CYP-13A12 of the nematode *Caenorhabditis elegans* is a PUFA-epoxygenase involved in behavioural response to reoxygenation

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A specific behavioural response of *Caenorhabditis elegans*, the rapid increase of locomotion in response to anoxia/reoxygenation called the O2-ON response, has been used to model key aspects of ischaemia/reperfusion injury. A genetic suppressor screen demonstrated a direct causal role of CYP (cytochrome P450)-13A12 in this response and suggested that CYP-eicosanoids, which in mammals influence the contractility of cardiomyocytes and vascular smooth muscle cells, might function in *C. elegans* as specific regulators of the body muscle cell activity. In the present study we show that co-expression of CYP-13A12 with the NADPH-CYP-reductase EMB-8 in insect cells resulted in the reconstitution of an active microsomal mono-oxygenase system that metabolized EPA (eicosapentaenoic acid) and also AA (arachidonic acid) to specific sets of regioisomeric epoxy and hydroxy derivatives. The main products included 17,18-EEQ (17,18-epoxyeicosatetraenoic acid) from EPA and 14,15-EET (14,15-epoxyeicosatrienoic acid) from AA. Locomotion assays showed that the defective O2-ON response of C_{20}-PUFA (polyunsaturated fatty acid)-deficient, Δ^{-12} and Δ^{-6} fatty acid desaturase mutants (*fat-2* and *fat-3* respectively) can be restored by feeding the nematodes AA or EPA, but not ETYA (eicosatetraynoic acid), a non-metabolizable AA analogue. Short-term incubation with 17,18-EEQ was sufficient to rescue the impaired locomotion of the *fat-3* strain. The endogenous level of free 17,18-EEQ declined during anoxia and was rapidly restored in response to reoxygenation. On the basis of these results, we suggest that CYP-dependent eicosanoids such as 17,18-EEQ function as signalling molecules in the regulation of the O2-ON response in *C. elegans*. Remarkably, the exogenously administered 17,18-EEQ increased the locomotion activity under normoxic conditions and was effective not only with C_{20}-PUFA-deficient mutants, but to a lesser extent also with wild-type worms.

Key words: *Caenorhabditis elegans*, cytochrome P450, 17,18-epoxyeicosatetraenoic acid (17,18-EEQ), eicosanoid, polyunsaturated fatty acid, reoxygenation.

INTRODUCTION

Oxygen deprivation upon restriction of blood supply followed by reperfusion and concomitant reoxygenation causes tissue injury and is involved in the initiation of various human pathologies including ischaemic stroke, myocardial infarction and acute kidney injury [1]. The nematode *Caenorhabditis elegans* can survive at a wide range of oxygen concentrations, but exhibits an aerotaxis behaviour prompting the worms to migrate to their preferred oxygen concentration of approximately 10 % [2]. Upon anoxia/reoxygenation, *C. elegans* shows the so-called 'O2-ON' behavioural response that is characterized by a rapidly increased locomotion speed [3,4]. Recent studies suggest that evolutionarily conserved pathways contribute to ischaemia/reperfusion injury in mammalian cells and to the O2-ON response in *C. elegans* [5].

Mammals and *C. elegans* share the expression of an evolutionarily conserved family of O_2-dependent prolyl hydroxylases, EGLN2 (egg laying defect nine 2) and EGL (egg laying defect)-9 respectively. Prolyl hydroxylation regulates HIF (hypoxia-inducible factor) levels in *C. elegans* as well as in

mammals and thus links hypoxia to HIF-mediated physiological responses [6–10]. EGL-9 inactivation blocks the O2-ON response of *C. elegans* [3,4]. A screen for mutations restoring the defective O2-ON response in an EGL-9-deficient strain, *egl-9(n586)*, identified a gain-of-function allele of the *cyp-13A12* gene that encodes a CYP (cytochrome P450) enzyme [5]. *C. elegans* harbours several CYP genes that are homologous with mammalian CYP isoforms [11]. CYP3A4, a CYP enzyme predominantly expressed in the liver, but also in the brain and other extrahepatic tissues [12,13], is the most closely related human homologue of CYP-13A12 (32 % amino acid identity). Human CYP3A4 has been primarily known for its important role in liver microsomal drug metabolism, but also contributes to the metabolism of a wide variety of endogenous substrates, including the epoxidation of AA (arachidonic acid; $C_{20:4,\ n-6}$) and anandamide [14,15]. Further work also revealed the *emb-8* gene, encoding the worm's homologue of mammalian CPRs (NADPH-CYP reductases) [16], as essential for the O2-ON response [5]. Taken together, these results of genetic analysis indicated that a microsomal mono-oxygenase system composed of a CYP and CPR component is

involved in mediating the locomotion behaviour of *C. elegans*; however, the substrate specificity of this enzyme as well as the potential role of its metabolites in eliciting the O2-ON response remained unclear.

Suggesting an important role for C_{20}-PUFAs (polyunsaturated fatty acids) and/or their metabolites in the O2-ON response, Δ^{-12} and Δ^{-6} fatty acid desaturase mutants (*fat-2* and *fat-3* respectively) completely lack this behaviour [5]. EPA (eicosapentaenoic acid; $C_{20:5,\ n-3}$) and AA are the main PUFAs in the wild-type strain, whereas neither EPA nor AA can be synthesized in the desaturase-deficient mutants. Concomitantly, only the wild-type, but not the *fat-2* and *fat-3* mutants, contain EPA- and AA-derived epoxy and hydroxy metabolites as produced by CYP mono-oxygenases when metabolizing these C_{20}-PUFAs [17,18]. We reported previously that the EGL-9-deficient strain also displayed very low levels of free CYP-eicosanoids and that this deficiency was partially overcome by the gain-of-function mutation in the *cyp-13A12* gene [5].

On the basis of these findings, we hypothesized an essential role for C_{20}-PUFA-derived CYP-eicosanoids in mediating the O2-ON response and the locomotion behaviour of *C. elegans*. To address these questions, we cloned and heterologously co-expressed CYP-13A12 and EMB-8, analysed the substrate and reaction specificity of the recombinant mono-oxygenase system, and tested the effect of the major EPA-derived metabolite on the O2-ON response and locomotive activity of *C. elegans*.

EXPERIMENTAL

Nematode strain and cultivation condition

The *C. elegans* wild-type strain Bristol N2 and the mutant strains *fat-2(wa17)*, *fat-3(ok1126)* and *fat-3(wa22)* were used throughout the present study. The nematodes were grown at 20°C on NGM (nematode growth medium) agar plates inoculated with *Escherichia coli* OP50 as the food source [19] and were incubated under similar conditions as described previously [17].

Chemicals

Non-labelled AA, EPA, ETYA (eicosatetraynoic acid) and 17,18-EEQ (17,18-epoxyeicosatetraenoic acid) were purchased from Cayman Chemicals; all radiolabelled fatty acids were purchased from Hartman Analytical. The compound used as 17,18-EEQ agonist was synthesized as described previously [20]. To prevent auto-oxidation, all stock solutions were prepared in an oxygen evacuated nitrogen chamber. DMSO (Sigma) was used as vehicle.

