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The Role of Long Chain Fatty Acids and Their Epoxide Metabolites in Nociceptive Signaling

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Abstract

Lipid derived mediators contribute to inflammation and the sensing of pain. The contributions of omega-6 derived prostanoids in enhancing inflammation and pain sensation are well known. Less well explored are the opposing anti-inflammatory and analgesic effects of the omega-6 derived epoxyeicosatrienoic acids. Far less has been described about the epoxidized metabolites derived from omega-3 long chain fatty acids. The epoxide metabolites are turned over rapidly with enzymatic hydrolysis by the soluble epoxide hydrolase being the major elimination pathway. Despite this, the overall understanding of the role of lipid mediators in the pathology of chronic pain is growing. Here we review the role of long chain fatty acids and their metabolites in alleviating both acute and chronic pain conditions. We focus specifically on the epoxidized metabolites of omega-6 and omega-3 long chain fatty acids as well as a novel strategy to modulate their activity *in vivo*.

Keywords

Omega-3 fatty acids; epoxy fatty acids (EpFAs); epoxyeicosatrienoic acids (EETs); epoxydocosapentanoic acids (EDP, EpDPEs); soluble epoxide hydrolase (sEH); pain

Introduction

The role of arachidonic acid (ARA) metabolites in pain and inflammation has received great attention over the last 45 years. This has expanded the understanding of lipids as cell signaling molecules with biological actions not limited to their role as energy stores. The exploration of bioactive lipid metabolites has largely remained focused on the omega-6 polyunsaturated fatty acid (PUFA) arachidonic acid ARA. Recently, this has extended to investigating the biological fate of the omega-3 fatty acids through the same enzymatic

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Conflict of interest: The University of California holds patents on the sEH inhibitors used in this study as well as their use to treat inflammation, inflammatory pain, and neuropathic pain. BD Hammock and B Inceoglu are co-founders of Eicosis L.L.C., a startup company advancing sEH inhibitors into the clinic. K Wagner and S Vito have no conflicts to declare.

pathways of the ARA cascade. The omega-6 and omega-3 PUFAs differ in the position of the double bonds from the methyl end of the molecules. Nevertheless, both are formed from the essential fatty acids linoleic acid and α-linolenic acid respectively which cannot be synthesized *de novo* by humans or other mammals. However, the conversion of α-linolenic acid to long chain omega-3 PUFAs is limited, and they are more efficiently taken in through dietary supplementation [1]. The PUFAs affect membrane fluidity, modulate inflammation, hemostasis and vascular tone [2]. Roles for omega-3 PUFAs in the central nervous system (CNS) and CNS development have been well described [3, 4] and more recently the role in CNS pathology has attracted focus [5, 6]. Here we examine the bioactivity of these omega-3 PUFAs compared to omega-6 ARA and their metabolites with special attention paid to their role in modulating nociceptive signaling and pain. While new evidence is emerging regarding the role of omega-3 PUFA metabolites of lipoxygenase enzymes in pain [7], this review will focus on the epoxidized PUFA metabolites (epoxy fatty acids, EpFAs) formed by cytochrome P450 enzymes.

PUFA metabolism

The ARA cascade is typically simplified and described as three enzymatic pathways including the cyclooxygenase (COX), lipoxygenase (LOX) and cytochrome P450 (CYP450) enzyme families that convert the parent PUFAs to multiple bioactive lipid metabolites. It is now established that the omega-3 fatty acids, both eicosapentaenoic acid (EPA, 20:5 n-3) and docosahexaenoic acid (DHA, 22:6 n-3), in addition to omega-6 ARA (20:4 n-6), are substrates for each of the major catalyzing enzymes of these three branches of the cascade [8, 9]. While use of the parent PUFAs as dietary intervention has been more often investigated, there is a broadening exploration of the activity of individual metabolite species. The COX produced prostaglandins from ARA are well studied and prostaglandin E_2 specifically is known to have a prominent role in pain and inflammation [10]. The COX-2 enzyme acts on EPA and DHA to form the respective E and D series resolvins in the presence of aspirin, and the LOX enzymes form neuroprotectin-1 from DHA [11, 12]. The LOX produced leukotrienes from ARA have roles in inflammation and lung related pathologies such as asthma [13]. The CYP450 produced metabolites from ARA are less well described and their biological activity is still being explored. The CYP450 oxidases also act on the omega-3 substrates forming multiple species of EpFAs [14] as well as numerous hydroxylated metabolites. Thus, a new nomenclature such as the PUFA cascade is more applicable.

