Identification of two epoxide hydrolases in *Caenorhabditis elegans* that metabolize mammalian lipid signaling molecules

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Abstract

We have identified two genes in the genomic database for *Caenorhabditis elegans* that code for proteins with significant sequence similarity to the mammalian soluble epoxide hydrolase (sEH). The respective transcripts were cloned from a mixed stage cDNA library from *C. elegans*. The corresponding proteins obtained after recombinant expression in insect cells hydrolyzed standard epoxide hydrolase substrates, including epoxyeicosatrienoic acids (EETs) and leukotoxins (EpOMEs). The enzyme activity was inhibited by urea-based compounds originally designed to inhibit the mammalian sEH. *In vivo* inhibition of the enzymes using the most potent of these compounds resulted in elevated levels of the EpOMEs in the nematode. These results suggest that the hydrolases are involved in the metabolism of possible lipid signaling molecules in *C. elegans*.
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Soluble epoxide hydrolase (sEH)[1], an enzyme first characterized in mammals, hydrolyzes an epoxide moiety through the addition of water [1,2]. Interestingly, the mammalian soluble epoxide hydrolase possesses two catalytic activities localized to distinct regions of the enzyme [3]. The epoxide hydrolase active site is located on the globular C-terminal region of sEH, while an active site on the N-terminal region has been found to display phosphatase activity [4]. These domains are expressed separately in plants, giving rise to the hypothesis that invertebrates may also express the N- or C- terminal domain separately [5,6].

In mammals, the epoxide hydrolase activity of sEH has been implicated in the metabolism of several epoxide-containing lipid signaling molecules [7,8]. This creates a diol species, which is more water-soluble than the epoxide and more readily eliminated through excretion, thereby depleting the supply of the epoxide signal. Also, in some cases the diols may be biologically active [9]. The epoxyeicosatrienoic acids (EETs) are epoxide-containing compounds produced by the metabolism of arachidonic acid by cytochrome p450 oxygenases [10]. The EETs have anti-inflammatory, as well as vasoconstrictive or vasodilatory properties, depending on the physiological context [11–13]. sEH has also been shown to metabolize 9,10-epoxy-12-octadecenoate (called leukotoxin, coronaric acid, or 9,10-EpOME) and 12,13-epoxy-9-octadecenoate (called isoleukotoxin, vernolic acid or 12,13-EpOME), cytochrome p450 metabolites of linoleic acid [14–16]. These compounds were identified as having a role in the development of acute respiratory distress syndrome (ARDS) [17].

Several recent studies have explored the signaling in *Caenorhabditis elegans*. C18 and C20 polyunsaturated fatty acids (PUFAs) have been shown to effect directed sperm movement towards the spermatheca and the formation of synaptic vesicles in neuronal tissues [18,19]. FAT-3 mutants lack the delta6 fatty acid desaturase and so cannot convert linoleic acid into C20 PUFAs because this desaturase activity is a prerequisite to subsequent elongase activity [20]. These mutants show a number of defects which are rescued by dietary supplementation of C20 and C18 PUFAs, including smaller brood size, alterations in the defecation cycle, and defects in chemotaxis [20,21]. These findings are exciting, because the nematodes amenability to genetic manipulation and the wealth of knowledge concerning its physiology make it an excellent model of lipid signaling. Thus the nematode may have implications for the study of such signaling pathways in mammalian disease models.

There are many similarities between fatty acid synthesis and metabolism in mammals and nematodes. Several desaturases and elongases in the fatty acid synthetic pathway of *C. elegans* have mammalian counterparts with parallel activities [22–26]. The cytochrome p450 monooxygenases responsible for production of the EETs and EpOMEs are present in the genome, though few have been characterized [27–29]. Finally, *C. elegans* contain arachidonic acid and linoleic acid, the precursors of these signaling molecules [30,31].

The genome of *C. elegans* contains five enzymes which display significant sequence similarity to the mammalian sEH. Of the five enzymes, two align with the C-terminal region of sEH. This raises the possibility that epoxide hydrolases are involved in the metabolism of epoxide-containing lipids in *C. elegans*. We report the cloning and characterization of both hydrolases which display significant sequence similarity to the C-terminal region of soluble epoxide hydrolase, and their functional disruption *in vivo* using a small molecule inhibitor.

Materials and methods

Nematode culture

The N2 (Bristol) strain of *C. elegans* was used. Plated nematodes were cultured on agar plates at 20 °C and fed the OP50 strain of *Escherichia coli* according to standard technique [32]. Liquid cultures of nematodes were grown in S-basal media and fed the NA22 strain of *E. coli* according to standard technique [32]. For the AUDA–BE liquid culture experiments, the worms were fed OP50.

Rapid amplification of cDNA ends

Details of worm extract preparation and RNA extraction can be found in supplementary materials. CEEH1 and CEEH2 3′RACE experiments were performed on the total RNA sample with the SMART RACE cDNA Amplification Kit (Clontech, Palo Alto, CA) using the gene specific primers 3CEEH11: 5′-GGGGGAGGTCTTGTTGCGTGGCAATTCGCGG-3′ and 3CEEH12: 5′-CTGGGGAACTGCGGACGGAGCATTGGAC-3′ for CEEH1 and 3CEEH21: 5′-GGGTCAAAAGCTGGAATCCGG

AATTCGG-3′ and 3CEEH22: 5′-CAGTCAGCCAGGCGGAACAACTGGTCC-3′ for CEEH2. CEEH2 5′RACE experiments were performed on the total RNA sample with the SMART RACE cDNA Amplification Kit (Clontech, Palo Alto, CA) using the gene specific primer 5CEEH21: 5′-GCTCCCCAATCATGAGCAGCAAGTG-3′. The 5′UTR of CEEH1 was determined by nested PCRs using the primers 5CEEH1F1: 5′-CCACTGTCACCTGGTTGGACG-3′ and 5CEEH1R1: 5′-CCTTCCAAAACGTTTGGCTTCTCCCGCTGC-3′, followed by the primers 5CEEH1F2: 5′-TATAACGCGTTTGGAATCACT-3′ and 5CEEH1R2: 5′-CGAACCGAACGCAAGGTCGTGACGGGAGAG-3′ using cDNA from a ProQuest *C. elegans* mixed stage library (Invitrogen, Carlsbad, CA).

Cloning

A ProQuest mixed stage cDNA library from *C. elegans* was purchased (Invitrogen, Carlsbad, CA). Primers for CEEH1 were designed to add BglII endonuclease sites on both ends, and a six histidine tag on the 3′ end of the coding sequence. Primers for CEEH2 were designed to add EcoRI endonuclease sites on both ends, and a six histidine tag on the 3′ end of the coding sequence. The primer pair for CEEH1 was 5′-AGATCTATGTTGTTTGAAAGTATATACATACAAATGT-3′ and 5′-AGATCTTTAGTGATGGTGATGGTGATGCTGATACTTATTCAAAAATTTT-3′. The primer pair for CEEH2 was 5′-GGATCCATGGGATTCTTTGCCGACTTGTTCA-3′ and 5′-GGATCCCTAGTGATGGTGATGGTGATGCAAGTGGGACTTGAAAGTTTTG-3′.

