Formation of 14,15-Hepoxilins of the A<sub>3</sub> and B<sub>3</sub> Series through a 15-Lipoxygenase and Hydroperoxide Isomerase Present in Garlic Roots*

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Denis Reynaud‡, Muslim Ali§, Peter Demin‡, and Cecil R. Pace-Asciak‡¶¶

From the ‡Research Institute, The Hospital for Sick Children, Toronto, Canada M5G 1X8, the ¶Department of Biochemistry, Faculty of Science, Kuwait University, 13060 Safat, Kuwait, and the ¶¶Department of Pharmacology, Faculty of Medicine, University of Toronto, Toronto, Ontario, Canada M5A 1S8

We report herein for the first time the formation by freshly grown garlic roots and the structural characterization of 14,15-epoxide positional analogs of the hepoxilins formed via the 15-lipoxygenase-induced oxygenation of arachidonic acid. These compounds are formed through the combined actions of a 15(S)-lipoxygenase and a hydroperoxyeicosatetraenoic acid (HPETE) isomerase. The compounds were formed when either arachidonic acid or 15-HPETE were used as substrates. Both the “A”-type and the “B”-type products are formed although the B-type compounds are formed in greater relative quantities. Chiral phase high performance liquid chromatography analysis confirmed the formation of hepoxilins from 15(S)- but not 15(R)-HPETE, indicating high stereoselectivity of the isomerase. Additionally, the lipoxygenase was of the 15(S)-type as only 15(S)-hydroxyeicosatetraenoic acid was formed when arachidonic acid was used as substrate. The structures of the products were confirmed by gas chromatography-mass spectrometry of the methyl ester trimethylsilyl ether derivatives as well as after characteristic epoxide ring opening catalytically with hydrogen leading to dihydroyx products. That 15(S)-lipoxygenase activity is of functional importance in garlic was shown by the inhibition of root growth by BW 755C, a dual cyclooxygenase/lipoxygenase inhibitor and nordihydroguaiaretic acid, a lipoxygenase inhibitor. Additional biological studies were carried out with the purified intact 14(S),15(S)-hepoxilins, which were investigated for hepoxilin-like actions in causing the release of intracellular calcium in human neutrophils. The 14,15-hepoxilins dose-dependently caused a rise in cytosolic calcium, but their actions were 5–10-fold less active than 11(S),12(S)-hepoxilins derived from 12(S)-HPETE. These studies provide evidence that 15(S)-lipoxygenase is functionally important to normal root growth and that HPETE isomerization into the hepoxilin-like structure may be ubiquitous; the hepoxilin-evoked release of calcium in human neutrophils, which is receptor-mediated, is sensitive to the location within the molecule of the hydroxyepoxide functionality.

Hepoxilins (11(S),12(S)-type) are formed through the coupling of a 12(S)-lipoxygenase and a 12(S)-HPETE<sup>2</sup> isomerase (hepoxilin synthase) discovered in the rat pancreatic islets of Langerhans (1, 2), the pineal gland (3), and brain (4–6). They have been shown to exert a variety of biological actions likely mediated via their actions on ion fluxes, namely calcium (5, 7–11) and potassium (12, 13) within the cell. The hepoxilins cause the release of insulin (14), potentiate vascular contraction (15, 16), block neurotransmitter release (17), regulate cell volume (12), and provoke skin vascular permeability (18). Hepoxilin actions, at least in the human neutrophil, have been shown to be mediated via a hepoxilin-specific receptor (19, 20). In an attempt to learn more about the enzymatic formation of the hepoxilins, we investigated other sources for the “hepoxilin synthase” which may provide an abundant supply for the purification of the enzyme. Herein, we report the formation of hepoxilins via the 15-lipoxygenase pathway which is abundant in freshly grown roots of garlic. The isolated compounds (14,15-hepoxilins) constitute structural analogs of the parent 11,12-hepoxilins and provide further information on the specificity of the hepoxilin receptor in human neutrophils toward hepoxilins derived from 12-lipoxygenase.

EXPERIMENTAL PROCEDURES

Materials—Garlic bulbs were purchased from a local grocery store. They were placed individually in glass beakers in contact with tap water for 2–3 weeks until sufficient roots emerged, water being changed every day. The roots were cut off, rinsed with tap water and stored at 70° until enough tissue was collected for the experiments. Ionomycin, Dextran T-500, and Ficoll-Paque were purchased from Amersham Pharmacia Biotech, Sweden, and Indo-1-AM from Calbiochem. ADAM reagent was purchased from Research Organics Inc., Cleveland, Ohio. Racemic 15(S/R)-HPETE was prepared by photo-oxidation as described previously for 12(S/R)-HPETE (3). RPMI 1640 medium, hemin (bovine), and all reagent grade chemicals for buffers and NDGA were purchased from Sigma. BW 755C was a gift of Dr. Salvador Moncada (Burroughs-Welch, UK).