EpFA synthesis

The enzymes responsible for the *de novo* production and degradation of EpFAs are broadly distributed through tissues and are present in neurons and glial cells in both rodents and humans [15, 16]. The omega-6 ARA and omega-3 EPA and DHA PUFAs are cellular membrane lipids esterified primarily at the sn-2 position of glycerophospholipids [9, 17, 18]. All three classes are released from these stores by phospholipases [19] and other enzymes. They are subsequently transformed by CYP450 enzymes into regioisomers of the EpFAs, the epoxydocosapentanoic acids (EDPs also known as EpDPEs), eicosatetraenoic acids (EEQs also known as EpETEs) and epoxyeicosatrienoic acids (EETs) from DHA, EPA and ARA respectively. The CYP450 oxidases convert all of these substrates with higher catalytic

turnover of EPA and DHA to ARA [20, 21]. CYP2C8, CYP2C9 and CYP2J2 are reported to be largely responsible for the formation of the EpFAs. However several other P450 oxidases thought to be principally responsible for the oxidative metabolism of the lipophilic xenobiotics may also produce EpFAs. All of these CYP450's make several regioisomers of EpFAs and all of them are involved to varying degrees in allylic, omega, and omega-1 hydroxylation in addition to epoxidation. The PUFA substrates can be epoxidized at any of several double bonds resulting in several regioisomer metabolites and the corresponding enantiomers for each class of PUFAs. Thus, there is a large metabolite pool when all possible outcomes are considered. EETs and omega-3 EpFAs are also esterified in phospholipids present in cellular membranes and plasma lipoproteins [22–24]. Therefore, they are immediately available upon release from these membranes.

EpFA metabolism

EETs are endogenous substrates of the soluble epoxide hydrolase (sEH, *EPHX2*, EC 3.3.2.10) an enzyme downstream of the CYP450s in the ARA cascade. The sEH appears primarily responsible for the hydration of EpFAs to their corresponding diols when it is present. These endogenous substrates are efficiently degraded by the sEH making it an important regulatory enzyme (Fig. 1). In his classical review of the epoxide hydrolases Oesch pointed out that the microsomal epoxide hydrolase (mEH, *EPHX1*, EC 3.3.2.9) can hydrolyze epoxysteric acid [25]. The hydrolysis of fatty acid epoxides by the mEH has been observed by numerous subsequent workers [26, 27], however compared to sEH the K_m is higher, the V_{max} or k_{cat} values are much lower, and in general the abundance of the mEH is much lower. Thus, except possibly in rare tissues where the sEH level is exceptionally low and mEH level is high, the sEH is the predominant enzyme responsible for the hydrolytic degradation of fatty acid epoxides. However, other mechanisms such as reincorporation into the cellular membrane and beta oxidation also limit the *in vivo* residence time of EETs. Several studies have attempted to determine the maximum possible hydrolysis of EpFAs in tissues that is not due to the sEH ranging from the early work of Moody to more recent investigations [28, 29]. The conclusion is that no substantial hydrolysis of EpFAs is due to enzymes other than the sEH. There are several genes that could code for proteins with sEH activity, with EH3 and EH4 the most well studied [30]. While there are reports of EpFA hydrolysis by EH3 [31] many laboratories have difficulty in reproducing this work possibly due to expression problems.

The sEH converts EpFAs of all three classes into the vicinal diols. There is evidence of substrate preference, for example sEH has the strongest preference for the 14,15 isomer of the EETs, and increasing the distance between the terminal carbon and the epoxide reduces this preference [23]. However, omega-3,4 epoxides of the EDPs and EEQ series are slowly metabolized [32]. Despite this selectivity, sEH turns over all of the regioisomers. The EETs have been shown to regulate vascular tone and blood pressure as well as being anti-inflammatory and antihyperalgesic in rodent models of disease [33–36]. The hydrated diol products produced from ARA by sEH, the dihydroxyeicosatrienoic acids (DHETs), are thought to have little or no biological activity [20, 37]. The diols are more polar, they exit the cells more quickly and are rapidly conjugated [38]. However, there is evidence that the DHETs facilitate chemoattraction of monocytes [39]. Diols of linoleic acid (leukotoxin and

isoleukotoxin diols) increase vascular permeability, cause edema, and appear to be powerful proinflammatory chemical mediators [40]. Clear biological activity for the diols of the omega-3 EpFAs has not been documented [32], and they are expected to demonstrate a similar increase in solubility and elimination to the omega-6 dihydroxy products.

Lipids in nociception

Here we examine the role of these lipids in antinociception examining evidence in both acute and chronic pain conditions. Nociception is the action of the nervous system in encoding noxious stimuli that results in the sensation of pain and antinociception is blocking this action. The differences between acute tissue injury or inflammation and unresolved neuropathic pain are examined in several reviews [41–43]. It is known clinically that some pharmacological therapies for pain such as NSAIDs and opiates that are active against inflammatory hyperalgesia (an increased pain sensation from normally painful stimuli) have no or limited efficacy in chronic pain [44]. This difference in analgesic efficacy is not dependent on peripheral pharmacology because many agents that act centrally against nerve injury such as gabapentinoids and anticonvulsants are also effective against tissue injury. The strategy of treating painful conditions by modulating lipids has great therapeutic potential because they are active against both acute and chronic pain. Additionally, altering substrate pools of bioactive molecules to obtain pain relief seems to have few adverse side effects [45, 46] and therefore the ability to chronically dose pain conditions. So far the loss of efficacy (tachyphylaxis) often seen with some modulators of receptors has not been observed with the modulation of lipid chemical mediators.