Amplification and cloning of *cyp-13A12* and *emb-8* cDNAs

To perform reverse transcription, 10 μg of total RNA, isolated from a *C. elegans* culture according to [21], was mixed with 0.8 μl of oligo(dT) and incubated for 5 min at 70°C. Then, 3 μl of 5× RT-buffer, 0.8 μl of 10 mM dNTPs and 0.4 μl of MMLV (Moloney murine leukaemia virus) revertase (Promega) were added and incubated for 90 min at 42°C. The reaction was stopped by incubating at 94°C for 4 min. Subsequently, PCR amplification of the *cyp-13A12* and *emb-8* cDNAs were performed using the Phusion® Flash PCR Mastermix (New England Biolabs) and the following primer pairs: 5′-TAGGCTTACACGG-TCCGATCATGGCAATTATATTTCTTGC-3′ and 5′-CAGAA-GCTGCTCGAGTCAGTGATGATGGTGATGATTATCATCCC-TCGGCGTC-3′ for *cyp-13A12* and 5′-ATGCGTAGTAC-GGTCCGATCATGCTGGCGTGGATTGTGTC-3′ and 5′-CT-

ACGTTACCTCGAGTCAGTGATGATGATGATGGTGTGAC-CACACATCAGCTTGG-3′ for *emb-8*. Both reverse primers contained a His_6-encoding codon block directly in front of the stop codon to allow an immunological detection of all heterologously expressed proteins using a His_6 epitope tag antibody. The cDNAs obtained were cloned into the pFastBac™1 vector (Invitrogen), and RsrII and XhoI (New England Biolabs) were used as corresponding restriction enzymes. The identity of all cloned cDNAs was confirmed by full-length sequencing of both DNA strands, performed by LGC Genomics.

Generation of recombinant baculoviruses

Recombinant baculoviruses containing the cloned cDNAs under control of the strong polyhedrin promoter were produced using the Bac-to-Bac® baculovirus expression system from Invitrogen. After re-amplification in *Spodoptera frugiperda* Sf9 cells, a virus titre of approximately $1–3 \times 10^9$ was obtained.

cyp-13A12/emb-8 co-expression in insect cells and preparation of enzymatically active microsomes

Sf9 cells were grown in Insectomed SF express medium (Biochrom) supplemented with 50 units/ml penicillin, 50 μg/ml streptomycin and 10% heat-inactivated FBS. To generate a complete CYP-CPR mono-oxygenase system, Sf9 cultures were co-infected with recombinant baculoviruses of *cyp-13A12* and the *C. elegans* CPR gene *emb-8* after they reached a cell density of 2×10^6 cells/ml. Control groups were infected with an empty baculovirus or with either *cyp-13A12* or *emb-8* virus alone. At 24 h after infection, the Sf9 culture was supplemented with 5 μM haemin chloride and 100 μM riboflavin to support the production of CYP and CPR holoenzymes. The cells were harvested after 60 h, and resuspended in ice-cold 0.1 M potassium phosphate buffer, pH 7.4, containing 20% (v/v) glycerol, 1 mM EDTA, 100 μM PMSF and 0.5 mM DTT. The insect cells were homogenized by sonication three times for 20 s on ice. The microsomal fraction was prepared at 4°C through differential centrifugation: 5 min at 3000 *g*, 20 min at 10000 *g* and 65 min at 100000 *g*. Subsequently, the microsomes were resuspended and homogenized in 0.1 M potassium phosphate buffer, pH 7.4, containing 20% (v/v) glycerol, 1 mM EDTA and 0.5 mM DTT. Several aliquots were shock-frozen in liquid nitrogen and stored at −80°C.

The microsomal protein concentration was measured according to Lowry et al. [22]. The CYP content was determined by COD (carbon monoxide difference) spectra using a difference absorbance coefficient of 91 mM^{-1}·cm^{-1} for the wavelength pair 450 nm minus 490 nm [23]. The CPR content was estimated as NADPH-cytochrome *c* reductase activity using an absorbance coefficient at 550 nm of 21 mM^{-1}·cm^{-1} [24].

Western blot analysis

For Western blot analysis microsomal protein (3 μg per lane) was separated by 10% SDS/PAGE and transferred to Immobilon-P PVDF membrane (Millipore) [25]. A polyclonal anti-(His_6 epitope tag) antibody raised in rabbits (Novus Biologicals) was used as the primary antibody against recombinant tagged CYP-13A12 and EMB-8. The peroxidase-conjugated anti-rabbit secondary antibody was purchased from Sigma. The blots were developed with Amersham ECL prime Western blotting detection reagent and finally visualized by using Amersham Hyperfilm™ ECL (GE Healthcare).

Microsomal fatty acid metabolism

[1-^{14}C]-labelled AA (53.8 mCi/mmol) and EPA (53.9 mCi/mmol) (Hartman Analytical) had radiochemical purities of >99%. To analyse the whole range of primary and secondary metabolites produced by CYP-13A12, reactions were performed in 400 μl of 0.1 mM potassium phosphate buffer, pH 7.2, containing 50 pmol of recombinant CYP-13A12 and the substrate at a final concentration of 10 μM. At first, the appropriate volume of microsomal sample was pre-incubated with the substrate for 10 min at 25°C. Subsequently, the reaction was started with NADPH (1 mM final concentration) and terminated after 10 min of shaking at 25°C by adding 40 μl of 0.4 M citric acid on ice. The reaction products were extracted with ethyl acetate, evaporated under nitrogen and re-dissolved in 50 μl of 99.8% ethanol. Controls included omission of NADPH from the reaction mixtures and the use of microsomes lacking CYP-13A12 or EMB-8. The reactions were performed for each substrate at least in triplicate.

Analysis of the metabolite profiles

The reaction products formed from the [1-^{14}C]-labelled substrates were analysed by RP (reverse-phase)-HPLC (Shimadzu LC 10 Avp) using a Nucleosil 100-5C18HD column (250 mm×4 mm; Macherey-Nagel) and a linear solvent gradient of acetonitrile/water/acetic acid (50:50:0.1, by vol.) to acetonitrile/acetic acid (100:0.1, v/v) over 45 min at a flow rate of 1 ml/min. For detection and quantification of the metabolites, an online radio flow detector (LB 509; Berthold) was used. Authentic standard compounds were prepared and used as described previously [26,27]. The AA and EPA hydroxylase activities were derived from the sum of 19-/20-HETE (19-/20-hydroxyeicosatetraenoic acid) and 19-/20-HEPE (19-/20-hydroxyeicosapentaenoic acid) respectively, and total AA and EPA epoxygenase activities as the sum of all detectable EETs (epoxyeicosatrienoic acids) and EEQs respectively. All data were calculated per minute and nmol of CYP protein, as determined by the corresponding COD. The metabolite profiles were also analysed by LC-MS/MS using a triple quadrupole tandem mass spectrometer Agilent 6460 combined with an Agilent 1200 HPLC-system as described previously [28]. For these experiments, microsomal incubations were performed exactly as described above; however, unlabelled AA and EPA were used as substrates.