Baculovirus expression

Recombinant baculoviruses harboring the CEEH1 or CEEH2 cDNA sequence were generated by co-transfection of Sf21 cells derived from *Spodoptera frugiperda* with the recombinant transfer vector plasmid pACUW21 and Bsu36I-cleaved BacPAK6 viral DNA (Clontech, Palo Alto, CA) as previously described [33].

Protein purification

Infected high five cells (250 mL) were pelleted and resuspended in phosphate buffer with 10 mM imidazole. The cells were homogenized with an Ultra-Turrax T25 homogenizer (IKA Works, Wilmington, NC) rotating at 17,500 rpm for three 30 s intervals, with 15 s rests on ice between each grinding. The homogenate was centrifuged at 100,000g for 1 h at 4 °C. Ni-NTA HisBind Resin (EMD Biosciences, Inc., Madison, WI) was used to purify the enzymes according to manufacturer's protocol. Details can be found in supplementary materials.

Protein analysis

Protein concentration measurements were made using the BCA assay (Pierce, Rockford, IL) with BSA fraction V protein (Sigma–Aldrich, St. Louis, MO) to derive a standard curve. Polyacrylamide gel electrophoresis was performed using Novex gels (Invitrogen, Carlsbad, CA) for both SDS–PAGE analysis and isoelectric focusing.

Radiotracer based epoxide hydrolase activity assay

Epoxide hydrolase activity was measured using racemic [^{3}H]-*trans*-1,3-diphenylpropene oxide (*t*-DPPO) as substrate as previously described at a final concentration of 50 μM [34]. *t*-DPPO was previously synthesized and purified [35]. Details of the kinetics and IC$_{50}$ determination can be found in supplementary materials.

Fluorescent epoxide hydrolase assays

Assays were performed with the fluorescent substrate (3-phenyl-oxiranyl)-acetic acid cyano-(6-methoxy-naphthalen-2-yl)-methyl ester (PHOME) at a final concentration of 50 μM. Assays were performed as

described [36] with the following exception. An enzyme concentration of 0.57 μg/mL for CEEH1 was used in the assay.

Non-radioactive and non-fluorescent epoxide hydrolase assays

A 5 mM solution of each substrate was made in ethanol for the EETs, 9,10-EpOME and 12,13-EpOME. The substrate solution (1 μL) was added to 100 μL of enzyme preparation in sodium phosphate buffer (0.1 M, pH 7.4) containing 0.1 mg/mL of BSA ($[S]_{final} = 50$ μM). The enzyme was incubated with the substrate at 30 °C for 10 or 30 min, and the reaction quenched by addition of 400 μL of methanol. The EET reaction products were analyzed by HPLC–MS/MS as previously described [37].

Lipid extraction

Pellets of nematodes were resuspended in 1:1 (vol:vol) methanol:water spiked with internal standards and homogenized using an Ultra-Turrax T8 roto-stator grinder (IKA Works, Wilmington, NC) rotating at 25,000 rpm for 45 s. Chloroform (2 vol) was then added to the sample. The solution was vortexed 2 min, then centrifuged at 14,000g for 5 min at room temperature. The bottom organic layer was removed with a glass pipette and set aside and another two volumes of chloroform added to the remaining aqueous phase. This extraction was repeated two more times, and the resulting chloroform fractions combined and evaporated under a nitrogen stream until almost dry then reconstituted in methanol containing 10,11-dihydroxyheptadecanoic acid (10,11-DHHep) and d_8 11,12-EET as internal standards.

In vivo inhibition using AUDA–BE

Worms were staged and 100 mL liquid cultures grown at 20 °C. AUDA–BE (0.5 mL of 10 mM in DMSO) was added to the worms 24 and 36 h after adding the staged L1 s to the culture to a final concentration of 1% DMSO and 10 μM AUDA–BE, and worms collected when the reached the adult stage as judged by examination of aliquots. Control group was treated with vehicle alone, to a final concentration of 1% DMSO.

LC–MS/MS analysis of worm extract

Lipid extracts were analyzed using Shimadzu ASP10 HPLC system (Shimadzu, Pleasanton, CA) and Quattro Ultima tandem mass spectrometer (Waters, Milford, MA). Details can be found in supplementary materials.

Results

Cloning of the hydrolases

A tBLASTx search of the NCBI genomic database of *C. elegans* employing *Gallus gallus* (GenBank Accession No. Q120010) and *Xenopus tropicalis* (GenBank Accession No. BC078066) sEH nucleotide and translated amino acid sequences returned five soluble epoxide hydrolase hits. Two of the predicted enzymes, GenBank Accession Nos. NM_064867 and NM_073261, displayed significant sequence similarity to the C-terminus of mammalian sEH, while the other three, GenBank Accession Nos. NM_072133, NM_063993 and NM_072107.3 aligned with the N-terminal domain (Table 1). Primers were constructed based on the genomic sequence of enzymes which aligned with the C-terminal domain, and the full length transcripts were verified by 5′ and 3′ RACE experiments. The RACE experiments were performed using both a purchased mixed

stage cDNA library, and a cDNA archive prepared in the lab from a mixed stage pellet of Bristol-Meyer worms. The reconstructed full-length mRNA sequences were submitted to Genbank. The enzyme corresponding to the GenBank Accession No. EU151493 was designated CEEH1, while the enzyme corresponding to the GenBank Accession No. EU151492 was designated CEEH2. Primers based on these results were designed and the sequences were cloned (Figs. 1 and 2).

Expression and purification of the hydrolases

The constructs encoded for a six histidine tag on the C-terminal end of the enzymes. The recombinant enzymes were expressed in a baculovirus expression system and purified on a nickel chelation column (see supplementary materials). The experimentally determined molecular weight for CEEH1 and CEEH2 were 48 and 44 kDa, respectively, in close agreement with the calculate values of 48.7 and 42.9 kDa. The experimentally determined isoelectric points for CEEH1 and CEEH2 were between the ranges of 8.7–8.8 and 6.7–6.8, respectively, also near the calculated values of 8.8 and 6.8.

Characterization of recombinant enzyme activity

The epoxide hydrolase activities were initially assayed with *t*-DPPO, a substrate for the mammalian sEH. The half life for CEEH1 at 37 °C was between 1 and 2 h, the half life at 24 °C was approximately 8 h, and the half life at 4 °C was 24 h. The half life for CEEH2 was approximately 2 h at 37 °C, 24 h at 24 °C and over six days at 4 °C.

