the geminal deuterium from C11 and C12, respectively, and replacement of a deuterium with hydrogen would produce a concomitant net decrease in *m/z* 399 ([$^2H_8$]HETE) and an increase in *m/z* 398 ([$^2H_7$]HETE). In fact, the results show complete retention of the heavy atoms, and the isotopic

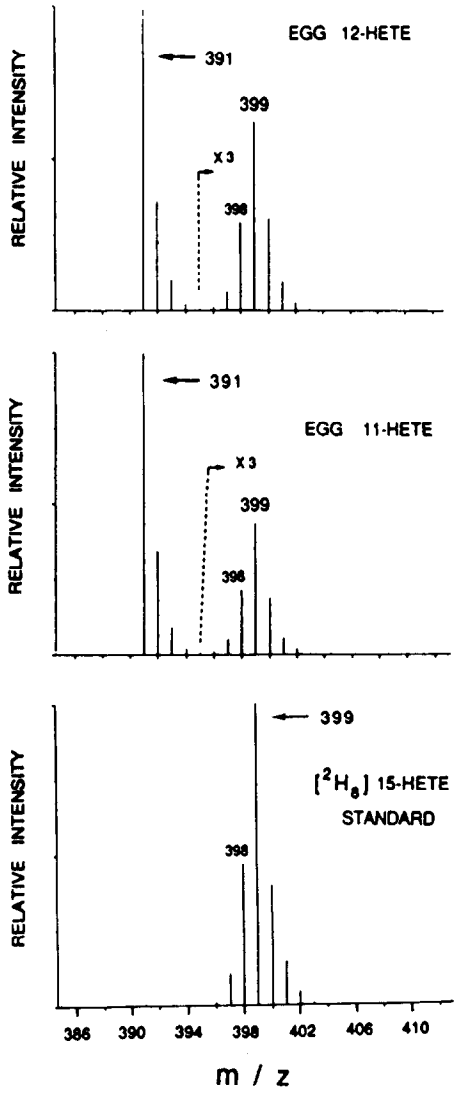

Fig.3. NI-CI GC-MS analyses of egg 12-HETE (top), 11-HETE (middle) and soybean lipoxygenase product synthesized from [$^2H_8$]arachidonate.

compositions closely match that of the soybean lipoxygenase product.

4. DISCUSSION

It has been hypothesized that 12(*R*)-HETE could originate from 12(*S*)-HPETE by forming a 12-keto-eicosatetraenoic acid intermediate which then might undergo conversion to 12(*R*)-HETE [4,5], e.g. via a stereospecific NADPH-dependent

keto-reductase [5]. The formation of keto-dienes by breakdown of lipid hydroperoxides is well recognized [14] and recently the formation of 12-keto compounds from 12-HPETE has been described in platelets [4]. At least superficially consistent with the possibility that egg 11- and 12-HETE are synthesized via keto-intermediates, we have noted the presence of traces of typical conjugated dienone chromophores during reversed-phase HPLC analyses of the sea urchin egg eicosanoid metabolites; these products chromatograph as slightly more polar than 11- and 12-HETE. However, the results of experiments with [$^2H_8$]arachidonate allow us to exclude the possibility that 11- or 12-keto intermediates were involved in egg HETE synthesis.

An NADPH-dependent cytochrome P450 mono-oxygenase is believed responsible for the synthesis of 12(*R*)-HETE by microsomal preparations of liver [2] and corneal epithelium [3] although the precise mechanism of these transformations is not yet elucidated. Egg 12(*R*)-HETE biosynthesis was not stimulated by addition of NADPH to the desalted egg $(NH_4)_2SO_4$ fraction. The activity in the egg is calcium-dependent, which is a known feature of several lipoxygenases (e.g. [15,16]). Finally, isolation of the egg 11(*R*)- and 12(*R*)-hydroperoxy-eicosanoids is consistent with biosynthesis via a lipoxygenase, with subsequent reduction giving the HETE products. The biological role of the H(P)ETEs in sea urchin eggs is not known, although in some types of starfish 8(*R*)-HETE potently induces the maturation of oocytes [17].

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