Preparation of assay plates and treatment

For long-term incubations, C_{20}-PUFA stocks were mixed with living OP50 bacteria and seeded on to NGM plates at a final concentration of 80 μmol in the bacterial lawn. Plates were dried in the dark at room temperature. Worms from a mixed culture were chunked to assay plates and incubated for a couple of days to ensure that the next generation was fed its whole life with dietary PUFA. Then, age-synchronized nematodes were obtained by bleaching gravid nematodes, disintegrating all of the worms, but leaving the eggs [29]. This synchronized progeny was incubated on freshly supplemented NGM/OP50 agar plates for three further days prior to use in the locomotion assay. Vehicle control experiments were included by mixing only solvent, 0.3% (v/v) DMSO, with the bacteria. Only in the case of the multi-worm tracker experiments were C_{20}-PUFA salts dissolved in ethanol at 1 mg/ml and 50 μl were spread evenly on to NGM plates before drying briefly and cultivating *E. coli* OP50 on to the plates.

For short-term incubations, two synchronized young adults (>15 trials per strain) were spread on to NGM/OP50 plates supplemented with either 10 μM of EPA, 17,18-EEQ, a 17,18-EEQ agonist, or only 0.3% (v/v) DMSO as a vehicle control. In this case, time of treatment was limited to 40 min.

Locomotive behaviour

The locomotive behaviour was measured by two different methods based on experience and equipment of the involved laboratories. The O2-ON response of young adult *fat-2(wa17)* (*n* > 50 for each treatment) was continuously measured using a multi-worm tracker with a gas-flow chamber system and quantified by customized MatLab algorithms as described previously [4]. For characterizing the locomotive behaviour of wild-type and *fat-3(wa22)*, we determined the worm's speed and body bend frequency according to Hart [30]. At least 30 worms per treatment were examined for each replicate by using a VHX-600 digital microscope (Keyence). To determine the move length, two nematodes per trial were transferred on to a fresh plate and the length of the 20 s crawler lane left in the OP50 lawn was measured for each individual. In addition, body bends as change in the direction of *C. elegans* movement were counted. A binocular-capable custom-built acrylic glass chamber equipped with an oxygen manometer (DO-100 from Voltcraft) and a gas-flow system of pure N_2 was used for experiments involving anoxia conditions. Reoxygenation was achieved by setting open plates back to ambient air for 2 min.

Determination of the endogenous EPA and 17,18-EEQ content

The amount of free EPA and the CYP-derived metabolite 17,18-EEQ was determined by LC-MS/MS for the wild-type under normoxia, anoxia (5 min) and reoxygenation (2 min) conditions. To ensure anoxia conditions during the process of harvest as long as possible, liquid nitrogen was carefully filled at the bottom of the anoxia chamber, but without freezing the agar inside the Petri dishes. Then nematodes were washed with ice-cold M9 buffer from the plates and prepared for LC-MS/MS analysis essentially as described previously [17,18].

Statistical analysis

The locomotion assay and the endogenous EPA and 17,18-EEQ datasets were analysed by one-way ANOVA to test for significant differences between treatments followed by the Bonferroni test to identify treatments that were significantly different from the control. All statistical tests were performed using Sigma Stat 3.5 (Systat Software). The error bars in the Figures are + S.D.

RESULTS

Cloning and heterologous expression of *cyp-13A12* and *emb-8*

The coding sequences of *emb-8* and *cyp-13A12* were amplified from *C. elegans* N2 and showed 100% identity with the corresponding mRNA sequences (NM_065702.5 and NM_067304.3) as available from NCBI Nucleotide. Heterologous expression of both components was successfully achieved in a baculovirus/Sf9 insect cell system. Microsomes isolated from insect cells transfected with the recombinant *cyp-13A12* baculovirus displayed reduced COD spectra as characteristic for CYP proteins (Figure 1A). The Soret peak at 450 nm indicated that the majority of the recombinant CYP protein retained the haem-thiolate co-ordination in the ferrous–CO complex. Only a very small amount of a P420 form, which could reveal an inactive form of the enzyme, was detectable. A maximal expression
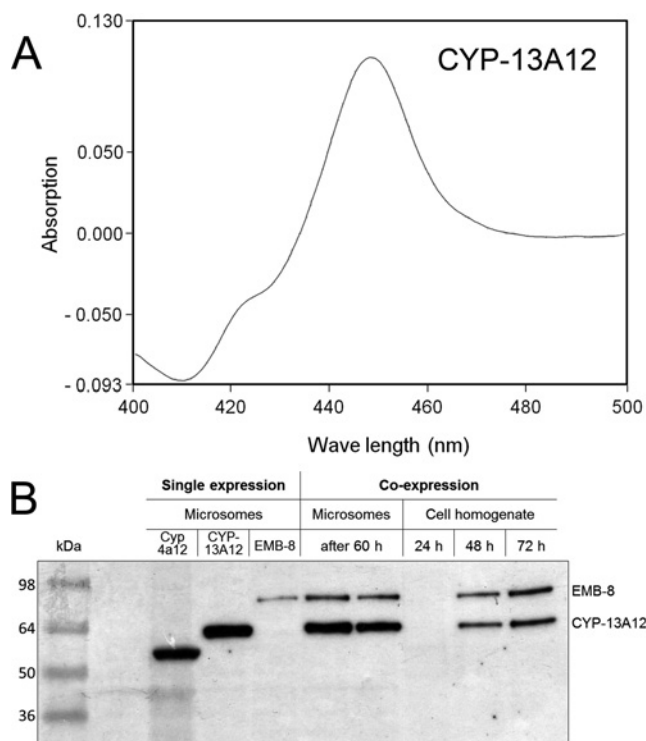
Figure 1   Detection of recombinant CYP-13A12 and CPR

(**A**) COD spectra of sodium dithionite-reduced microsomal samples isolated from 60 h old Sf9 insect cell cultures, expressing CYP-13A12 (0.59 nmol/mg of protein). (**B**) Representative Western blot analysis showing recombinant His-tagged CYP-13A12 and EMB-8 expressed alone and together. Shown are microsomal preparations and three corresponding homogenate samples isolated at the indicated times. Each lane contains 3 μg of protein. Besides the marker, recombinant His-tagged CYP4A12 served as a control. A anti-(His$_6$ epitope tag) antibody was used as a primary antibody.

level of 0.59 nmol of CYP-13A12 per mg of microsomal protein was obtained 60 h after infection. Control microsomes, isolated after transfection with an empty baculovirus, were free of any spectrally detectable CYP protein (results not shown). Microsomes containing recombinant EMB-8 showed NADPH-dependent reduction of cytochrome c with a reductase activity of 43.3 m-units/ mg of protein.

For metabolic studies, the required co-expression of *cyp-13A12* with *emb-8* was achieved by co-infection of Sf9 cells with both recombinant baculoviruses. The microsomes obtained from these co-infected cells contained 0.34 nmol of spectrally active CYP-13A12/mg of protein and 53.1 m-units of EMB-8/mg of protein. Successful co-expression of both the His-tagged recombinant CYP-13A12 and EMB-8 was also confirmed by Western blotting. The expression of both components became visible 48 h post-infection and reached a maximum after 60 h (Figure 1B). The samples showed the presence of a prominent 58 kDa protein band for CYP-13A12 and a band at 79 kDa for EMB-8, both enriched in the microsomal fraction (Figure 1B).