Incubations—Separate incubations were carried out with arachidonic acid (Cayman Chemical Co., Ann Arbor, MI) or with 15(S/R)-HPETE as substrates. Typically, 1 g of garlic roots was homogenized in 2 ml of phosphate-buffered saline of the following composition: NaCl (140 mM), Na<sub>2</sub>HPO<sub>4</sub> (10 mM), and Na<sub>2</sub>HPO<sub>4</sub> (10 mM), pH 7.2. The

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1 The abbreviations used are: HPETE, hydroperoxy-eicosatetraenoic acid; HETE, hydroxy-eicosatetraenoic acid; Hz, hepxiolin; 14,15-hepoxilin A<sub>1</sub>, 11-hydroxy-14,15-epoxyeicosa-5,8,11-trienoic acid; ADAM, allyl diazomethane; Indo-1-AM, 1-2-amino-5-(6-carboxyindole-2-yl)-phenoxy-2-[(2'-amino-5'-methylphenoxy)ethane-N,N,N',N'-tetraacetic acid pentaceatoxymethyl ester; RP16040, Roswell Park Memorial Institute medium 1640; GCMS, gas chromatography-mass spectrometry; EI, electron impact; HPLC, high performance liquid chromatography; TMSI, trimethylsilyl ether; BW 755C, (3-amino-1-(trifluoromethyl-phenyl)-2-pyrazoline); NDGA, nordihydroguaiaretic acid; Rt, retention time.
homogenate was centrifuged at 2000 rpm for 5 min, and 400-μl aliquots were added to silicized tubes containing either arachidonic acid (1 μg) or 15(S/R)-HPETE (1 μg) and made up with buffer to a total volume of 1 ml. The samples were incubated at 37 °C for 60 min. The incubation was terminated by cooling on ice and addition of 1 ml of methanol and 2 ml of 10% phosphoric acid containing ADAM reagent (140 μg/sample). The samples were stirred at 23 °C in the dark for 60 min to form the ADAM esters, and the ethyl acetate phase was separated and evaporated to complete dryness with a stream of N₂ gas. The residue containing the ADAM esters of the products was analyzed directly by HPLC. To generate standards of 14,15-hepoxilins, 15(S/R)-HPETE (1 μg) was treated in phosphate-buffered saline containing homogenate of freshly grown garlic roots and was used as with the garlic root incubations.

**HPLC Analysis**—The ADAM derivatives of the incubates were analyzed by HPLC on different columns using the intrinsic fluorescence of ADAM esters (mercury lamp with cut-off filters at 254 nm excitation and 400 nm emission) and an on-line fluorescent detector (Kratos) as described previously (21). First, the crude samples were analyzed by reverse phase-HPLC on a Nova-Pak C18 column (Waters Corp. Milford, MA, 3.9 × 300 mm) using acetonitrile/water (75:25, v/v, flow rate 1.5 ml/min) as running solvent. The appropriate fractions in which the 14,15-hepoxilin metabolites (A₃ and B₃) were eluted, as well as the fractions containing 15-HETE, were collected. These fractions were further analyzed and purified by SP-HPLC on a µPorasil column (Waters Corp. Milford, MA, 3.9 × 300 mm) using hexane/isopropanol (99.7/0.3, v/v, flow rate 2 ml/min). The purified 15-HETE and 14,15-hepoxilin A₃ and B₃ fractions were next separately analyzed on chiral phase HPLC using a Chiralcel-OD (J. T. Baker, Phillipsburg, NJ, 4.6 × 250 mm, flow rate 1.5 ml/min) column eluted isocratically with hexane/isopropanol (96/4, v/v) for 15-HETE and hexane/isopropanol (95/5, v/v) for the 14,15-hepoxilins (22).

**GCMS**—Samples were analyzed as the MeTMSi derivatives in the EI mode. Because the ADAM esters of the metabolites are unsuitable for hydrolysis and conversion into the methyl ester form for GCMS analysis, separate large scale experiments using 100 μg of 15-HETE were performed in which the crude ethyl acetate extracts were converted directly into the methyl ester form (instead of the ADAM esters) with a solution of ethereal diazomethane. The methyl esters were then purified by SP-HPLC on a µPorasil column (hexane/isopropanol 99.7/0.3, v/v, detection 210 nm). The purified fractions were converted into the TMSi ether derivatives by reaction with TriSilZ (Chromatographic Specialties, Brockville, Ontario, Canada, 20 μl), 5 min at 60 °C. Aliquots were injected into the GCMS directly. Samples were also analyzed after hydrogenation (platinum oxide/methanol, 30 s) of the methyl esters and subsequently conversion into the TMSi ether derivatives. A DB-1 methyl silicone capillary column (Chromatographic Specialties, Brockville, Ontario, Canada, 30 m length by 0.25 mm ID, 0.25 μm film thickness) was used; the column temperature was programmed from 200 °C initially to 300 °C at 10 °C/min.

