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Characterization and cDNA cloning of a clofibrate-inducible microsomal epoxide hydrolase in *Drosophila melanogaster*

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In order to understand the roles of the epoxide hydrolases (EHs) in xenobiotic biotransformation in insects, we examined the induction of EHs by exogenous compounds in *Drosophila melangaster* third instar larvae. Among the chemicals tested, clofibrate, a phenoxyacetate hypolipidermics drug, increased EH activity towards *cis*-stilbene oxide approximately twofold in larval whole-body homogenates. The same dose of clofibrate also induced glutathione *S*-transferase activity. The effect of clofibrate on EH induction was dose-dependent and the highest activity occurred with a 10% clofibrate application. Three other substrates conventionally used in EH assays (*trans*-stilbene oxide, *trans*-diphenylpropene oxide and juvenile hormone III) were poorly hydrolysed by larval homogenates, with or without clofibrate administration. Because the increased EH activity was localized predominantly in the microsomal fraction, we synthesized degenerate oligonucleotide primers with sequences corresponding to conserved regions of known microsome EHs from mammals and insects in order to isolate the gene. The 1597 bp putative cDNA of *D. melanogaster* microsomal EH (*DmEH*) obtained from a larval cDNA library encoded 463 amino acids in an open reading frame. Northern blot analysis showed that the transcription of *DmEH* was increased in larvae within 5 h of clofibrate treatment. Recombinant DmEH expressed in baculovirus hydrolysed *cis*-stilbene oxide (23 nmol·min^{-1}·mg protein^{-1}) and was located mainly in the microsomal fraction of virus-infected Sf9 cells. There was no detectable EH activity toward juvenile hormone III. These observations suggest that *DmEH* is involved in xenobiotic biotransformation, but not in juvenile hormone metabolism, in *D. melanogaster*.

Keywords: detoxification; *Drosophila melanogaster*; epoxide hydrolase; induction; insect.

Numerous studies have demonstrated the important roles of epoxide hydrolases (EHs) (E.C. 3.3.2.3) in xenobiotic biotransformation in mammals [1,2]. Mutagenic and carcinogenic alkene and arene epoxides are often generated from environmental aliphatic and aromatic hydrocarbons, respectively, by oxidation catalysed by mono-oxygenases, including cytochrome p450s, in the body. The generated electrophilic epoxides can bind irreversibly to cellular micromolecules or readily alkylate nucleic acids. The EHs convert such harmful xenobiotic epoxides to electrophilically unreactive, water-soluble diols, which can be easily excreted. Endogenously produced epoxides, such as steroid and fatty acid epoxides, are also metabolized by EHs. There are five classes of EH in mammals: soluble (sEH), microsomal (mEH), hepoxilin A_3 hydrolase, leukotriene A_4 hydrolase, and cholesterol 5,6-oxide hydrolase [3]. Both sEH and mEH have been shown to degrade xenobiotics, but mEH appears to be by far the most important of the two enzymes. The activities of rodent sEH and mEH in liver are induced by many different compounds. Increased mEH activity has been detected in response to phenobarbital, *trans*-stilbene oxide (TSO), 3-methyl cholanthene [4–6], clofibrate [7–9], clofibric acid, isosafrole, β-naphthoflavone [10], tamoxifen [11], nitrosamines [12], and benzil [13]. The sEHs were induced by the peroxisome proliferator agents (*p*-chlorophenoxyacetic acid, 2,4-dichlorophenoyacetic acid, clofibrate) [9,14,15], chlorinated paraffins, and di(2-ethylhexyl)phthalate [8]. Some inductions were confirmed to occur at the transcriptional level [11,12]. The induction of mEH in rats is coordinated with the induction of other xenobiotic metabolizing enzymes. For example, the same compounds that induce mEH also induce UDP-glucuronosyltransferases and glutathione S-transferase (GST) [8,10]. The induction of these detoxification enzymes by xenobiotics is an important self-defence mechanism that enables the rapid elimination of harmful exogenous compounds.

In contrast with the established roles of mammalian EHs, the roles of insect EHs in xenobiotic metabolism are poorly understood. As in mammals, EH activities toward TSO and

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Abbreviations: EH, epoxide hydrolase; mEH, microsomal EH; sEH, soluble EH; DmEH, *Drosophila melanogaster* microsomal EH; GST, glutathione S-transferase; CE, carboxylesterase; CSO, *cis*-stilbene oxide; TSO, *trans*-stilbene oxide; tDPPO, *trans*-diphenylpropene oxide; JHIII, juvenile hormone III; CDNB, 1-chloro-2,4-dinitrobenzene; DIG, digoxigenein; ARE, antioxidant response element; PPRE, peroxisome proliferator response element.

Enzyme: epoxide hydrolases (EHs) (E.C. 3.3.2.3).

Note: The nucleotide sequence data reported in this paper will appear in the DDBJ Nucleotide Sequence Database with accession number AB107959.

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cis-stilbene oxide (CSO) have been detected in adult *Drosophila melanogaster* [16,17]. One could argue that insect EH activities were first clearly described using a cyclodiene analogue as a substrate [18]. Insect hydrolases including EHs metabolize cyclodiene insecticides [19]. However, EH activities in DDT-resistant *D. melanogaster* and insecticide-resistant houseflies, *Musca domestica* were equivalent to those in the respective susceptible strains [20,21]. The involvement of EH activity in insecticide resistance has not been clearly demonstrated, but this is not surprising, because the epoxide-containing insecticides used commercially are so sterically hindered that they resist all EH activity.