Metabolism of AA by recombinant CYP-13A12/EMB-8

The recombinant microsomal CYP-13A12/EMB-8 system metabolized AA to a complex product pattern predominantly consisting of epoxy and hydroxy metabolites (Figure 2A). AA was metabolized only in the presence of NADPH and none of the metabolites occurred in incubations with control microsomes

lacking CYP-13A12 or EMB-8. The main primary epoxy metabolite produced by CYP-13A12 was 14,15-EET, followed by 11,12-EET. The main primary hydroxy metabolites produced by CYP-13A12 co-migrated with 19-/20-HETE. A group of minor reaction products eluted in the RP-HPLC between 19-/20-HETE and 14,15-EET, indicating the formation of monohydroxy derivatives originating from CYP-13A12-catalysed mid-chain oxidations.

LC-MS/MS analysis was used to validate the identity of the obtained metabolites (Figure 2B). The primary epoxy products were confirmed as 14,15-EET (47.4 % of total products) and 11,12-EET (13.9 % of total products). In addition, low amounts of 8,9-EET (1.0 % of total products) were detectable. 19-HETE (24.5 % of total products) was identified as the main primary monohydroxy product. In addition, minor amounts of 15-HETE, 12-HETE and 11-HETE (together 4.2 % of total products) were identified, but none of the other potential mid-chain HETEs were produced (Figure 2B). The ratio of epoxy to monohydroxy products was approximately 2:1. Moreover, small amounts of 14,15- and 11,12-HEET (hydroxyepoxyeicosatrienoic acid) were detectable that were presumably formed as secondary metabolites, e.g. by secondary ω-hydroxylation of the primary EETs. At a substrate concentration of 10 μM, AA was metabolized by the CYP-13A12/EMB-8 mono-oxygenase system with a rate of 0.43 nmol/nmol per min to the primary epoxy metabolites and with a rate of 0.26 nmol/nmol per min to primary hydroxy metabolites.
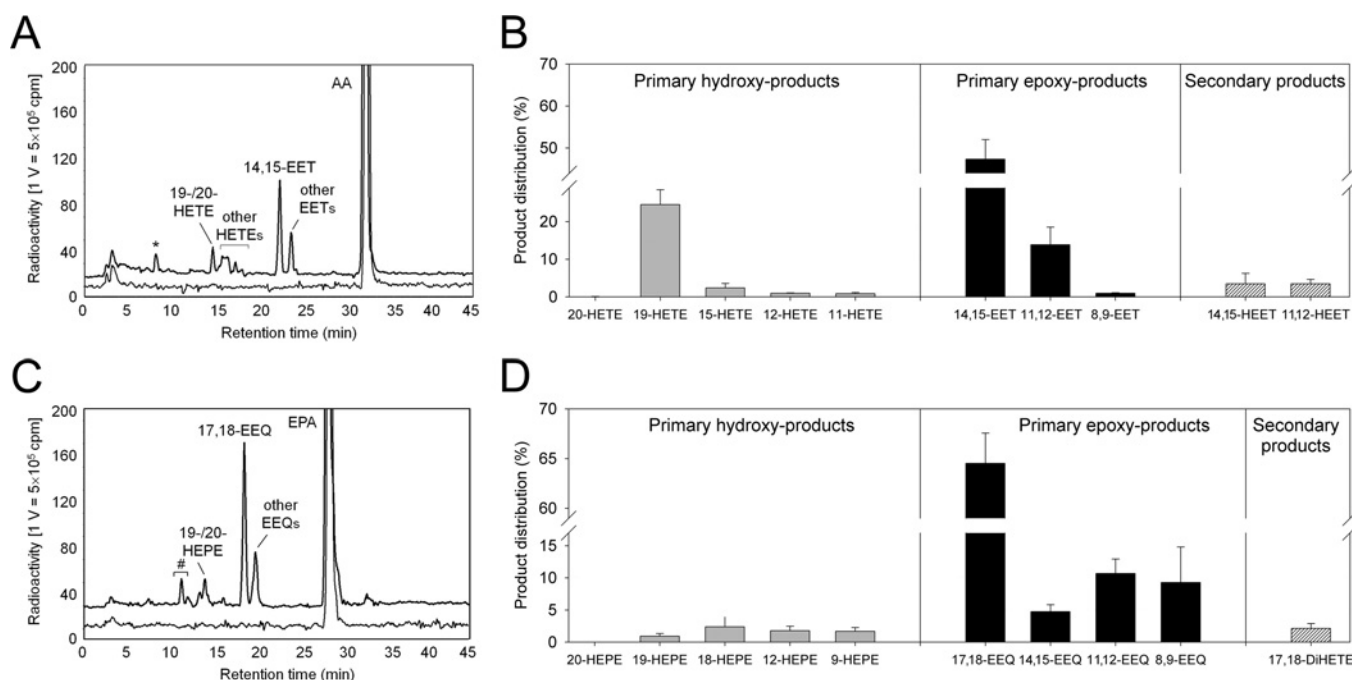
Metabolism of EPA by recombinant CYP-13A12/EMB-8

The CYP-13A12/EMB-8 system also accepted EPA as a substrate and converted this $n-3$ PUFA to a set of epoxy as well as monohydroxy metabolites. 17,18-EEQ represented the main primary epoxy metabolite, whereas the main primary monohydroxy product co-migrated with 19-/20-HEPE (Figure 2C). No product formation occurred in control experiments, when either NADPH was omitted or microsomes lacking CYP-13A12 or EMB-8 were used.

The identities of the EPA metabolites produced by CYP-13A12 were also confirmed by LC-MS/MS analysis (Figure 2D). The main primary epoxy products were identified as 17,18-EEQ (64.5 % of total product) and 11,12-EEQ (10.7 % of total product; Figure 2D). Also including 14,15-EEQ and 8,9-EEQ, the epoxy metabolites represented 89.2 % of the total products. As the main primary monohydroxy product, 18-HEPE was approximately 2.4 % of total products; 20-, 19-, 15-, 12-, 9-, 8- and 5-HEPE represented approximately 5.6 % of the total products (Figure 2D). The ratio of epoxy to monohydroxy metabolites was approximately 11:1. In addition, 17,18-DiHETE (17,18-dihydroxyeicosatetraenoic acid) originating from the hydrolysis of 17,18-EEQ was present in minor amounts (2.1 % of total product). Formation of total primary metabolites from EPA occurred with a rate of 0.45 nmol/nmol per min for epoxy metabolites and 0.06 nmol/nmol per min for hydroxy metabolites.