CEEH1 had a specific activity of 3000 nmol min^{-1} mg^{-1}, comparable to the human sEH (Table 2). However, CEEH2 had a specific activity of 14 nmol min^{-1} mg^{-1} (Table 2). Although this was much lower than the activity displayed by CEEH1, insect cells infected with a baculovirus containing the CEEH2 sequence displayed significantly higher activity than insect cells infected with a control virus containing the sequence of an alpha/beta hydrolase fold enzyme which does not possess epoxide hydrolase activity (see supplementary material). This provided evidence that the observed EH activity after partial purification was not due to a co-purified enzyme from the insect cells. K_m and k_{cat} were then determined for *t*-DPPO and CEEH1. The K_m was found to be 160 μM, and the k_{cat} 12 s^{-1} (Table 3).

Next, natural products were assayed with the recombinant enzymes. The specific activities with the compounds were first determined. 14,15-EET was found to have a value of 615 nmol min^{-1} mg^{-1}, 11,12-EET had a value of 202 nmol min^{-1} mg^{-1}, and 8,9-EET had the lowest value at 44.2 nmol min^{-1} mg^{-1} (Table 2). The specific activities for 9,10-EpOME and 12,13-EpOME were found to be 137 nmol min^{-1} mg^{-1} and 132 nmol min^{-1} mg^{-1}, respectively (Table 2). CEEH2 showed a greater than twentyfold lower activity, compared to CEEH1 (Table 2). K_m and k_{cat}

Table 1
Protein BLAST results

Query	Subject	Subject residues aligned	Segment identity	Score	e-Value
CEEH1	sEH from *H. sapiens*	241–342	27%	305	$7e{-}26$
	sEH from *G. gallus*	248–547	26%	303	$1e{-}25$
	sEH from *X. tropicalis*	249–548	28%	289	$5e{-}24$
	HLD from X. autotrophicus	34–148	30%	131	$1e{-}05$
CEEH2	sEH from *H. sapiens*	238–548	24%	282	$3e{-}23$
	sEH from *G. gallus*	238–547	22%	237	$5e{-}18$
	sEH from *X. tropicalis*	226–556	26%	294	$1e{-}24$
	HLD from *X. autotrophicus*	34–148	33%	156	$1e{-}08$
NM_063993	sEH from *H. sapiens*	100–222	39%	225	$5e{-}17$
NM_072107.3	sEH from *H. sapiens*	1–237	27%	205	$1e{-}13$
NM_072133	sEH from *H. sapiens*	84–221	31%	170	$1e{-}10$

The protein sequences were aligned using the program 'Blast 2 sequences' with a κ value of 0.267 and a λ value of 0.041. The GenBank Accession Nos. were as follows: sEH from *H. sapiens* = L05779; sEH from *G. gallus* = DQ120010; sEH from *X. tropicalis* = BC075370; HLD from *X. autotrophicus* = M26950.

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   1    gaccaaATGTTGTTTGAAAGTATATACATACAATGTTTGAACAAATTTTCTAAATACAAGAAGTTTGTGTGCCACACTAC
   1         M   L   F   E   S   I   Y   I   Q   C   L   N   K   F   S   K   Y   K   K   F   V   C   H   T   T

  81    ATGTAATTGTCCCAATTACAAAATTATTGGTTATCTTCTTTACTTTTTGTGTCTGTGTCGGCCGATAAATTGTAGTCTTC
  26         C   N   C   P   N   Y   K   I   I   G   Y   L   L   Y   F   L   C   L   R   P   I   N   C   S   L

 161    CTCTCTCCCGTCACGACCTTGCGTTCGGTTCGTTTTCTTATTACTTGACAATGTTTCTCTATAGAATACTCGTCCGCCTC
  52         P   L   S   R   H   D   L   A   F   G   S   F   S   Y   Y   L   T   M   F   L   Y   R   I   L   V   R   L

 241    TTACAATTTTATTATTTTGTCAAATTCTCGGCAATTCTATTCCTGGGATTTGCGGTTAAAGGAAGATCATTGTTTGAGAA
  79         L   Q   F   Y   Y   F   V   K   F   S   A   I   L   F   L   G   F   A   V   K   G   R   S   L   F   E   K

 321    AAAGCAGCGGGAGAAGCCAAaCgTTTTGGAAGGATGGGACAGCAGGTACATCAAGTTAAAGAAAGTGCGGCTTCATTATG
 106         K   Q   R   E   K   P   N   V   L   E   G   W   D   S   R   Y   I   K   L   K   K   V   R   L   H   Y

 401    TCCAAACTGGATCCGATGACAAACCATTAATGCTATTTATTCATGGATACCCCGAGTTTTGGTATTCTTGGAGATTTCAG
 132         V   Q   T   G   S   D   D   K   P   L   M   L   F   I   H   G   Y   P   E   F   W   Y   S   W   R   F   Q

 481    TTGAAAGAATTTGCAGATAAATACAGATGTGTGGCAATCGATCAACGTGGCTACAATCTTTCCGATAAGCCAAAACACGT
 159         L   K   E   F   A   D   K   Y   R   C   V   A   I   D   Q   R   G   Y   N   L   S   D   K   P   K   H   V

 561    GGATAATTATTCAATTGATGAGCTAACTGGAGATATTCGAGATGTCATTGAAGGTCTTGGTTATGACAAAGCTATTGTAG
 186         D   N   Y   S   I   D   E   L   T   G   D   I   R   D   V   I   E   G   L   G   Y   D   K   A   I   V

 641    TTGCTCATGACTGGGGAGGTCTTGTTGCGTGGCAATTCGCGGAACAGTATCCAGAGATGGTGGATAAGTTGATTTGTTGC
 212         V   A   H   D   W   G   G   L   V   A   W   Q   F   A   E   Q   Y   P   E   M   V   D   K   L   I   C   C

 721    AACATTCCAAGACCCGGGTCTTTTAGAAAACGAATTTATACCAGCTGGTCACAGTTCAGAAAAAGCTGGTACATGTTCTT
 239         N   I   P   R   P   G   S   F   R   K   R   I   Y   T   S   W   S   Q   F   R   K   S   W   Y   M   F   F

 801    CTACCAAAACGAGAAAATACCAGAAATGCTGTGTTCGGCTGATGATATGAAATGTTGGAGcTcTGCTTTAGAGCCAAAG
 266         Y   Q   N   E   K   I   P   E   M   L   C   S   A   D   D   M   K   M   L   E   L   C   F   R   A   K

 881    AAATTGGAATACAAAACAACAAGAACTTCACGGATGAGGATTTGGAAGCATGGAAGTATTCATTTTCAATGAATGGAGCC
 292         E   I   G   I   Q   N   N   K   N   F   T   D   E   D   L   E   A   W   K   Y   S   F   S   M   N   G   A