An EH that metabolizes juvenile hormone (JH) has been well studied in several insect species. The JHs, analogues of methylfanesoate 10,11-epoxides, are crucial in insect development and reproduction. *D. melanogaster* also reportedly produces a bis-epoxide of methylfanesoate [22]. The titre of JHs in the haemolymph fluctuates during the development of an insect's stadium and correlates with specific developmental events, such as molting and metamorphosis [23]. At the late stage of the last stadium, JH production is reduced, and JH is inactivated via catabolism by JHEH and JH-specific esterase. JHEH was purified from *Manduca sexta* [24], and the JHEH gene was isolated from *M. sexta* [25], *Tricoplusia ni* [26] and *Ctenocephalides felis* [27]. The activity and expression of JHEH during different developmental stages were examined in *T. ni* [26]. JHEH activity was very low at the beginning of the last larval stadium, but it gradually increased, reaching a peak at the wandering stage late in the last larval stadium. At the prepupal stage, EH activity declined to a level equal to that in the early time of the stadium. Northern blot analysis revealed that this pattern was regulated at the transcriptional level. Thus, the production of JHEH is regulated inversely to the JH titre. It is not known whether JHEH is induced by xenobiotics or is involved in detoxification.

To elucidate the roles of insect EHs in xenobiotic biotransformation, we first examined induction of EH activity by several chemicals in the larvae of a standard *D. melanogaster* strain, Canton-S. We found that exogenous chemicals altered EH activity and that clofibrate was a potent inducer of mEH. We also isolated a cDNA clone that potentially encodes a xenobiotic-metabolizing mEH, which differs from the JH metabolizing EH.

Materials and methods

Insects

D. melanogaster (Canton-S) were reared on a diet containing corn meal (9% w/v), sucrose (10% w/v), nutritional yeast (4% w/v), agar (0.9% w/v) propionic acid (0.3% v/v) and butyl *p*-hydroxybenzoate (0.2% w/v, dissolved in 70% ethanol) with a 16-h light/8-h dark cycle at 25 °C. On day 1 of the third instar, larvae were collected and used for induction experiments.

Chemicals and administration

Clofibrate [2-(*p*-chlorophenoxy)-2-methylpropionic acid ethyl ester], clofibric acid [2-(*p*-chlorophenoxy)-2-methylpropionic acid], *cis*-9,10-epoxystearic acid, laminarin were purchased from Sigma, and fenvalerate was from American Chemical Service. Clofibrate, clofibric acid and epoxystearic acid were dissolved in acetone at 10% (w/v), respectively. Fenvalerate was dissolved in acetone at 1% (w/v), and laminarin was dissolved in water at 0.5% (w/v). One hundred microlitres of each solution was spread on a 35 mm-diameter filter paper (Whatmann No.1). This procedure delivered 41 μmoles clofibrate, 47 μmoles clofibric acid, 34 μmoles epoxystearic acid, 2.4 nmoles fenvalerate, 500 mg laminarin in the total assay, respectively. After the solvent was evaporated, the paper was wetted with 200 μL water in a 35 mm Petri-dish (Falcon). Ten larvae were allowed to crawl on the paper for 2 h, then a piece of diet was supplied, the dishes were covered with parafilm and incubated at 25 °C for 18 h. Thus the total exposure was 20 h. Time-dependence experiments began when larvae were placed on the filter paper.

Enzyme preparation

Third instar larvae were homogenized in 0.3 mL cold homogenizing buffer (50 mM Tris/HCl pH 8.0, 1 mM EDTA, 0.01% phenyl thiourea) using Kontes pellet pestle (749515) motorized by a Hand-Tite Drill (Black and Decker). The supernatant of 10 000 ***g*** centrifugation for 10 min at 4 °C was kept at −80 °C until used for enzyme assays. To separate cytoplasmic and microsomal fractions, the supernatant was further centrifuged at 100 000 ***g*** for 60 min at 4 °C. Protein concentrations were determined by Bradford assay using a Protein assay reagent (Bio-Rad) with BSA (Sigma) as a standard.

EH assay

EH activities were measured by the radiometric partition assay using four different tritiated-substrates, *cis*- and *trans*-stilbene oxides ([^{3}H]CSO and [^{3}H]TSO) [28], *trans*-diphenylpropene oxide ([^{3}H]tDPPO) [29] or juvenile hormone III ([^{3}H]JHIII; NEN Life Science Products). Serial dilution of enzyme samples were prepared using homogenization buffer. To inhibit GST activity, diethyl maleate was added to the samples at 1 mM final concentration. In the assays with JHIII as a substrate, 3-octyl-thio-1,1,1-trifluoropropan-2-one was added at 0.1 mM final concentration to inhibit JH specific esterase activity [30]. Reactions were initiated by the addition of 1 μL substrate (0.5 mM final concentration) to 0.1 mL each sample, and incubated for 30 min at 30 °C in a shaking water bath. To stop the reaction, 0.25 mL isooctane was added then vortexed for 30 s. followed by centrifugation at 2793 ***g*** for 5 min. Thirty microlitres from the aqueous phase were mixed with 1 mL scintillation cocktail ACSII (Amersham), and radioactivity was counted using a WALLAC 1409-012. Assays were done in triplicate and all radioactive counts were corrected by nonenzymatic hydration.

GST and CE assays

GST and carboxyl esterase (CE) activities were assayed by spectrophotometric methods using a 96-well microtiter

plate [31]. GST activity was measured by adding 10 μL 0.36 mM 1-chloro-2, 4-dinitrobenzene (CDNB) to 300 μL enzyme which was equilibrated with 0.1 M Na_2HPO_4 buffer (pH 6.5) containing 5 mM reduced glutathione. To assay CE, 2 μL 4-nitrophenyl acetate (final concentration 0.5 mM) was added to 298 μL enzyme solution equilibrated with 0.1 M Tris/HCl buffer pH 7.5. Immediately after the addition of the substrate, increase of absorbance rate in the first 2 min was measured at 340 nm for GST and at 405 nm for esterase by Vmax Kinetic Microplate Reader (Molecular Devices). GST was also assayed by another radiometric partition assay using tritiated TSO as substrate. This method was basically the same as the EH assay described above. The enzyme sample was mixed with reduced glutathione (final concentration 5 mM), and extraction of the aqueous phase was carried out using *n*-hexanol which removes both epoxide and diol from the conjugate in the aqueous fraction. Assays were done in at least triplicate and the value was corrected for the nonenzymatic reaction.