A C$_{20}$-PUFA-metabolite is required for the normal O2-ON response

In line with previous studies [5], the O2-ON response was well pronounced in the *C. elegans* N2 wild-type strain, but did not occur in the C$_{20}$-PUFA-deficient *fat-2(wa17)* and *fat-3(ok1126)* mutants (Figures 3A–3C). As shown for the *fat-2(wa17)* mutant, this phenotypic impairment was rescued after feeding the worms AA or EPA for several days using the long-term incubation protocol with 80 μM C$_{20}$-PUFAs as described in the Experimental section

Figure 2   Metabolism of AA and EPA by recombinant CYP-13A12

(**A**) Representative RP-HPLC chromatogram showing the metabolite pattern formed after incubating 10 nmol of AA with 50 pmol of CYP-13A12 in a total volume of 400 μl for 10 min at 25°C in the presence of 1 mM NADPH (top line). None of the metabolites were produced in control samples, where NADPH was omitted (bottom line). The main metabolites co-migrated with authentic 19-/20-HETE (retention time = 15.4 min) and a set of regioisomeric monoepoxides at 23.5 min (14,15-EET) and, e.g. 24.9 min (11,12-EET). Metabolites with uncertain or unknown identity are marked (*). (**B**) The metabolite pattern was analysed by LC-MS/MS analyses ($n = 3$). The product distribution indicates the relative contribution of the respective individual metabolites as a percentage; moreover the metabolite class is indicated. Error bars indicate $+$S.D. (**C**) Representative RP-HPLC chromatogram showing the metabolite pattern formed after incubating 10 nmol of EPA with 50 pmol of CYP-13A12 in a total volume of 400 μl for 10 min at 25°C in the presence of 1 mM NADPH (top line). None of the metabolites were produced in control samples where NADPH was omitted (bottom line). The main metabolites co-migrated with authentic 19-/20-HEPE (retention time = 13.9 min), 17,18-EEQ (retention time = 19.5 min) and a set of other regioisomeric EEQs (retention time = 21–23 min). Metabolites with uncertain or unknown identity are marked (#). (**D**) The metabolite patterns were analysed by LC-MS/MS analyses ($n = 3$). The product distribution indicates the relative contribution of the respective individual metabolites as a percentage, moreover the metabolite class is indicated. The error bars are $+$S.D.

(Figures 3D and 3E). In contrast, ETYA, a non-metabolizable AA-analogue, failed to restore the O2-ON response under otherwise identical conditions (Figure 3F).
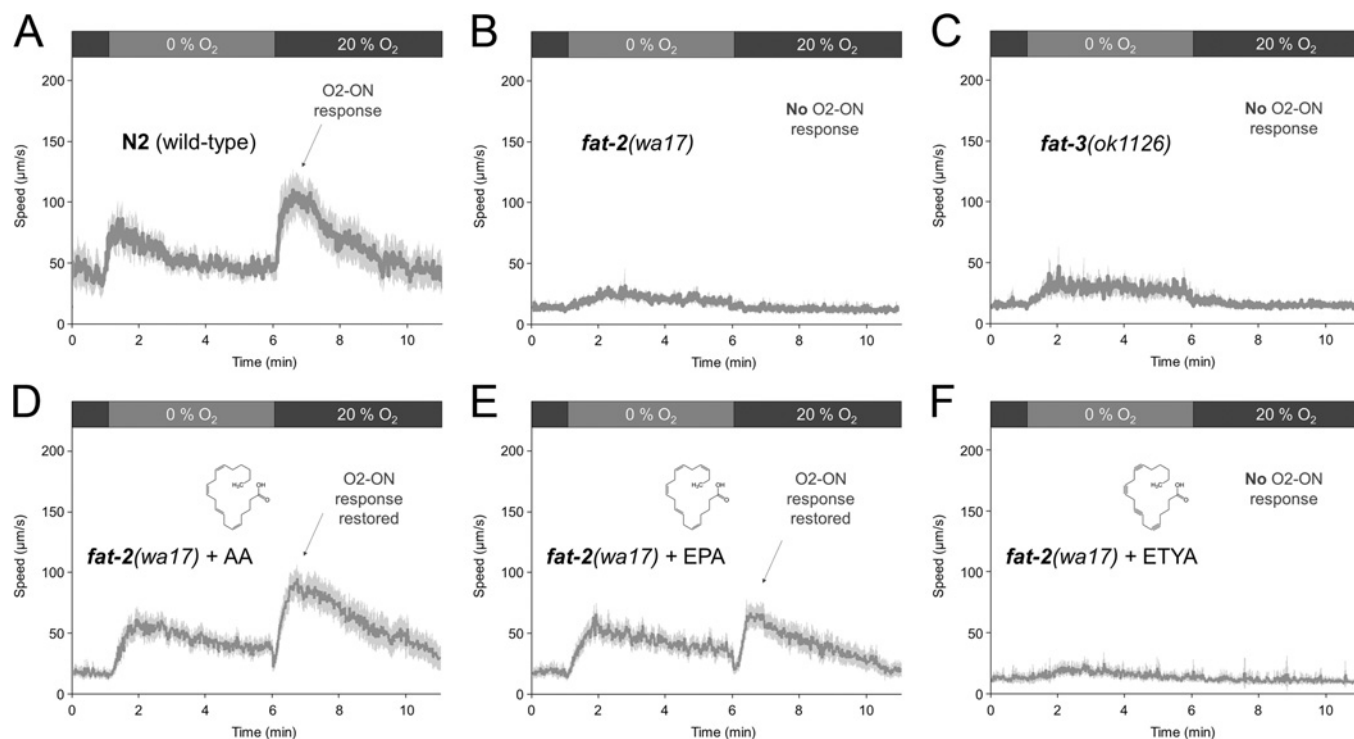
The normal O2-ON response of the wild-type strain (Figure 4A) was accompanied by significant changes in the endogenous levels of free EPA (Figure 4C) and its CYP-dependent metabolite 17,18-EEQ (Figure 4D) as revealed by LC-MS/MS analysis of worms harvested during normoxia, 5 min after anoxia, and 2 min after reoxygenation. Free EPA levels were already slightly higher after anoxia compared with normoxia and then increased almost 5-fold in the immediate reoxygenation phase. Free 17,18-EEQ levels declined during anoxia and were then rapidly restored in response to reoxygenation. Similar, but less pronounced, changes were observed regarding the release of free AA and the formation of its CYP-dependent metabolite 14,15-EET. However, the maximal levels of free AA and 14,15-EET reached only approximately 30 and 10% of the corresponding EPA and 17,18-EEQ levels (results not shown).

To test the hypothesis that 17,18-EEQ is required for the O2-ON response, we pretreated worms carrying the *fat-3(wa22)* mutation with this EPA-derived epoxy metabolite. In the presence of 10 μM 17,18-EEQ, we observed an almost 2-fold increase in the nematode's speed and number of body bends after a 5 min/2 min anoxia/reoxygenation event, compared with the non-treated control group (Figure 4B, right panel). However, the exogenously applied 17,18-EEQ was already effective during normoxia (Figure 4B, left panel), indicating that 17,18-EEQ has the capacity to increase the locomotion speed independently of anoxia/reoxygenation as shown in detail below.

17,18-EEQ increases the locomotive activity of *C. elegans*

In the last part of the present study, we compared the effects of C_{20}-PUFA- and 17,18-EEQ- supplementation on the locomotion behaviour of the *fat-3(wa22)* mutant and N2 wild-type strains; the chemical structures of compounds used for treatment are shown in Figure 5(A). In these experiments, we took advantage of the fact that C_{20}-PUFA deficiency does not only abolish the O2-ON response, but also impairs locomotion under normoxic conditions. Compared with their wild-type counterparts, untreated *fat-3(wa22)* worms showed an almost 50% reduction of migration speed and number of body bends per minute when kept on agar plates in ambient air (Figures 5B and 5C, left panels). The impaired locomotion behaviour of the C_{20}-PUFA-deficient *fat-3(wa22)* mutant strain was largely improved upon long-term incubation (3 days, 80 μM) with exogenous EPA or AA (Figure 5B, central panel). In contrast, neither EPA nor AA supplementation increased the normal locomotion activity of the N2 wild-type strain that is self-sufficient in producing C_{20}-PUFAs (Figure 5C, central panel). ETYA, the non-metabolizable AA analogue, significantly reduced the speed and body bending of wild-type worms, but did not rescue nor further decrease the impaired locomotion activity of the *fat-3(wa22)* mutant strain (Figures 5B and 5C, left panels).