 961    TCATTCAAATATCCTATTAACTACTATCGAAATATTTTTAATGCAAAGAAGCAACAGGCTGATTTAGTGTTGGAGATGCC
 319         S   F   K   Y   P   I   N   Y   Y   R   N   I   F   N   A   K   K   Q   Q   A   D   L   V   L   E   M   P

1041    CACACTTATCATCTGGGGAACTGCGGACGGAGCATTGGACATTGAAGCAGCCGTGGATAGTTTGAACACCCTCAAACAAG
 346         T   L   I   I   W   G   T   A   D   G   A   L   D   I   E   A   A   V   D   S   L   N   T   L   K   Q

1121    GAACAATGAAGAAAATCGAAGGAGCTTCACATTGGGTTCAACAGGATGAACCTGAGATGGTTAATGAGCATATAAAAAAA
 372         G   T   M   K   K   I   E   G   A   S   H   W   V   Q   Q   D   E   P   E   M   V   N   E   H   I   K   K

1201    TTTTTTGAATAAGTATCAGTAGttttttgttttttgagaatttgttttaaagctaatcatcaaaattaaaataaagaaattc
 399         F   L   N   K   Y   Q   *

1281    ttattgtgaaaaaaaaaaaaaaaaaaaaaaaaaaaa
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Fig. 1. Nucleotide sequence and translated protein sequence of the CEEH1 cDNA. This DNA sequence has been assigned GenBank Accession No. EU151493.

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   1    tttttttgttatttctattattattcaATGGGATTCTTTGCCGACTTGTTCAAAGTTGTCTATTCCACATGGAGACAATA
                                    M  G  F  F  A  D  L  F  K  V  V  Y  S  T  W  R  Q  Y

  81    TATTTACACTACTGGCGCTTTACTCACTCTCACGTGGAAATGGTTCACAGAAGGAAATGAGTATTTTGTGGAACACGTAT
  19     I  Y  T  T  G  A  L  L  T  L  T  W  K  W  F  T  E  G  N  E  Y  F  V  E  H  V

 161    ATCCTGAACCTGAGTGCTTGAAAAATTGGAATCACAAATTCGTGCAGTTGAAAAATATTCGAATGCATTATGTTGAAGAA
  45     Y  P  E  P  E  C  L  K  N  W  N  H  K  F  V  Q  L  K  N  I  R  M  H  Y  V  E  E

 241    GGTCCTGCAGATGGAGATGTTCTACTTATGGTTCATGGATTTCCAGAATTTTGGTATTCATGGAGATTTCAACTAGAACA
  72     G  P  A  D  G  D  V  L  L  M  V  H  G  F  P  E  F  W  Y  S  W  R  F  Q  L  E  H

 321    TTTCAAACATACTCACAGATGTATCGCAATCGACATGCGAGGTTACAATACAACTGATCGTCCATCTGGAATTTCTGATT
  99     F  K  H  T  H  R  C  I  A  I  D  M  R  G  Y  N  T  T  D  R  P  S  G  I  S  D

 401    ATAATCTAACCCACTTAGTGGAAGATATTCGTCAATTCATTGAAATTCTTGAATTAAAACGAGTAACACTTGCTGCTCAT
 125     Y  N  L  T  H  L  V  E  D  I  R  Q  F  I  E  I  L  E  L  K  R  V  T  L  A  A  H

 481    GATTGGGGAGCAATTGTTTGTTGGCGTGTTGCAATGTTACATTCAAATCTTATCGACCGACTTGTCATTTGTAATGTTCC
 152     D  W  G  A  I  V  C  W  R  V  A  M  L  H  S  N  L  I  D  R  L  V  I  C  N  V  P

 561    TCATCCATTTGCATTTTTTGAAGTCTACAATATGAGTAAAGAACAAAGAAATAAAAGCTGGTATATTTATCTGTTCCAGT
 179     H  P  F  A  F  F  E  V  Y  N  M  S  K  E  Q  R  N  K  S  W  Y  I  Y  L  F  Q

 641    CTCAATACATTCCAGAAATCGCAATGAGATCTAATAAAATGAAAATGTTAGAGGCAATGTTTAGAGGGTCAAAAGCTGGA
 205     S  Q  Y  I  P  E  I  A  M  R  S  N  K  M  K  M  L  E  A  M  F  R  G  S  K  A  G

 721    ATCCGGAATTCGGAAAACTTCACAGATGAGGATATGTTGGCATGGAAACATGTTTTCAGTCAGCCAGGCGGAACAACTGG
 232     I  R  N  S  E  N  F  T  D  E  D  M  L  A  W  K  H  V  F  S  Q  P  G  G  T  T  G

 801    TCCATTAAACTATTATCGAGATTTATTCAATGCTCCTGCTATTCCTCGAAAACTTCAAATTGTTCAGCCGAAAGTATTGA
 259     P  L  N  Y  Y  R  D  L  F  N  A  P  A  I  P  R  K  L  Q  I  V  Q  P  K  V  L

 881    TTCTTTGGGGTGATGAAGATGCTTTTTTTGGATAAAAAAGGAGCTGAATTATCTGTCCAGTTTTGCCGAGATTGTCGTGTT
 285     I  L  W  G  D  E  D  A  F  L  D  K  K  G  A  E  L  S  V  Q  F  C  R  D  C  R  V

 961    CAAATGATCCGCGGAGCATCTCATTGGGTTCAACAAGATCAGCCACAACTTGTCAATGTATATATGGAGCAATTCATGAA
 312     Q  M  I  R  G  A  S  H  W  V  Q  Q  D  Q  P  Q  L  V  N  V  Y  M  E  Q  F  M  N

1041    CGAAGATTCCTACAGACCTATTGGAGAAATCAAAACTTTCAAGTCCCACTTGTGAatagataattgtttatttacaatta
 339     E  D  S  Y  R  P  I  G  E  I  K  T  F  K  S  H  L  *

1121    agttttataaatttttaaacaaaataaatattttttatttcaaaaaaaaaaaaaaaaaaaaaaaaaa
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Fig. 2. Nucleotide sequence and translated protein sequence of the CEEH2 cDNA. This DNA sequence has been assigned GenBank Accession No. EU151492.

were then determined for the EpOMEs (Table 3). The K_m values fell in the 1–10 μM range, while the k_{cat} values ranged from approximately 0.2 to 0.002 s^{-1} (Table 3).

A series of fluorescent substrates has been developed to characterize human sEH epoxide hydrolase activity, and for the use in high-throughput assays [36]. In order to compare the CEEH1 to the human enzyme, a limited structure activity experiment was performed using these substrates. All of the substrates were hydrolyzed by CEEH1 (Table 4). CEEH2 did not display activity when assayed with these substrates.

Analysis of epoxide hydrolase inhibition

A select group of small molecule inhibitors of the mammalian sEH were assayed with recombinant CEEH1 (Table 5). The same inhibitors were used to inhibit *t*-DPPO activity in crude extract from a mixed stage liquid culture of *C. elegans*. AUDA was found to be the most potent inhibitor of the five assayed.