Calculations and statistics

Enzyme activities were calculated as specific activity·mg^{-1} protein. The significance of differences between chemical-treated and control groups was estimated by Student's *t*-test with $P < 0.05$ accepted as significant.

cDNA cloning of DmEH

Poly(A)$^+$ RNA was extracted from clofibrate-treated larvae using a QuickPrep Micro mRNA Purification Kit (Amersham Pharmacia Biotech), and first-strand cDNA was synthesized using a First-Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech). Four oligonucleotide primers were designed based on the amino acid sequences (GLDIHFI, KPDTVG, M/LV/LHGWP, I/VQGGDWG) which are highly conserved sequences between human mEH and *T. ni* JHEH. The primer sequences and the combinations are as follows: 5′-C/TTA/C/G/TGAC/TATA/C/TCAC/TTTC/TAT-3′ (first PCR forward) and 5′-CCA/C/G/TACA/C/G/TGTA/GTCA/C/G/TGGC/TTT-3′ (first PCR reverse), 5′-ATGA/GTA/C/G/TCAC/TGGA/C/G/TTGGCC-3′ (second PCR forward) and 5′-CCCCAA/GTCA/C/G/TCCA/C/G/TG/CCT/CTG-3′ (second PCR reverse). Second round PCR was carried out using the first round PCR product as a template. From the nucleotide sequence of the second PCR product, an additional primer corresponding the internal partial nucleotide sequence was synthesized to amplify the 3′ region of the cDNA combined with the oligo-$(dT)_{18}$ primer. To obtain 5′ end sequence of the cDNA, we searched Flybase (http://flybase.bio.indiana.edu/), a *Drosophila* expressed sequence tag library and obtained a clone covering 5′ partial sequence of the *DmEH*. The full-length *DmEH* ORF was amplified by PCR from first strand cDNAs using *Pfu* polymerase (Promega). The forward primer corresponded to the 5′-end of the ORF and contained a *Sal*I site (5′-CTACGTCGACGATGGCGAACATCTGGCCACGAATC-3′); the reverse primer corresponded to the 3′-end of the ORF and contained an *Xba*I site (5′-AGGCTCTAGATTTATGAGAAATTGGCTTTCTGGAC-3′).

Expression of the DmEH

Recombinant EH was expressed using BAC-TO-BAC Baculovirus Expression System (Gibco BRL) following the manufacture's protocol. Briefly, the *DmEH* ORF was subcloned into a pFASTBAC plasmid and the nucleotide sequence and correct orientation were confirmed. Competent DH10BAC cells were transformed with the plasmid, and the EH gene was inserted into a bacmid DNA. The resultant recombinant bacmid was harvested from the *Escherichia coli* cells, and the DNA was purified by the alkaline lysis method. To obtain a control virus, pFASTBAC without insert was used and the bacmid DNA was purified in the same way. Sf9 cells were transfected with the recombinant and the control bacmid DNAs using CELLFECTIN (Gibco BRL). After 4 days, the cell culture supernatant was harvested and stored at −80 °C as a stock virus. For virus amplification, Sf9 cells were infected with the stock virus, and supernatant was collected then virus concentration was determined by plaque forming assay.

SDS/PAGE

The virus-infected cells (1×10^6) were harvested 72 h after infection and washed by centrifugation with 100 mM phosphate-buffered saline containing 2 mM EDTA. The cell pellets were suspended directly in two volumes of sample-treatment buffer (10% urea, 2.5% SDS, 5% 2-mercaptoethanol, 0.005% bromophenol blue) then boiled for 5 min. Samples were loaded onto a 12% polyacrylamide gel and electrophoresed at 100 V, constant voltage. The gel was stained with Coomassie Brilliant Blue R-250.

Northern blot analysis

Forty larvae on day 1 of the third larval instar were treated with 10% clofibrate and 10 larvae were collected each time after 0, 5, 8 and 14 h. Poly(A)$^+$ RNA was purified from the larvae using QuickPrep Micro mRNA Purification Kit (Amersham Pharmacia Biotech). Three hundred nanograms of each mRNA sample were electrophoresed on a 1.2% (w/v) agarose gel containing 6.6% (v/v) formaldehyde, and then transferred onto a GeneScreen Plus membrane (DuPont). Digoxigenein (DIG)-labelled probes were prepared using a PCR DIG Labelling Mix (Roche). To prepare a DmEH probe, primers (5′-ATGGCGAACATCTGGCCACGAATC-3′ and 5′-TTATGAGAAATTGGCTTTCTGGAC-3′) were used, and to prepare Actin 5C probe as an internal marker, primers (5′-GTTCGAGACCTTCAACTCGC-3′ and 5′-TTCGAGATCCACATCTGCTG-3′) were used. The nucleotide sequence of the Actin C5 was obtained from the database (Accession No. K00667). Hybridization and detection were carried out by using a DIG system (Boehringer Mannheim). The membranes were incubated in a hybridization solution [50% (v/v) formamide, 5 × NaCl/Cit, 7% (w/v) SDS, 50 mM sodium-phosphate, pH 7.0, 0.1% (v/v) *N*-lauroylsarcosine, 2% (w/v) blocking reagent) at 48 °C for 2 h. Each DIG-labelled probe was added to the solution then incubated at 48 °C overnight. The membranes were rinsed twice with 2 × NaCl/Cit, 0.1% (w/v) SDS, and then washed twice with 0.5 × NaCl/Cit, 0.1% (w/v) SDS at 68 °C. After

the reaction with alkaline phosphatase-conjugated anti-DIG Ig and initiation of the luminescence reaction with substrate, the chemiluminescent signal was detected by exposure of the membrane to X-ray film. The X-ray film was scanned using a ScanJet 4C (Hewlett Packard).