**Measurement of Intracellular Free Calcium**—Neutrophils were prepared according to procedures previously published by our group (7). Intracellular free calcium concentrations were monitored continuously in a Perkin-Elmer fluorescence spectrophotometer (Model 650–40) using Indo-1 (Diamed Labs., Canada) at 37 °C with constant stirring. Compounds were added in 1 ml of cell medium in a temperature controlled plastic cuvette (Diamed Labs., Canada) at 37 °C with constant stirring. Compounds were added in 1 ml of MeSO. Each measurement was followed by a calibration for maximum and minimum calcium release with ionomycin (1 μM final concentration) and MnCl₂ (3 mM final concentration), respectively, according to Grinstein and Furuya (23). Responses were recorded on a chart recorder (LKB model 2210, Amersham Pharmacia Biotech, Sweden) at 1 cm/min chart speed.

**Measurement of Garlic Root Growth**—Garlic bulbs were purchased from a local grocery store, care being taken to select bulbs of similar size and weight. They were cleaned and placed on glass beakers (one/beaker) filled with tap water (100 ml) with the bottom of the bulb touching the water. Four groups of 6 bulbs/group were set up. Two groups contained the two inhibitors which were dissolved in MeSO and added to selected containers at a final concentration of 4 mg/100 ml. Two other groups contained only MeSO and served as control. The water was replaced every third day for two weeks. At the end of the study period, the roots were cut off with scissors, dried on paper, and weighed.

**RESULTS AND DISCUSSION**

Incubation of arachidonic acid with a cell-free homogenate of freshly grown garlic roots was incubated with arachidonic acid (AA) (panel E), or 15(S/R)-HPETE (panel D). Also shown are chromatograms from samples in which 15(S/R)-HPETE was incubated with hemin only (panel C) and garlic root homogenate incubated without any substrate (panel B). Authentic 11,12-hepoxilins derived from 12(S)-lipoxygenase are shown in panel A. Products were analyzed as ADAM esters by fluorescence detection (see “Experimental Procedures” for details).
Fig. 1 C), a condition previously shown by us to lead, in the 12-HPETE series, to the formation of 11(S),12(S)- and 11(R),12(R)-hepoxilins A3 and B3.

Despite the nonenzymatic (hemin-catalyzed) conversion of 12-HPETE into the hepoxilins, we have previously observed that an enzyme system exists in the pineal gland which forms exclusively the 11(S),12(S)-hepoxilins (3). This was demonstrated by the selective utilization of 12(S)- but not 12(R)-HPETE during hepoxilin formation. In the present experiments, we employed a similar approach with 15(S/R)-HPETE to investigate whether a selective consumption of 15(S)-HPETE could be observed during incubation with garlic root homogenates to indicate the presence of an HPETE isomerase. Hence HPLC fractions corresponding to 15-HETE and the 14,15-hepoxilins were isolated and subjected to chiral phase analysis.

**Fig. 2.** Chiral phase HPLC analysis of 15-HETE fractions collected from the chromatograms in Fig. 1, after further purification on SP-HPLC. Note the selective formation of 15(S)-HETE from the incubations of garlic roots with arachidonic acid (AA) (panel D), indicating the presence of a 15(S)-lipoxigenase. Also note in the experiments with 15(S/R)-HPETE substrate that both HETE enantiomers are present in the incubate when hemin was used as catalyst (i.e. nonenzymatic reaction) (panel B), whereas in the presence of garlic roots only 15(R)-HETE accumulates (panel C), indicating the selective utilization of the 15(S) enantiomer of the racemic 15-HPETE substrate by the HPETE isomerase in the garlic root system. Panel A shows the resolution of 15(S) and 15(R)-enantiomers of 15-HETE substrate.

Fig. 2 shows that garlic roots convert arachidonic acid exclusively into the 15(S)-HETE which accumulates in the incubation (Fig. 2D); conversely, when 15(S/R)-HPETE is used as substrate, the 15(S)-enantiomer is selectively consumed for further conversion into hepoxilin products while the 15(R)-enantiomer remains unmetabolized (Fig. 2C). When a nonselective reaction of 15-HPETE is carried out, as with hemin, both 15(S)- and 15(R)-HETE are detected at the end of the incubation (Fig. 2B). These experiments indicate the stereospecificity of 15-HPETE formation in garlic roots as well as the selective utilization of the 15(S)-enantiomer by HPETE isomerase to form the 14,15-hepoxilins. Confirmation of these findings was obtained when 14,15-hepoxilins were analyzed by chiral-HPLC. Fig. 3 shows that while 14,15-hepoxilin B3 (the major hepoxilin formed by garlic roots) affords chiral specificity (compare panels C and D) when an enzyme system is used, note that the corresponding hepoxilin A3 does not appear to, as its pattern is similar to that obtained during hemin catalysis (Fig. 3).