Small molecule inhibition in vivo

Finally, AUDA–BE was used to inhibit the enzymes *in vivo*. AUDA and its butyl ester are of similar inhibitory potency. The butyl ester is anticipated to penetrate tissues rapidly and be hydrolyzed to the free AUDA by esterases. Synchronized worms grown in liquid culture were treated with the inhibitor 24 and 36 h after the first larval stage was transferred from plates to the culture. The worms were harvested at the adult stage and the lipids extracted for LC–MS/MS analysis. It was found that 9,10-EpOME and 12,13-EpOME levels increased by approximately 200% in the treated populations relative to the controls (Fig. 3). Changes in 9,10-DiHOME levels were not significantly different from controls. Interestingly, the 12,13-DiHOME levels decreased, rather than increased in the treated populations (Fig. 3). The epoxide-to-diol ratio for 9,10-EpOME and 12,13-EpOME both increased. The 9,10-EpOME ratio increased by 174% while the 12,13-EpOME ratio increased tenfold (Fig. 3).

Table 2
Specific activity with *t*-DPPO and natural substrates

Compound name	Structure	Specific activity (nmol min^{-1} mg^{-1})	
		Recombinant CEEH1	Recombinant CEEH2
t-DPPO		3000 ± 230	14 ± 2.6
9,10-EpOME		137 ± 1.77	$6.77 \pm .0033$
12,13-EpOME		132 ± 1.16	$4.75 \pm .085$
14,15-EET		615 ± 10.8	$7.63 \pm .72$
11,12-EET		202 ± 12.3	4.10 ± 0.32
8,9-EET		44.2 ± 1.00	2.00 ± 0.14
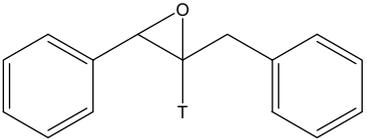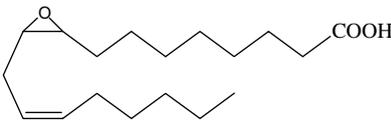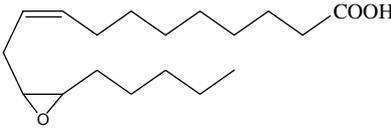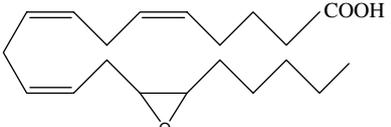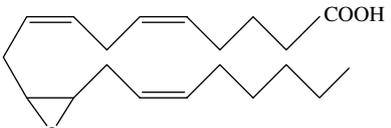
Recombinant CEEH1 and CEEH2 were partially purified as described. Assay conditions are described in Materials and methods. Results are presented as the mean ± standard deviation of two to three separate experiments performed in triplicate. *Note.* The 5,6-EET rapidly cyclizes on storage.

Discussion

The mammalian sEH is an approximately 60 kDa enzyme composed of two globular regions connected by a short proline-rich linker [2]. Mammalian soluble epoxide hydrolase is thought to be a product of the fusion of two ancestral bacterial genes [5]. The C-terminal region contains the epoxide hydrolase active site and is descended from haloalkane dehalogenase (HLD), while the N-terminal region contains a phosphatase active site and is descended from haloacid dehalogenase (HAD) [5,38]. When sequences from the nematode enzymes, vertebrate sEH homologs, and the bacterial gene HLD are compared by BLAST, one sees that CEEH1 and CEEH2 score higher with the vertebrate sEH homologs than with HLD and the aligned segments fall within the C-terminal region, or epoxide hydrolase domain, of the vertebrate sEH homologs (Table 1).

Certain important characteristics of soluble epoxide hydrolases can be identified in the CLUSTALW alignment (Fig. 4). The alpha/beta hydrolase fold family catalytic triad is composed of a catalytic nucleophile and a histidine–aspartate pair that activates a water molecule [3]. This triad is shared by all five enzymes, though the spacing between the amino acids is not conserved in the lower organisms. In addition to these residues, the mammalian soluble epoxide hydrolases have two tyrosines which are thought play a role in the orientation and activation of the epoxide moiety [3]. These amino acids are not found in the haloacid dehalogenase, but align in both CEEH1 and CEEH2.

In addition to the C-terminal homologs CEEH1 and CEEH2, three enzymes were identified that display significant sequence similarity to the N-terminus of vertebrate sEH (Table 1), which may make *C. elegans* a fascinating case in which both C-terminal and N-terminal precursors of mammalian sEH are expressed individually, as they are in the plants so far examined [6,39]. The CEEH2 sequence confirmed by RACE agreed with the WormBase

Table 3
K_m and k_{cat} with *t*-DPPO and EpOMEs

Name structure	Kinetic parameter	Recombinant CEEH1	Recombinant CEEH2	Recombinant human sEH
t-DPPO 	K_m (µM) k_{cat} (s^{-1}) k_{cat}/K_m (µM^{-1} s^{-1})	160 ± 21 12 ± 0.5 0.07	N.D. N.D. .0004	6.2 ± 0.6 4.3 ± 0.3 0.7
9,10-EpOME 	K_m (µM) k_{cat} (s^{-1}) k_{cat}/K_m (µM^{-1} s^{-1})	7.5 ± 3.9 0.22 ± 0.035 0.029	8.4 ± 3.6 0.01 ± 0.004 0.0012	6.15 ± 1.0 2.78 0.452
12,13-EpOME 	K_m (µM) k_{cat} (s^{-1}) k_{cat}/K_m (µM^{-1} s^{-1})	11.4 ± 5.1 0.11 ± 0.022 0.0096	1.9 ± 0.72 0.002 ± 0.0004 0.001	5.17 ± 0.56 1.93 0.37

Recombinant CEEH1 and CEEH2 were partially purified as described. Results for *t*-DPPO are presented as the means ± standard deviation of two to three separate experiments performed in triplicate. In the case of the EpOMEs, the error is the standard error from a nonlinear regression fitting the results of an experiment performed in triplicate to the Michaelis–Menten equation. The k_{cat}/K_m ratio for CEEH2 and *t*-DPPO was estimated under the assumption of $[S] << K_m$. N.D. means not determined. Values for *t*-DPPO and the human enzyme are from Morisseau [6]. Values for the EpOMEs and the human enzyme are from Greene [40] and were determined with the methyl ester of these compounds.