Results

Altered EH activity after chemical treatment

EH activity toward CSO was increased 2.25-, 1.35-, and 1.43-fold in the larvae treated for 20 h with 10% clofibrate, 10% clofibric acid, and 0.5% laminarin, respectively (v/v/v) (Fig. 1). In contrast, epoxy-stearic acid treatment suppressed EH activity by ≈75%, suggesting that this compound might be toxic to the larvae. Treatment with 1% (w/v) fenvalerate, which caused no apparent damage to the larvae, did not affect EH activity. When acetone was spread directly on the larval cuticle, EH activity was reduced by approximately half (data not shown). However, using our filter paper exposure method, there was no difference in EH activity between naive and acetone-treated larvae. Therefore, solvent effects were negligible under our experimental conditions.

Induction of EH activity by clofibrate

Because clofibrate induced the highest EH activity, the dose–response and time course of induction by clofibrate were examined. EH activity increased in a dose-dependent manner with 0, 0.1, 0.5, 1, 5 and 10% clofibrate (Fig. 2).

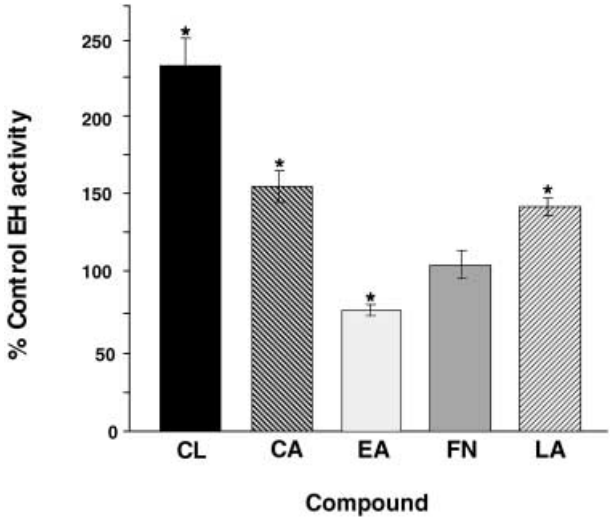

Fig. 1. Altered EH activity in *D. melanogaster* larvae after exposure to five different compounds. Ten larvae were treated with five different compounds and collected after 20 h. The larvae were homogenized and EH activity was assayed with a radiometric partition assay as described in Materials and methods. CL, clofibrate (41 μmoles); CA, clofibric acid (47 μmoles); EA, epoxystearic acid (34 μmoles); FN, fenvalerate (2.4 nmoles); LA, laminarin (500 mg). Data represent means ± SD of three independent replications. Stars indicate significant differences ($P > 0.05$) from control (acetone treatment for CL, CA, EA and FN; water treatment for LA).

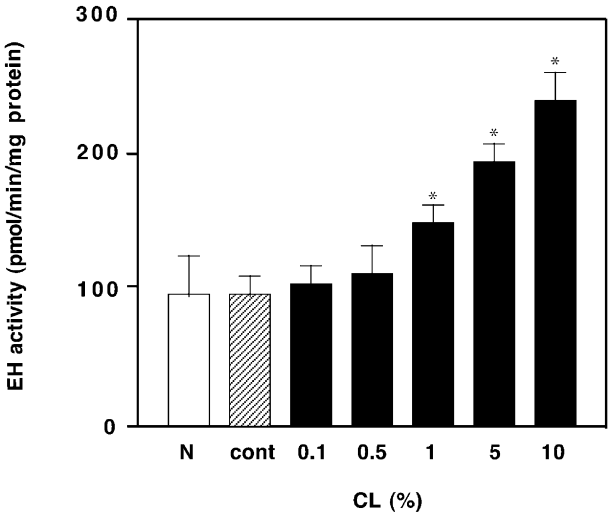

Fig. 2. Dose-dependent induction of EH activity by clofibrate. Larvae were treated with the indicated concentrations of clofibrate, and EH activities of whole-body homogenates were assayed. Bars represent SDs. Starred values are significantly different ($P > 0.05$) from controls. N, No treatment; Cont, acetone treatment. A 10% solution of clofibrate delivers 41 μmoles of compound in the filter disc assay.

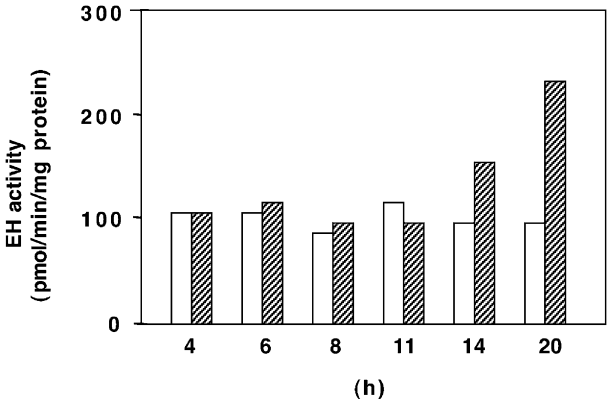

Fig. 3. Induction time-course of EH activity in larvae treated with clofibrate. At the indicated times after treatment with acetone (white bars) or 10% clofibrate (striped bars), larvae were homogenized, and EH activity was assayed. Data represent mean activities from duplicate experiments.

Significant increases were observed with treatments of 1–10% exposure. Treatment with > 10% clofibrate resulted in extensive melanization of the larvae, and all larvae died at 20% clofibrate (data not shown). At each time point after exposure to 10% clofibrate (4, 6, 8, 11, 14 and 20 h), EH activity was measured. The activity did not change until 11 h after exposure and increased to 1.6-fold and 2.25-fold at 14 and 20 h postexposure, respectively (Fig. 3).

Substrate selectivity of EH activity in larvae

[^{3}H]TSO, [^{3}H]tDPPO, and [^{3}H]JHIII were used to determine the substrate selectivity of larval EH activity.

Table 1. Substrate selectivity of EH activity in *D. melanogaster*. Data represent mean activity (pmol·min^{-1}·mg protein^{-1}) ± SD based on triplicate assays. n.d., No detectable activity greater than nonenzymatic hydration.