Characterization of the products was achieved through...
GCMS analysis both as the intact metabolites and after catalytic hydrogenation. Hydrogenation serves to stabilize the hepxilin structure and leads to characteristic reductive opening of the epoxide group depending on whether the hydroxyl group is adjacent to it (B-like) or allylic to it (A-like) (25). Fig. 4 compares the mass spectrum of the two types of metabolites isolated from garlic roots; the top panel shows the 14,15-hepoxilin B₃ (panel A) with its reconstructed ion chromatogram (panel B) and 14,15-hepoxilin A₃ (panel C) and its reconstructed ion chromatogram (panel D).

Fig. 5. EI mass spectra of the isolated products after catalytic hydrogenation (platinum oxide/methanol). The mass spectra of hydrogenated products derived from 14,15-hepoxilin B₃ are shown in panels A (mass spectrum) and B (reconstructed ion chromatogram) and of the hydrogenated 14,15-hepoxilin A₃ in panels C (mass spectrum) and D (reconstructed ion chromatogram).
between two vicinal OH groups) and 173 (C15–C20) (Fig. 5A for the former product); and m/z 173 (C15–C20), 317 (C11–C20), 287 (C1–C11) (Fig. 5C for the latter product). Selected ion chromatograms for these spectra are shown in the accompanying Fig. 5, B and D.

The products isolated in this study are derived from activation of 15(S)-lipoxygenase (see Scheme 1). That this enzyme activity is of importance to the rooting system of garlic was demonstrated by the addition of two inhibitors of lipoxygenases, BW 755C and NDGA, prior to initiation of rooting. Both inhibitors blocked the appearance of roots which was abundant in the control groups (Fig. 6).

11(S),12(S)-Hepoxilin A3 has been shown to evoke a dose-dependent rise in intracellular calcium in human neutrophils (7, 9). We therefore examined the isolated 14,15-hepoxilins in this bioassay. Fig. 7 shows typical calcium profiles for the A-type and the B-type 14(S),15(S)-hepoxilins in comparison with the profiles seen with the 11,12-type hepoxilins A3 and B3 (top panels). It is clear that the 14,15-hepoxilins are able to dose-dependently cause a rise in intracellular calcium although they are less active than 11,12-hepoxilin A3.

Garlic is widely used as a health supplement for a variety of conditions. It has been reported to have anti-platelet (26, 27), anti-cancer (28–30), and anti-atherogenic (31) properties although active ingredients additional to the selenium-containing compounds, allicin (32) and ajoene (32, 33), have not been systematically investigated. A lipoxygenase pathway has recently been identified from garlic with the isolation of some unique divinyl ether metabolites of linoleic acid (34). The presence of a 15(S)-lipoxygenase in garlic may afford relevance to atherosclerosis. Reported evidence suggests that 15-lipoxygenase oxidizes LDL to a pro-atherogenic form (35). However, evidence with transgenic rabbits that overexpress 15-lipoxygenase also indicates that these animals are resistant to the development of atherosclerosis when fed a cholesterol-rich diet (36, 37). Hence 15-lipoxygenase may act in both a pro- and anti-atherogenic manner depending on the time course of atherosclerosis (38). In the early stages, 15-lipoxygenase may serve an anti-atherogenic role, whereas in the later stages, it may act in a pro-atherogenic fashion (39). The products described herein are major products derived from 15-lipoxygenase present in garlic. Their actions on neutrophils in terms of intracellular calcium release may provide an insight into the antiinflammatory actions of these compounds as hepoxilins derived from the 12-lipoxygenase have been shown to inhibit the actions of inflammatory mediators (9). Whether the anti-atherogenic actions of garlic are related to the formation of the 14,15-hepoxilins remains to be established.

In conclusion, we have demonstrated the presence in garlic roots of 15(S)-lipoxygenase activity, and this is coupled with an HPETE isomerase (see Scheme 1). The products are analogs of the 11,12-hepoxilins that we described previously from the pancreas, pineal gland, and brain formed through the enzymatic isomerization of 12(S)-HPETE. The present results show that 15(S)-lipoxygenase is functionally important to the rooting system of garlic although we do not know whether the isolated 14,15-hepoxilins play a role. The present study also provides further information into the structural specificity of the hepoxilin receptor for 11,12-hepoxilin A3 in human neutrophils.
and the potential ubiquity of the HPETE isomerase (or hepxi-
lin synthase).

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