Figure 3   A metabolizable PUFA is required for the normal O2-ON response

(**A**) Speed graph of N2 wild-type showing an intact O2-ON response, whereas (**B**) *fat-2(wa17)* and (**C**) *fat-3(ok1126)* mutants show in each case a defective O2-ON response. Average speed values ± 2 S.E.M. (light grey) of animals ($n > 50$) are shown with step changes of O_2 between 20 % and 0 % at the indicated times. (**D**) Speed graph of *fat-2(wa17)* mutants with the O2-ON response rescued by AA supplementation, and (**E**) speed graph of *fat-2(wa17)* mutants with the O2-ON response rescued by EPA supplementation. (**F**) Speed graph of *fat-2(wa17)* mutants with the defective O2-ON response not rescued by ETYA supplementation. Only with wild-type as well as AA and EPA exposure of *fat-2(wa17)*, the mean speed within 0–120 s after O_2 restoration is increased relative to that before O_2 restoration ($P < 0.01$, one-sided unpaired Student's t test).

17,18-EEQ clearly rescued the *fat-3(wa22)* mutant strain from locomotion impairment when administered for 40 min at a concentration of 10 μM (Figure 5B, right panel). In contrast, the same treatment regime was not sufficient for achieving any effect with EPA, the precursor of 17,18-EEQ. Remarkably, however, the short-term exposure to 17,18-EEQ was almost as effective as long-term EPA or AA feeding. Similar to 17,18-EEQ, a synthetic 17,18-EEQ agonist also accelerated the migration of the worms (Figure 5B, right panel). Complementing studies with the N2 strain revealed that wild-type worms also respond with increased locomotion activity when exposed to 17,18-EEQ or its synthetic agonist (Figure 5C, right panel).

DISCUSSION

The present study demonstrates that CYP-13A12 and EMB-8 constitute a microsomal mono-oxygenase system that metabolizes C_{20}-PUFAs and thereby generates signalling molecules regulating the locomotion behaviour of *C. elegans*. The identity of the mono-oxygenase system and its function are in line with and directly explain previous genetic data implicating the *cyp-13A12* and *emb-8* genes as well as C_{20}-PUFAs into the O2-ON response of *C. elegans* [5].

The recombinant CYP13A12/EMB8 mono-oxygenase system functioned predominantly as an epoxygenase when metabolizing AA and EPA. The main product produced from AA was 14,15-EET, whereas EPA was preferentially epoxidized at its (ω-3) double bond to yield 17,18-EEQ as the main metabolite. Moreover, CYP-13A12 showed (ω-1)-hydroxylase activity with

AA, and also catalysed mid-chain oxidations resulting in a series of regioisomeric hydroxy metabolites as minor products. The metabolite patterns of CYP-13A12 resemble those produced by CYP-33E2 from *C. elegans* [18]; CYP2J2, an epoxygenase highly expressed in the human heart [31,32]; and also human CYP3A4 [14]. CYP-13A12, CYP-33E2 and CYP2J2 have in common that they prefer EPA over AA as substrate and show a regioselectivity in favour of 17,18-EEQ as the main EPA-derived metabolite. Compared with CYP-13A12, CYP3A4 displays a lower regioselectivity and produces considerably higher amounts of various mid-chain oxidation products [33]. Among the human counterparts of CYP-13A12, CYP2J2 has attracted particular interest for its protective role in ischaemia/reperfusion injury. CYP2J2 overexpression in transgenic mice mediates improved post-ischaemic functional recovery of the heart and reduces infarction size [34,35]. CYP2J2 overexpression also protects against hypoxia reoxygenation injury in cultured endothelial cells [36]. Most recently, CYP2J2 was shown to exert marked neuroprotective effects in a mouse model of cerebral ischaemia [37].

EPA and AA are the main C_{20}-PUFAs in *C. elegans*, where EPA represents approximately 30 % and AA approximately 5 % of total fatty acids [17]. As exemplified in the present study by the *fat-2* and *fat-3* mutant strains, any inability to synthesize C_{20}-PUFAs is associated with impaired locomotion activity under normoxic conditions and a lack of the O2-ON response to anoxia/reoxygenation. Long-term feeding with EPA or AA, but not with ETYA, rescued this impaired locomotion behaviour. ETYA harbours triple instead of double bonds [38] and has been used as a non-metabolizable analogue of AA and
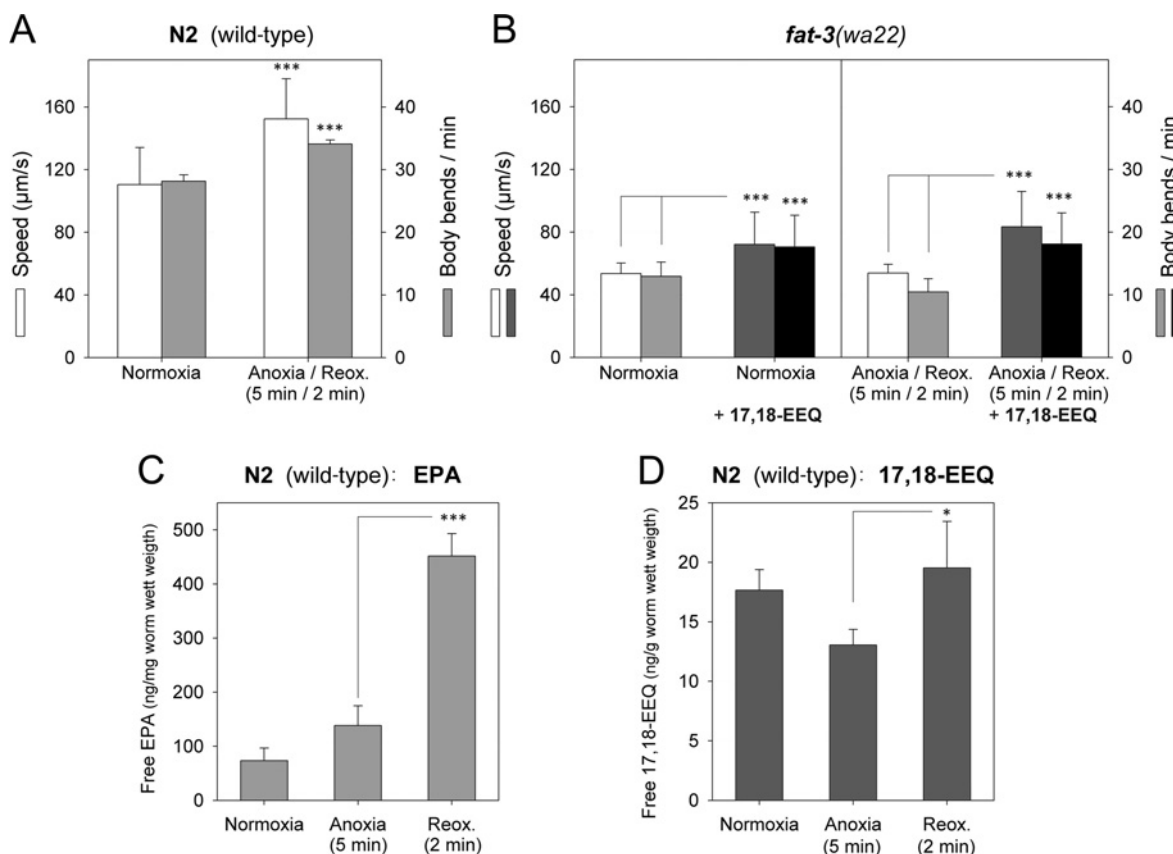
Figure 4   Impact of 17,18-EEQ on the O2-ON response