Substrate	Control	Clofibrate (10%)
CSO	130.0 ± 7.7	226.6 ± 4.8
TSO	2.6 ± 1.0	4.9 ± 1.3
tDPPO	n.d.	n.d.
JHIII	0.28 ± 0.22	0.38 ± 0.32

Table 2. Cellular distribution of induced EH activity. Whole larval bodies were homogenized after a 20-h treatment with 10% clofibrate. EH activities were assayed using CSO as a substrate, and data represent mean activity (pmol min^{-1} mg protein^{-1}) ± SD based on three different homogenates.

	Control	Clofibrate	Induction (fold)
Crude	95.5 ± 17.6	230.3 ± 30.2	2.4
100 000 ***g*** supernatant	103.9 ± 9.9	174.7 ± 12.6	1.7
100 000 ***g*** pellet	818.7 ± 30.8	1871.5 ± 175.6	2.3

Activities toward CSO were 130.0 and 226.6 pmol·min^{-1}·mg protein^{-1} in control and 10% clofibrate-treated larvae, respectively (Table 1). Little activity was detected toward TSO (2.6 and 4.9 pmol·min^{-1}·mg^{-1}) or JHIII (0.28 and 0.38 pmol·min^{-1}·mg^{-1}), and no activity toward tDPPO was detected in either control or clofibrate-treated larvae. Thus, the physiological change in the JHEH activity of the third instar larvae was negligible under our experimental conditions.

Localization of the increased EH activity

Whole larval bodies were treated with acetone or clofibrate, homogenized, and separated into soluble and microsomal fractions by ultracentrifugation. Each fraction was tested for EH activity. The fold induction of EH activities in crude, soluble, and microsomal fractions were 2.4, 1.7 and 2.3, respectively (Table 2), supporting a predominantly microsomal localization.

Induction of GST activity by clofibrate

The aliquots of the same crude larval homogenates were used for EH, GST, and CE assays, and the induction of each by clofibrate was compared (Fig. 4). GST activity was measured using two different methods, a radiometric assay with TSO as a substrate and a spectrophotometric assay with CDNB as a substrate. The same fold induction (1.4) was obtained in both GST assays, and was significant at the 5% level. CE activity was measured by spectrophotometry. Clofibrate increased CE activity in each experiment, but the increase was not significant at the 5% level, as compared with controls, based on four replications. Thus, GST activity was induced by clofibrate, but the level of induction was lower than that of EH activity.

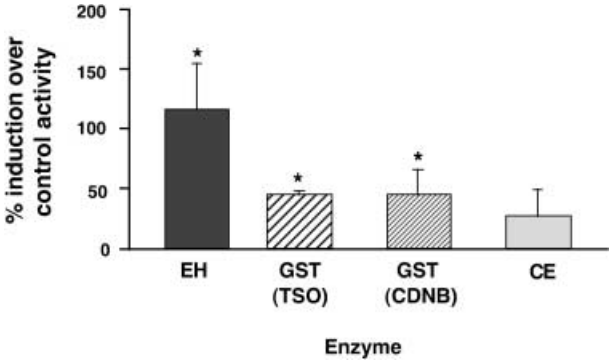

Fig. 4. Effect of clofibrate on three enzyme activities. Larvae were homogenized after a 20-h clofibrate administration, and aliquots of the same sample were used for four different assays. EH activity toward CSO and GST activity toward TSO were assayed with a partition method. GST activity toward CDNB and CE activity toward nitrophenyl acetate were assayed spectrophotometrically. Data represent the mean of induced activity (%) over each control activity. Bars indicate SDs based on three to five replications; stars denote significant induction.

Cloning of a *DmEH* gene

Based on its substrate selectivity and localization, the induced EH activity was speculated to be due to an mEH. Using PCR-based cDNA cloning, we isolated a cDNA clone (designated as *DmEH*) of 1597 bp containing an ORF that encoded 463 amino acids (Fig. 5). The deduced amino acid sequence contains the catalytic triad characteristic of mEHs and displays a high sequence similarity to four other mEHs (Fig. 6).

Expression of DmEH in baculovirus

We isolated four recombinant virus clones, rEH1, rEH2, rEH3 and rEH4, which were used to inoculate Sf9 cells. The cells were harvested after 72 h, and the cellular proteins were analysed in a 12% (w/v) gel. A distinct band of 43 kDa was observed in all four samples, and no band of this size was seen in the control sample taken from cells infected with nonrecombinant baculovirus (Fig. 7). We collected the cells infected with the rEH4 clone and the cell culture medium separately to assay for EH activity. The cells were homogenized and separated into cell debris, cytosol, and microsomal fractions. EH activity expressed in Sf9 cell culture was found in the cell debris (42 nmol·min^{-1}·mg protein^{-1}) and microsomal (23 nmol·min^{-1}·mg protein^{-1}) fractions. No activity was found in the medium or cytosolic fractions. There was no EH activity toward JHIII in any of the cell fractions.