Table 4
Specific activity with fluorescent substrates

No.	Structure R =	% of highest specific activity	
		Recombinant CEEH1	Recombinant human sEH
1		80 ± 5.9	26
2		43 ± 2.2	15
3		52 ± 1.9	38
4		24 ± 3.2	<1
5		100 (2300 ± 240 nmole/min/mg)	100 (2700 ± 44 nmole/min/mg)
6		41 ± 2.0	13
7		68 ± 2.8	15
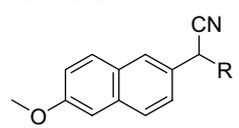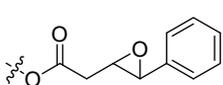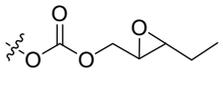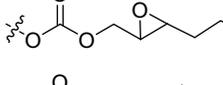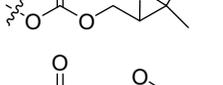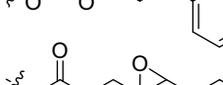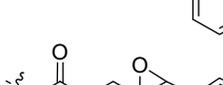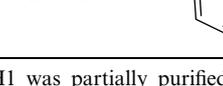
Recombinant CEEH1 was partially purified as described. Assay conditions are described in Materials and methods. Results are presented as the mean ± standard deviation of three separate experiments performed in triplicate. Values for the recombinant human enzyme are from Jones [36].

Table 5
IC$_{50}$ s with urea-based inhibitors

Name	Structure	Mixed stage crude extract IC$_{50}$ (nM)	Recombinant CEEH1 IC$_{50}$ (nM)	Recombinant Human sEH IC$_{50}$ (nM)
CEU		>50000	>50000	42000 ± 2000
DCU		>50000	41000 ± 1200	160 ± 10
CDU		6900 ± 66	4300 ± 320	100 ± 10
AUDA		650 ± 25	530 ± 44	100 ± 10
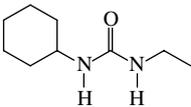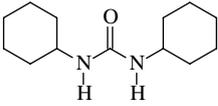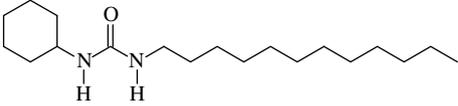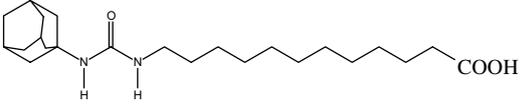
IC$_{50}$ values for the urea-based inhibitors N-cyclohexyl-N′-ethylurea (CEU), N,N′-dicyclohexylurea (DCU), N-cyclohexyl-N′-dodecylurea (CDU), and 12-(3-adamantane-1-yl-ureido)-dodecanoic acid (AUDA). Recombinant CEEH1 was partially purified as described. Assay was performed using [^{3}H] t-DPPO as substrate. Conditions are described in Materials and methods. Values for the human enzyme are from Jones (2005), which also used [^{3}H] t-DPPO as substrate [36]. Error bars represent the standard deviation for three separate experiments performed in triplicate.
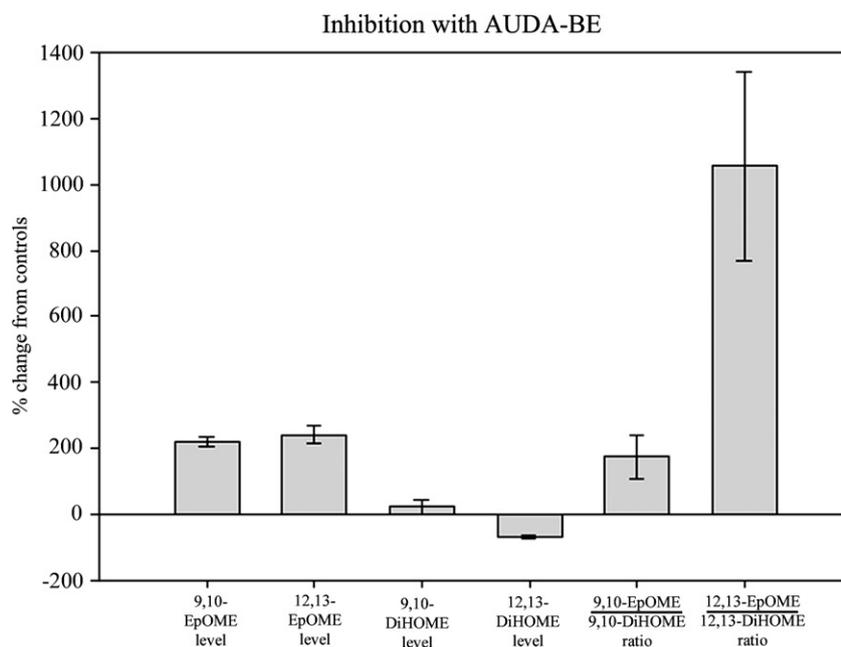
Fig. 3. Inhibition of epoxide hydrolase activity in vivo using the urea-based small molecule inhibitor AUDA–BE. Worms grown in suspension culture were fed the bacterial strain OP50. Inhibited populations were treated twice with AUDA–BE in DMSO to a final inhibitor concentration of 10 μM. Controls were treated with vehicle alone. Error bars represent the standard deviation.

predicted mRNA from gene model K06C5.5 (Fig. 2). The CEEH1 sequence corresponded to the predicted mRNA from gene model K02F3.6 (Fig. 1).

When the enzymes were assayed with t-DPPO, a substrate used to measure mammalian sEH epoxide hydrolase activity, CEEH2 was found to have less than 1/100 the activity of CEEH1 despite the fact that CEEH2 shares the conserved catalytic triad as well as the two orienting tyrosines (Table 2). CEEH2 could, of course, be optimized to hydrolyze very different substrates from CEEH1 or even have quite a different role in the nematode. CEEH1 was found to be less stable than CEEH2. This instability may

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HSEH      1   -MTLRGAVFDLDGVLALPAVFGVLGRTEEALALPRGLLNDAFQKGGPEGATTRLMKGEITLSQWIPLMEENCRKCSETAK
GGSEH     1   -MARRFALFDLGGVLFGPGLQHFLGSCERSYALPRNFLRDVLFAGGSDSPHAKVMRGQITLSQLFLEVDEGCRQHASTAG
XTSEH     1   MAARRFVLFDLGGVLLTPGPQVAFQRLERSLSLPSGFLQNVFVRSGSEGPFARAERGQIPFSKFIAEMDKECKAFAEESG
CEEH1     1   ------------------------------------------MLFESIYIQCLNKFSKYKKFVCHTTCNCPNYK----
CEEH2     1   ------------------------------------------------MGFFADLFKVVYSTWR---------
XAHLD     1   --------------------------------------------------------------------------------


HSEH     80   VCLPKNFSIKEIFDKAISARKINRPMLQAALMLRKKGFTTAILTNTWLDDRAERDGLAQLMCELKMHFDFLIESCQVGMV
GGSEH    80   ITLPTTFSIAHVFEEMAAKGTLNAPLLRAASMLRRNGFKTCVFTNNWVDDSMGRLFTSSLLTVVQRHFDLLIESCRVGLH
XTSEH    81   VSLPDSFSLEQTFHGMFESGGINKPMLKAAVTLRHHGFKTCVLTNNWIDDSPQRSHSAELFSSLNRHFDLVVESCRTGMR
CEEH1    32   -----IIGYLLYFLCLCRPINCSLPLSRHDLAFGSFSYYLTMFLYRILVRLLQFYYFVKFSAILFLGFAVKGRSLFEKKQ
CEEH2    16   -------------------------------------------QYIYTTGALLTLTWKWFTEGNEYFVEHV
XAHLD     1   --------------------------------------------------MINAIRTPDQRFSNLDQYPFSPNY
                                                                                :      .