(**A**) Nematode speed and the number of body bends increases in the wild-type in response to a 5 min anoxia/2 min reoxygenation stimulus. (**B**) Supplementation with 10 μM 17,18-EEQ restored the locomotion of *fat-3(wa22)* mutants both in anoxia/reoxygenation and normoxia conditions. (**A** and **B**) $n \geqslant 30$ ($\geqslant 15$ trials with $n = 2$), $+$S.D. (***$P < 0.001$, one-way ANOVA). (**C**) An anoxia/reoxygenation stimulus caused a strong increase in the amount of free EPA in the wild-type. (**D**) The free 17,18-EEQ content is reduced during anoxia, but significantly restored in response to reoxygenation. (**C** and **D**) LC-MS/MS analysis of in each case 5000 synchronized worms (24 h post-L4), $n = 3$, $+$S.D. (*$P < 0.05$ and ***$P < 0.001$, one-way ANOVA).

an inhibitor of AA-derived eicosanoid formation [39–41]. It is noteworthy that C_{20}-PUFAs are also essential for touch sensation of *C. elegans*. Unlike impaired locomotion, this phenotype can be rescued both by AA and its non-metabolizable analogue ETYA [42]. These findings suggested that C_{20}-PUFAs modulate touch sensation while being incorporated into membrane phospholipids [42], whereas EPA- and AA-derived metabolites, rather than the parental C_{20}-PUFAs themselves, regulate the locomotion activity of the nematodes.

Indeed, our subsequent experiments revealed that 17,18-EEQ, the major EPA-derived metabolite produced by the CYP-13A12/EMB-8 mono-oxygenase system, was itself sufficient to improve the impaired locomotion activity of the C_{20}-PUFA-deficient *fat-3(wa22)* strain. This effect occurred even after short-term exposure of the worms to 10 μM 17,18-EEQ, whereas long-term feeding and much higher concentrations were required to achieve similar effects with AA or EPA. Also, other authors found that high concentrations and a minimal time of approximately 24 h are required to restore the phenotype of *fat-3* mutants by C_{20}-PUFA supplementation [43–48]. Remarkably, the exogenously administered 17,18-EEQ increased the locomotion activity even under normoxic conditions and was effective not only with the mutant, but to a lesser extent also with wild-type worms. According to our LC-MS/MS data, 17,18-EEQ is also the leading candidate for mediating the O2-ON response of the wild-type strain. These data show that free 17,18-EEQ levels decline

during anoxia and are rapidly restored upon reoxygenation. Providing the substrate for increased *de novo* biosynthesis of 17,18-EEQ, free EPA levels were strongly increased upon anoxia/reoxygenation. Taken together, these results suggest a direct correlation between the free 17,18-EEQ levels and the locomotive activity of *C. elegans*. This correlation also explains how exogenous 17,18-EEQ was also effective under normoxic conditions, whereas the O2-ON response is obviously mediated by enhanced endogenous 17,18-EEQ biosynthesis following anoxia/reoxygenation. We cannot exclude the possibility that other EPA- and also AA-derived metabolites share the locomotion-promoting capacity of 17,18-EEQ. In particular, AA-derived metabolites such as 14,15-EET may take over the function of 17,18-EEQ, if EPA is not available as suggested by the normal locomotion behaviour of *fat-1* mutants that are unable to synthesize $n-3$ PUFAs [49]. Moreover, considering that the substrate and reaction specificities of CYP-13A12 and CYP-33E2 largely overlap, further genetic analysis is required to dissect the individual roles of these enzymes in the O2-ON response.

The postulated role of 17,18-EEQ as a mediator of the O2-ON response in *C. elegans* (Figure 6) is in line with the general concept of CYP-eicosanoid formation and action as established in mammalian cells [50–53]. CYP enzymes require molecular oxygen, NADPH and free C_{20}-PUFAs to catalyse epoxidation and hydroxylation reactions [54]. Accordingly, the biosynthesis of epoxy and hydroxy metabolites proceeds under normoxic
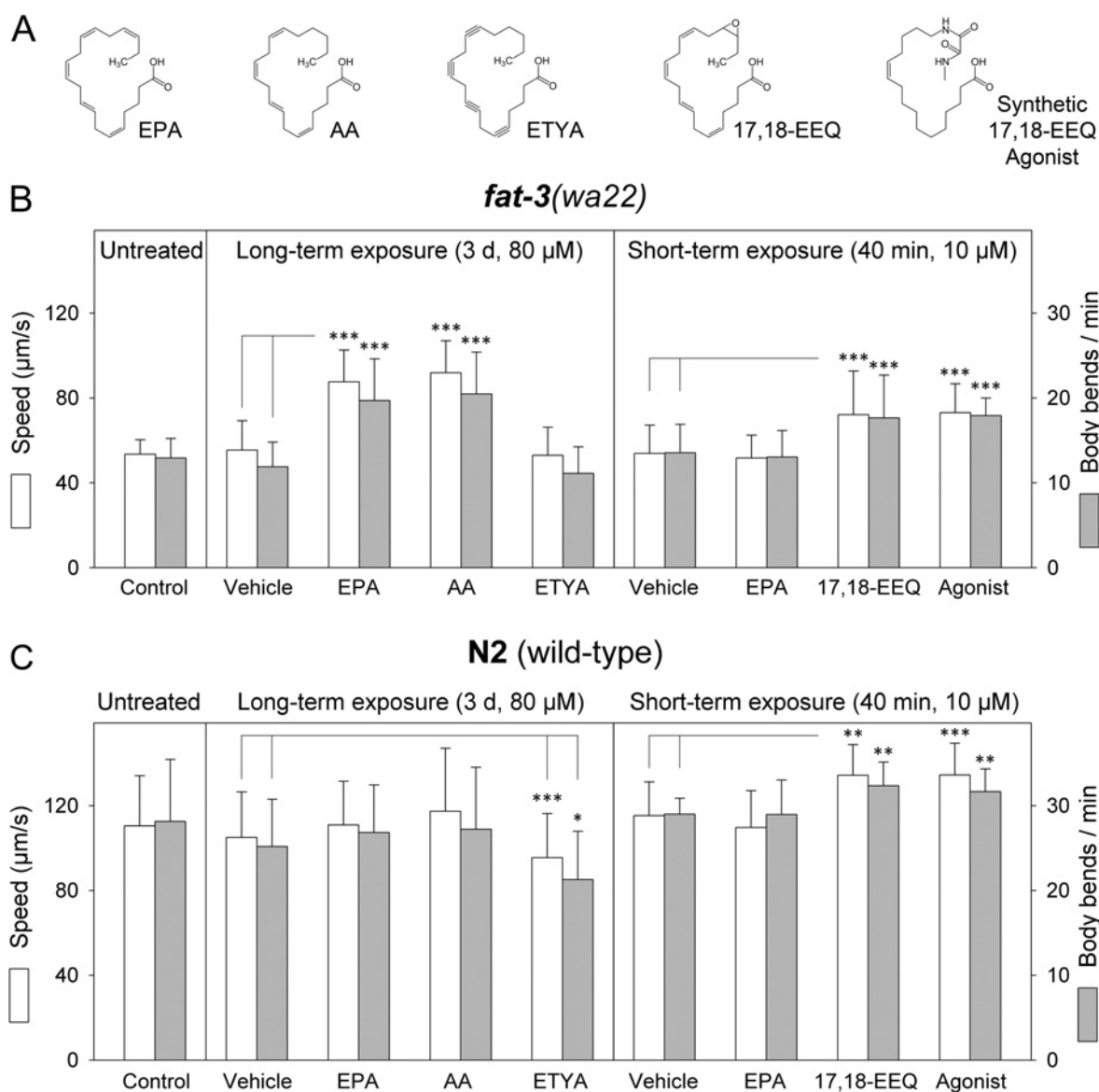
**Figure 5   17,18-EEQ rescues _fat-3(wa22)_ mutants from movement restriction under normoxia**