Transcriptional activation of *DmEH* with clofibrate

Northern blot analysis revealed that transcription of *DmEH* in larvae was enhanced within 5 h of clofibrate treatment and then declined to the control level by 8 h post-treatment (Fig. 8). The results demonstrate that induction of EH activity occurred at the transcriptional level and that the induction was transient. We also analysed larval mRNA

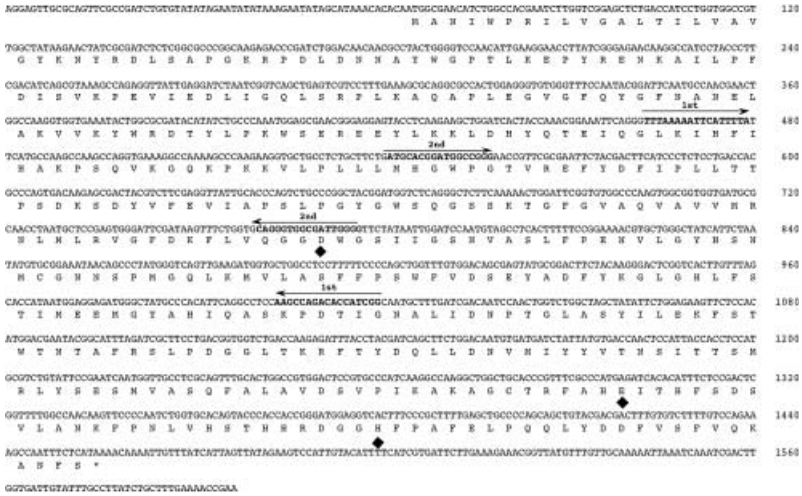

Fig. 5. Nucleotide and deduced amino acid sequences of the cloned *DmEH*. The oligonucleotide primers used in the PCR-based cDNA cloning are depicted in bold, and the arrows indicate orientation. Nucleotides numbers are shown to the right of the sequence, and the predicted amino acid sequence appears below. Black triangles signify the catalytic triad conserved in the microsomal EHs.

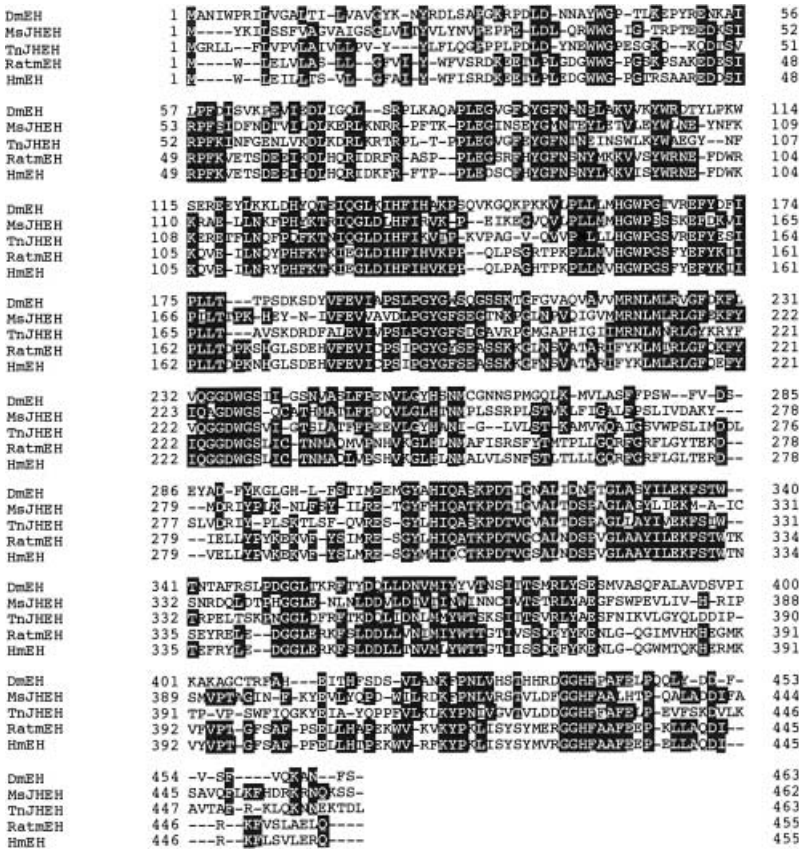

Fig. 6. Alignment of the five microsomal EH sequences. The deduced amino acid sequence of *DmEH* was compared with those of four other microsomal residential EHs. The abbreviations and accession number of each gene are: MsJHEH, *M. sexta* juvenile hormone EH (U46682); TnJHEH, *T. ni* juvenile hormone EH (U73680); RatmEH, rat microsomal EH (M15338); HmEH, human microsomal EH (BC008291). Black shadows indicate amino acids identical between at least three sequences. Dashes denote gaps.

after treatment with laminarin and found that the *DmEH* mRNA levels were equivalent at all post-treatment time points tested (1, 3, 5, 8 and 12 h) (data not shown). Therefore, the increase in EH activity induced by laminarin was produced via a mechanism different from that of clofibrate.

Discussion

We demonstrated that mEH activity was induced by clofibrate in *D. melanogaster* to a level similar to that induced by clofibrate in mice. In addition, we isolated one mEH-encoding gene (*DmEH*) from a cDNA library of clofibrate-treated larvae. Several experimental results suggest that this gene is responsible for the induced activity: *DmEH* expression was enhanced by clofibrate; recombinant DmEH was localized in the microsomal fraction; and the substrate selectivity of recombinant DmEH was similar to that of the induced mEH.

Recombinant DmEH with relatively high activity (42 nmol min^{-1}·mg $protein^{-1}$) was also detected in the 10 000 ***g*** pellet. The 10 000 ***g*** pellet should contain the

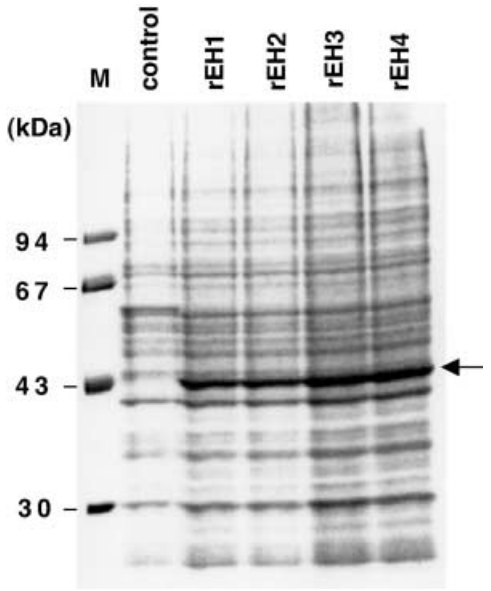

Fig. 7. SDS/PAGE of recombinant DmEH expressed in baculovirus. Sf9 cells infected with recombinant baculoviruses were harvested 3 days after infection. Cellular proteins were separated by SDS/PAGE on a 12% gel. rEH1, rEH2, eEH3, and rEH4 are recombinant virus clones. The arrow points to the expressed DmEH. Control, control baculovirus; M, molecular size markers.