HSEH    160   KPEPQIYKFLLDTLKASPSEVVFLDDIGANLKPARDLGMVTILVQDTDTALKELEKVTGIQLLNTPAPLPTSCNPSDMSH
GGSEH   160   KPDPRIYTYALEVLQAQPQEVIFLDDIGENLKPAREMGMATILVRDTDTVLKELQELSGVQLLQQEEPLPTTCDPATMSH
XTSEH   161   KPETQIYDYALKMLKANPKETIFLDDIGANLKPAREMGIATVLVKDTETALKELQALSGVPLFENEEVTPVPANPDNVTH
CEEH1   108   REKPNVLEG-------------------------------------------------------------------WDS
CEEH2    45   YPEPECLKN-------------------------------------------------------------------WNH
XAHLD    25   LDDLPGYPG-------------------------------------------------------------------
                 .


HSEH    240   GYVTVKPRVRLHFVELG----WPAVCLCHGFPESWYSWRYQIPALAQAGYRVLAMDMKGYGESSAPPEIEEYCMEVLCKE
GGSEH   240   GYVPIRPGVQLHFVEMGH---GPAICLCHGFPESWLSWRYQIPALADAGFRVIALEMKGYGESTAPPEIEEYSQEQICKD
XTSEH   241   GSVTVKPGVQLHYVEMGN---GPVICLCHGFPESWYSWRFQIPALADAGFRVIAFDMKGYGDSSAPQEIEEYSQEQICKD
CEEH1   120   RYIKLKK-VRLHYVQTGSD-DKPLMLFIHGYPEFWYSWRYQLKEFADK-YRCVAIDQRGYNLSDKPKHVDNYSIDELTGD
CEEH2    57   KFVQLKN-IRMHYVEEGPA-DGDVLLMVHGFPEFWYSWRFQLEHFKHT-HRCIAIDMRGYNTTDRPSGISDYNLTHLVED
XAHLD    33   --------LRAHYLDEGNSDAEDVFLCLHGEPTWSYLYRKMIPVFAESGARVIAPDFFGFGKSDKPVDEEDYTFEFHRNF
                          ::  *:::  *     .   ** *     :*   :  :  .    *  :*  :  *:. :   *    .:*


HSEH    316   MVTFLDKLGLSQAVFIGHDWGGMLVWYMALFYPERVRAVASLNTPFIPANPNMSPLESIKANPVFDYQLYFQEPGVAEAE
GGSEH   317   LTIFLDKLGIPQAVFIGHDWGGAVVWNMALFYPERVRAVASLNTPYRPADPTVDIVETMKSFPMFDYQFYFQEPGVAEAE
XTSEH   318   LVSFLDVMGISQASFIGHDWGGAVVWNMALFYPERVRAVASLNTPFFTSDPGVNALERIKANPIFDYQLYFQEPGVAEAE
CEEH1   197   IRDVIEGLGYDKAIVVAHDWGGLVAWQFAEQYPEMVDKLICCNVP-RPGSFRKRIYTSWSQFRKSWYMFFYQNEKIPEML
CEEH2   134   IRQFIEILELKRVTLAAHDWGAIVCWRVAMLHSNLIDRLVICNVP-HPFAFFEVYNMSKEQRNKSWYIYLFQSQYIPEIA
XAHLD   106   LLALIERLDLRNITLVVQDWGGFLGLTLPMADPSRFKRLIIMNACLMTDPVTQPAFSAFVTQPADGFTAWKYDLVTPSDL
                :  .::  :     .  .  :***. :   ..    ..  :   *        :      :   .  ..
                                      ▼                                    □


HSEH    396   LEQNLSRTFKSLFRASDE-----SVLSMHKVCEAGGLFVNSPEEPSLSRMVTEEEIQFYVQQFKKSG--FRGPLNWYRNM
GGSEH   397   LEKDIGRTLKALIRSTRPEDRLHSVPGLLGVQERGGLLVGFPEDIPESLILHGAELQYYIERFQRSG--FRGPLNWYRNM
XTSEH   398   LEKDLERTFKVFFRGSSEKDRLATSLTTMNVRERGGILVGTDEDPPLSSIINEADLQYYVAQFKKSG--FRGPLNWYRNM
CEEH1   276   CSADDMKMLELCFR------------------------AKEIGIQNNKNFTDEDLEAWKYSFSMNGASFKYPINYYRNI
CEEH2   213   MRSNKMKMLEAMFR------------------------GSKAGIRNSENFTDEDMLAWKHVFSQPGGTTG-PLNYYRDL
XAHLD   186   RLDQFMKRWAPTLT------------------------EAEASAYAAPFPDTSYQAGVRKFPKMV------AQRDQAC
                :  :     :                              .     .      *     .   :    . :
                                          ▼                         ▼                     □


HSEH    469   ERNWKWACKSLGRKILIPALMVTAEKDFVLVPQMSQHMEDWIPHLKR-GHIEDCGHWTQMDKPTEVNQILIKWLDSDARN
GGSEH   475   RPNWRWALSAKDRKILMPALMVTAGKDVVLLPSMSKGMEEWIPQLRR-GHLEACGHWTQMERPAALNRILVEWLEGLPPD
XTSEH   476   QRNSEWNISAHGWKILVPALMVTAGKDFVLLPIMTKGMENLIPNLSR-GHIEECSHWTQMERPAAVNGILIKWLAEVHN-
CEEH1   331   FNAK--KQQADLVLEMP-TLIIWGTADGALDIEAAVDSLNTLKQGTM-KKIEGASHWVQQDEPEMVNEHIKKFLN--KYQ
CEEH2   267   FNAP--AIPRKLQIVQPKVLILWGDEDAFLDKKGAELSVQFCRDCRV-QMIRGASHWVQQDQPQLVNVYMEQFMNEDSYR
XAHLD   234   IDISTEAISFWQNDWNGQTFMAIGMKDKLLGPDVMYPMKALINGCPEPLEIADAGHFVQEFGEQVAREALKHFAETE---
                     .::  .   *   *                  :   ..*:.*       .  .  .:


HSEH    547   --PPVVSKM---
GGSEH   554   GAMLRVSRL---
XTSEH   553   --LPVTSKL---
CEEH1   404   ------------
CEEH2   344   PIGEIKTFKSHL
XAHLD   310   ------------
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Fig. 4. Alignment of the vertebrate sEH homologs with the nematode enzymes and bacterial HLD. The mammalian epoxide hydrolase "catalytic triad" residues are marked by closed triangles. The tyrosines thought to polarize the epoxide moiety of the epoxide hydrolase substrate are marked by open squares. HSEH = sEH from *H. sapiens* (GenBank Accession No. L05779); GGSEH = sEH from *G. gallus* (DQ120010); XTSEH = sEH from *X. tropicalis* (BC075370); XAHLD = HLD from *X. autotrophicus* (M26950).

be due to the dilute state and of the enzyme, which does not reflect the intracellular environment. These data argue against instability accounting for CEEH2's low activity on the substrates tested. The k_{cat}/K_m ratio, or enzyme specificity, of CEEH1 for *t*-DPPO was lower than the human enzyme, having both a higher k_{cat} and K_m (Table 3). The ratio indicated that *t*-DPPO is a fair substrate for CEEH1 activity. The estimated k_{cat}/K_m ratio for CEEH2 shows that *t*-DPPO is a poor substrate for assaying CEEH2 activity (Table 3).

The structure of the lipid epoxide molecules thought to be endogenous substrates for mammalian sEH differ greatly from *t*-DPPO. The EETs are 20 carbon fatty acids, in which an epoxide lies near the center of the molecule. Like the human enzyme, CEEH1 hydrolyzed 14,15-EET at the highest rate, which has the epoxide moiety furthest from the carboxylic acid (Table 2). CEEH2 showed twenty-fold less activity when assayed with the molecules, but also hydrolyzed 14,15-EET at the highest rate. 9,10-EpOME and 12,13-EpOME are regioisomeric 18 carbon fatty acid

epoxides. The k_{cat}/K_m ratios for these compounds reveal that CEEH2 has a ten to twentyfold lower specificity for the EpOME when compared to CEEH1 (Table 3). CEEH2 has roughly the same or lower K_m for the EpOMEs as CEEH1, but a much lower k_{cat}. CEEH2 could influence the metabolism of the EpOMEs *in vivo* by competing with CEEH1 for substrate binding, resulting in a decrease in the rate of diol production.

To learn more about the substrate selectivity of CEEH1, fluorescent substrates recently developed for use in high-throughput screens of inhibitor libraries were next assayed (Table 4). The left side of the substrates consist of a cyanohydrin paired with a series of right sides, which contain either an ester or carbonate moiety. When released, the cyanohydrin rearranges to yield an intensely fluorescent aromatic aldehyde with a huge Stoke's shift. These right sides also have a range of steric properties, from a short alkyl chain to substituted phenyl rings, as well as one tri-substituted epoxide. CEEH1 hydrolyzed substrate 5 at the highest rate, like the human enzyme (Table 4), but did not show a marked preference for the aryl carbonate (substrate 5) over the ester (substrate 1). Also, the nematode enzyme showed less of a preference for the aryl carbonate over the alkyl carbonates, compared to the human enzyme. Interestingly, CEEH1 also hydrolyzed substrate 4, the trisubstituted epoxide, which is very slowly turned over by the mammalian enzyme. Substrates 6 and 7, which contain nitro- and chloro-substituted phenyl rings, were also fair substrates for CEEH1, when compared to the human enzyme. This may indicate that the nematode binding site lacks steric constraints found in the mammalian enzyme.

In order to test for inhibition of the enzyme, four inhibitors were chosen that have high, medium and low potency when assayed with the mammalian enzymes. Their structures are based on a urea pharmacophore, which has been shown to bind to the mammalian enzyme in a reversible manner as a putative transition state mimic. These same five inhibitors were also used to characterize the crude extract. The fluorescent probes are attacked by esterases and glutathione *S*-transferase, and so they cannot easily be used to measure epoxide hydrolase activity in the crude extract. To use these substrates in an epoxide hydrolase "specific assay" one must remove glutathione or inhibit glutathione *S*-transferase activity and inhibit or remove esterase activity. However, the *t*-DPPO assay can be made specific for epoxide hydrolase activity after a correction for glutathione transferase activity or elimination of glutathione. In this case, no GST activity was detected. Crude extract from a 20 k × g spin of a mixed stage population was prepared. Less than 10% of the total activity was recovered in the pellet, possibly the result of worm carcasses, which trap some soluble material. When the supernatant and recombinant CEEH1 were characterized with the five inhibitors, CEEH1 showed the same pattern and level of response as the crude extract (Table 5) suggesting that CEEH1 is similar or identical to the majority of the

enzyme accounting for *in vivo* activity. The difference in the IC_{50} values could be due to substrate binding or sequestering, another metabolic pathway for *t*-DPPO, or the presence of other hydrolases in the crude extract.

When worms were treated with the small molecule inhibitor AUDA–BE, the EpOME levels increased, and their diol levels did not change or increased slightly (Fig. 3), though no phenotypic effects were noted. In the simplest model, one might expect that a decrease in epoxide hydrolase activity would result in an increase in epoxides and a decrease in the corresponding diols, resulting in an increased epoxide-to-diol ratio. This assumes one pathway of diol production, which may not be the case in the nematode, and does not consider feedback regulation of other enzymes involved in the formation or elimination of the diols. The EpOMEs are relatively lipophilic compounds that will concentrate in tissues, while the DiHOMEs are dramatically more polar. They are anticipated to rapidly diffuse into the media or be conjugated. It is possible that compensatory mechanisms affected by the decrease in epoxide hydrolase activity, coupled with the rapid clearance of the diols resulted in a relatively unchanged level of diols, regardless of epoxide levels. More work will be required to dissect these metabolic pathways. A second experiment employing RNAi can be found in supplementary materials.

Taken together, the *in vitro* characterization of the enzymes and the small molecule inhibition results suggest that CEEH1, and possibly CEEH2 to a lesser extent are involved in the metabolism of these C18 lipids *in vivo*. In mammals, epoxide-containing lipids have been found to play numerous signaling roles. The metabolites of 9,10-EpOME and 12,13-EpOME have been shown to promote the onset of ARDS-like symptoms. The EETs have been implicated in a number of physiological processes, including the regulation of inflammation. The role of these epoxide-containing lipids have not been studied in *C. elegans*.

The demonstration of a nematode epoxide hydrolase with the ability to hydrolyze mammalian soluble epoxide hydrolase substrates offers the possibility of parallel signaling pathways in *C. elegans*. Their involvement in the metabolism of epoxide-containing lipids *in vivo* has impacts for the study of both lipid metabolism and lipid signaling in *C. elegans*, which is a model both for parasitic and phytophagus nematodes as well as a more general model for eukaryotic regulatory biology.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.abb.2008.01.016.

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