(**A**) Chemical structures of compounds used for treatment. (**B**) Long-term exposure with 80 μM EPA and AA, but not ETYA, a non-metabolizable AA analogue, rescued the impaired locomotion of the _fat-3(wa22)_ strain, detected under normoxic conditions. Even after addition for only 40 min (short-term exposure), 10 μM 17,18-EEQ significantly increased the locomotion of _C. elegans_ young _fat-3(wa22)_ adults and (**C**) in N2 wild-type. Note also the impairment in locomotive behaviour in response to ETYA treatment of the wild-type. Vehicle: 0.3 % DMSO; $n \geqslant 30$ (3–5 trials with $n = 10$), + S.D. (*$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$, one-way ANOVA).

conditions and is limited by the availability of free EPA and AA as substrates. Under basal conditions, the majority of intracellular EPA and AA are esterified into membrane phospholipids. Thus _de novo_ synthesis of CYP-eicosanoids is in general strictly coupled to the activation of phospholipases as triggered by diverse hormones and growth factors [51]. Importantly, hypoxia also results in phospholipase activation and thus provides free C_{20}-PUFAs for CYP-dependent metabolite formation in the subsequent reoxygenation phase. In mammalian cells, ischaemia activates the cytosolic calcium-dependent phospholipase A_2 that liberates AA and other PUFAs from the _sn_-2 position of glycerophospholipids [55–57]; however, the identity and substrate specificity of the phospholipases activated in the nematode during anoxia/reoxygenation remain to be elucidated. Once produced, the epoxides of C_{20}-PUFAs can be rapidly further metabolized

and inactivated by soluble epoxide hydrolases [58]. _C. elegans_ harbours two genes encoding soluble epoxide hydrolases [59]; however, their role in modulating the locomotive activity remains to be shown.

The cellular and molecular mechanisms of how 17,18-EEQ increases the locomotive activity of _C. elegans_ are unknown. In mammals, CYP-eicosanoids act in an autocrine or paracrine manner and serve as second messengers of diverse hormones regulating the contractility of vascular smooth muscle cells and cardiomyoctes [20,60,61]. In _C. elegans_, 17,18-EEQ mediates the O2-ON response by increasing the activity of body muscle cells. Surprisingly, we found that CYP-13A12 and also CYP-33E2, another 17,18-EEQ-generating CYP isoform, are predominantly located in the MCs (marginal cells) of the worm's pharynx [5,18]. MCs intercalate with pharyngeal muscles and might,
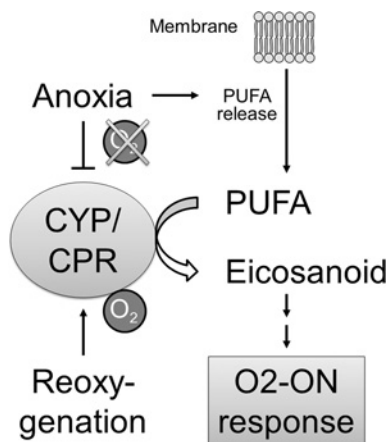
Figure 6 Proposed role of CYP-eicosanoids in mediating the O2-ON response

As shown in the present study CYP-eicosanoids such as 17,18-EEQ activate the locomotion behaviour of *C. elegans*. To produce the corresponding metabolites, CYP enzymes require molecular oxygen, NADPH and free C_{20}-PUFAs. Under normoxic conditions CYP-eicosanoid formation is limited by the availability of free C_{20}-PUFAs. Anoxia results in decreased CYP-eicosanoid synthesis due to oxygen limitation; however, anoxia activates phospholipases releasing free C_{20}-PUFAs from membrane stores that are then available for enhanced CYP-eicosanoid formation in the subsequent reoxygenation phase resulting in a rapidly increased locomotion speed of the nematodes.

on one hand, structurally reinforce these muscles [62]. On the other hand, MCs contain abundant mitochondria, suggesting that these cells might perform active non-structural roles [63] too. Nonetheless, it is currently largely unclear how CYP-eicosanoids produced in the pharynx are able to modulate the muscle activity in the worm's body. Possibly 17,18-EEQ is secreted by the MCs and then recognized as a signalling molecule by nearby sensory neurons that in turn trigger the O2-ON response via neural circuits [64,65] known to control forward/backward locomotion. Within this process, 17,18-EEQ might also stimulate the release of neuropeptides that act as neurohormones on to body muscles. Providing a first link between C_{20}-PUFA deficiency and neurotransmission, *fat-3(lg8101)* worms were shown to release abnormally low levels of neurotransmitters at cholinergic and serotonergic neuromuscular junctions and to be depleted of synaptic vesicles [43].

The O2-ON response of *C. elegans* and ischaemia/reperfusion injury of mammalian organs are quite different in terms of their final outcomes. However, as elaborated in the present study, they may share common mechanisms of CYP-eicosanoid formation and action. In particular, it appears that the epoxy metabolites, known to play a protective role in ischaemia/reperfusion injury, serve in *C. elegans* as mediators of the O2-ON response. A major open question concerning both research fields is the identity of the primary cellular targets of CYP-eicosanoids. Increasing evidence obtained in mammalian systems suggests the existence of GPCRs (G-protein-coupled receptors) that specifically interact with selected sets of epoxy and hydroxy metabolites derived from $n-6$ and $n-3$ PUFAs [52,66,67]. Supporting the hypothesis that 17,18-EEQ may interact with similar targets in mammals and *C. elegans*, we found that a metabolically robust synthetic analogue, developed to mimic the effect of 17,18-EEQ on cardiomyocyte contractility [20], was also effective in modulating the locomotive activity of *C. elegans*. Further genetic analysis of the components mediating the 17,18-EEQ effects in *C. elegans* may facilitate the search for the thus-far unknown receptors of CYP-eicosanoids.

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