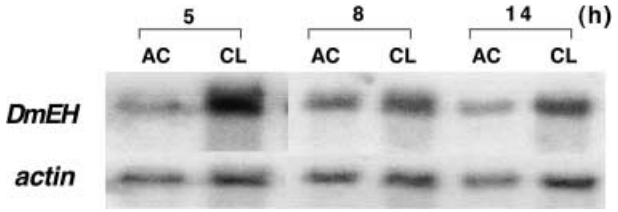

Fig. 8. Transcriptional induction of *DmEH* after treatment with clofibrate. Larvae were treated with 10% clofibrate and harvested at 5, 8 and 14 h post-treatment. The poly(A)-RNA was extracted from the larvae, and 300 ng mRNA of each sample was loaded on a gel. Actin mRNA served as an internal marker to equate mRNA quantities.

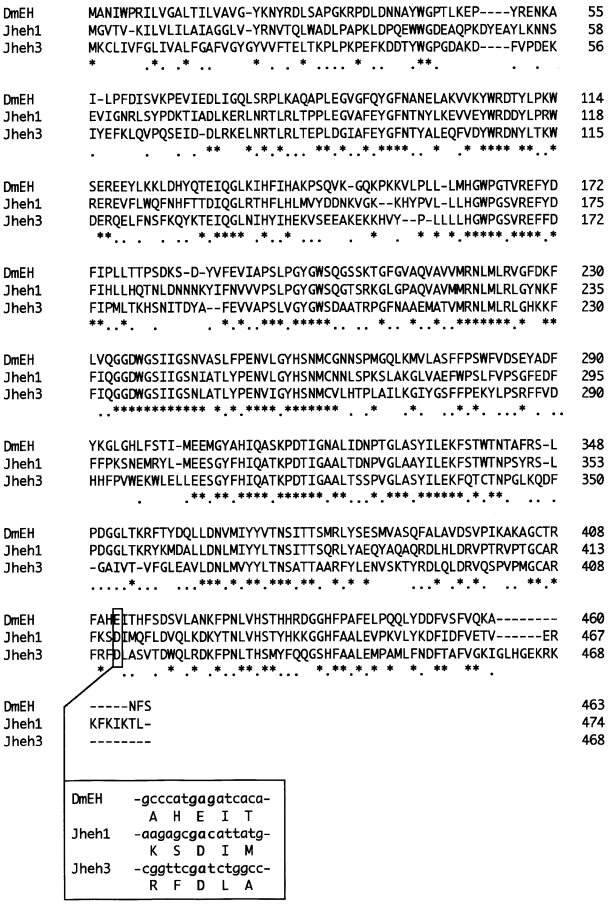

Fig. 9. Alignment of deduced amino acid sequences of DmEH and Jhehs. Asterisks signify amino acids identical among all three proteins; dots indicate amino acids identical between two proteins. The small box indicates the position of substituted amino acids within the mEH catalytic triad (Glu in DmEH; Asp in Jheh1 and Jheh3). The large box encloses the nucleotide sequences surrounding the substituted amino acids in the three genes.

nucleus, peroxisomes, mitochondria, and cell debris. The EH activity in this fraction might have been due to the presence of microsomes that were not completely homogenized. If DmEH distributed to sites other than microsome, it was probably localized to plasma and nuclear membranes, based on reports that mouse mEHs distribute to these membranes as well as to microsomes [32]. Recombinant DmEH was not detected in the soluble fraction of Sf9 cells, whereas clofibrate-inducible EH activity was seen in the soluble fraction of larval homogenates, although at a lower level than in microsomes (Table 2). The results suggest the existence of another clofibrate-inducible EH gene encoding an sEH with a substrate selectivity similar to that of DmEH.

The entire genomic sequence of *D. melanogaster* was unavailable when we began isolating this gene. After the genomic sequences became accessible, we searched for the map position of *DmEH* in the genome using a Flybase and found that *DmEH* is identical with *jheh2* at 55F8 on chromosome 2R. Only two nucleotide differences, which do not affect the deduced amino acid sequence, exist between the sequences of *DmEH* and *jheh2*, indicating that these two are the same gene. Three possible EH-encoding genes, designated *jheh1*, *jheh2*, and *jheh3* are located between 55F7 and 55F8 on chromosome 2R. The deduced amino acid sequences of all three genes were compared with those of two mammalian mEHs and two insect JHEHs (data not shown). All of the homology scores calculated using the Lipman–Peason method were similar (38.6–42.5% for mammalian mEHs and 40.2–45.1% for JHEHs). Because only JH-metabolizing mEH genes have been isolated from insects thus far, these genes were designated as *jheh*s in preliminary annotations. However, our results with the recombinant enzyme demonstrate that DmEH does not hydrolyse JHIII. Therefore, we propose that this gene be named *DmEH* (*D. melanogaster* microsomal epoxide hydrolase). The deduced amino acid sequence of *DmEH* possesses the

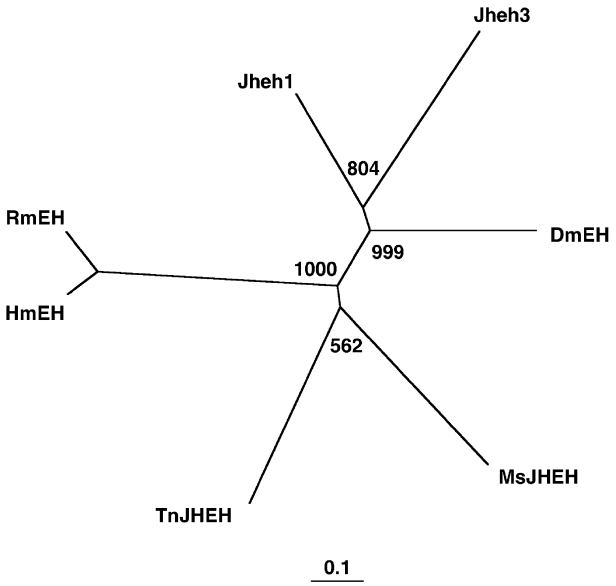

Fig. 10. Evolutional tree of seven mEHs. The CLUSTAL X program [39] was used to align amino acid sequences, and phylogenetic relationships were reconstructed using the Neighbor-Joining method [40]. Putative signal peptide sequences and gap positions were excluded because large differences in these regions can cause unreliable overestimations of distances. The numbers at the nodes are bootstrap probabilities estimated with 1000 replications. The scale bar represents relative evolutional distance.

conserved catalytic triad shared among all epoxide hydrolase of the alpha/beta hydrolase fold family (Asp237, Glu413, His430). However, in *jheh1* and *jheh3*, Glu is substituted with Asp at position 417 and 412, respectively. This one amino acid substitution is due to one nucleotide substitution at the third base of the amino acid codon in each gene (Fig. 9). The catalytic triad present in Jheh1 and Jheh3 (Asp-Asp-His) is more commonly seen in sEHs. Based on the phylogenic analysis of the deduced amino acid sequences of seven mEHs (Fig. 10), three of these (DmEH, Jheh1 and Jheh3) seem to be derived from a common ancestral gene via gene duplication that occurred after the divergence of Diptera and Lepidoptera. Therefore, it is possible that *jheh1* and *jheh3* are derived from *DmEH*. The tandem arrangement of the three genes along a short distance on the same chromosome in *D. melanogaster* (Fig. 11) supports this theory, although the relatively low level of amino acid sequence similarity among the three mEHs (Fig. 9) suggests the possibility of different substrate specificities and/or functions. Because JH-metabolizing mEH activity was detected in adult *D. melanogaster* [33,34], *jheh1* or *jheh3* might function as a JHEH. It will be interesting to determine whether Jheh1 and Jheh3 can metabolize JH, whether the genes are expressed differentially during development, and whether the genes are induced by xenobiotics or natural chemical mediators.

The activation of *DmEH* by clofibrate was rapid and transient, although the exact peak time of the expression was not precisely determined in this study. Similar rapid and transient activations of self-defence protein genes in insects occur when insects are infected with microorganisms [35], in which case antimicrobial peptide genes are activated within a few hours, and mRNA levels generally return to normal within a day. The rapid response through activation of defence protein genes, including those for detoxification enzymes and antimicrobial peptides against injurious

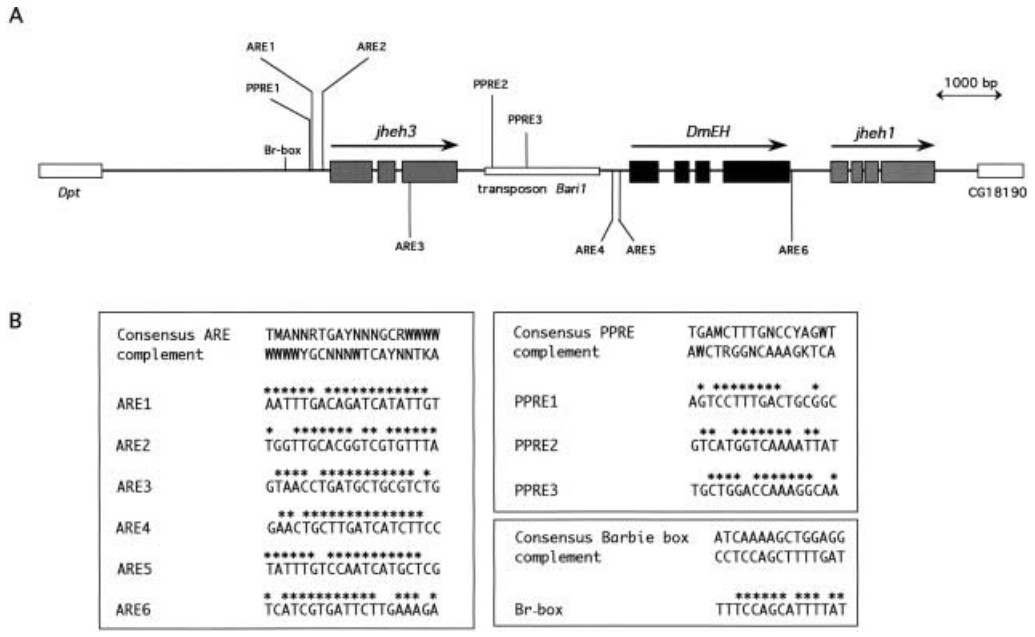

Fig. 11. *DmEH* and other two mEH genes on chromosome 2R. The orientation and structure of three mEH genes and the neighbouring genes on each side, nucleotides 222 627–236 435 in a genomic clone (accession no. AE003798), are depicted schematically. (A) The positions of putative xenobiotic response elements are shown. (B) A comparison of the putative *cis*-elements of *DmEH* with the consensus sequences of ARE, PPRE and Barbie box.

exogenous substances, is an important self-defence mechanism in insects.

The mechanisms by which xenobiotics activate mammalian mEH genes have not yet been elucidated. Several *cis*-acting xenobiotic-response elements have been characterized for other detoxification or β-oxidation enzyme genes, such as the antioxidant response element (ARE) in murine GST genes [36], the peroxisome proliferator response element (PPRE) in the rat acyl-CoA gene [37], and the Barbie-box in a bacterial p450 gene [38]. We found several similar sequences around *DmEH* and two other *jheh*s (Fig. 11). Based on the occurrence of multiple copies of PPRE- and ARE-like sequences in the promoter regions of these three EH genes, they are probably regulated by many different xenobiotics.

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