Parent PUFAs

Recently there is increasing interest in the historical change in lipid consumption revealing a heavily biased omega-6 to omega-3 Western diet [5, 47]. The bias was increased in favor of omega-6 lipids when omega-3 lipids were removed from industrial cooking oils to increase their thermal stability to oxidation. This dietary bias is important because dietary omega-6 and omega-3 PUFAs compete with each other both for incorporation into cellular membranes and as substrates for enzymes [48]. It has also been demonstrated that DHA and EPA omega-3 PUFAs cross into the brain efficiently by simple diffusion [49]. Therefore, omega-3 enriched diets have been examined for their effects on pathologies. The results demonstrate that dietary supplementation with the omega-3 PUFAs has several beneficial physiological effects including cardioprotection, reduced inflammation and neuroprotection [50–52]. In addition to these, omega-3 fatty acid supplementation has been shown to have an effect in various painful conditions including rheumatoid arthritis, inflammatory bowel disease, and dysmenorrhea though many of these effects were interpreted as mostly anti-inflammatory and thereby antihyperalgesic [2]. More recently, the omega-3 fatty acids have been investigated for their direct antinociceptive effects and possible mechanisms of the analgesic action, including the generation of active metabolites.

Antinociceptive activity of parent PUFAs

Earlier studies investigated dietary supplementation of omega-3 rich oils in humans to determine the anti-inflammatory effect of nutritional manipulation on conditions such as

rheumatoid arthritis [53, 54]. Joint pain and stiffness used as endpoints gave initial evidence that omega-3 intervention could be antihyperalgesic. More recently, the acute effect of omega-3 oil in experimental models has been evaluated. Oral dosing has demonstrated reduced writhing in normal mice in the acetic acid assay [55], including dose dependent effects after acute enteral administration [56]. These observations have been supported by central intracerebroventricular (i.c.v.) administration in mice which significantly reduced writhing behavior [55]. Administration of omega-3 fatty esters also blocked both early and late stages of pain behavior in the formalin assay in mice [56]. These studies using acute administration of omega-3 oils in naive mice offer compelling evidence that omega-3 fatty acids and specifically DHA have antihyperalgesic activity.

Proposed mechanisms of action of omega-3 PUFAs in biologies related to nociception include the change in cellular membrane lipids which alters fluidity and the production of cytokines and prostanoids [51]. There is evidence that the omega-3 lipids alter the activity of ion channels, alter voltage activated sodium current and displace high affinity ligands at TRPV1 receptors [57–59]. Additional results suggests that opioid signaling may be affected, although indirectly, with a proposed effect on the release of endogenous peptides [60]. In humans, dietary supplementation with omega-3 oil increased the downstream levels of EpFA metabolites in plasma [24]. Because the enzymatic metabolism of omega-3 fatty acids is rapid, it is likely that the bioactive metabolites appear quickly and may be driving the bioactivity of PUFA supplementation.

Inflammatory Pain

Inflammation is a complex immune response to high intensity stimulation that results in a release of biological active factors such as cytokines and peptides from local and migratory cells [61]. These factors are able to sensitize and also directly affect sensory neurons in a way which is recognized as pain [10]. Early work established the parent PUFA ARA has direct pain producing (algogenic) properties [62] and intradermal injection of ARA caused pain in naïve rats [63]. A more recent study found that intraplantar administration of ARA in inflamed rats did not further increase pain compared to the vehicle control [32]. The difference between injecting naïve rats and the inflamed rats may be significant, and it is likely that the effects observed in the naïve rats are overshadowed by the carrageenan induced inflammatory pain state. Intraplantar administration of DHA and EPA also fail to show significant change in the inflamed model [32]. However, oral omega-3 pretreatment in modeled inflammatory pain induced by carrageenan demonstrated both decreased thermal pain and edema [56]. There is also evidence of direct intraarticular administration of DHA in a murine model of knee arthritis reducing flinching behavior and knee edema [64]. Together the results of these inflammatory pain models support the efficacy of omega-3 oil in relieving inflammatory pain.

Neuropathic Pain

Neuropathic pain differs from injury or inflammatory pain because it is a chronic pain that lasts beyond injury resolution. One of the hallmarks of neuropathic pain is that it occurs in the absence of stimuli [65]. Diabetic neuropathy specifically is a chronic pain condition which is a major concern as the worldwide diabetic population increases, and it is the most

common secondary condition of diabetes mellitus [66]. The type 1 diabetic model of neuropathy uses the chemical ablation of pancreatic beta islet cells rendering the animals with diabetes and neuropathy resulting from unchecked hyperglycemia. In diabetic neuropathy, the activity of desaturase enzymes that produce long chain PUFAs are reduced resulting in diminished levels of DHA and ARA in cellular membrane phospholipids [67]. Na,K-ATPase activity, a transmembrane sodium potassium exchanger enzyme active in the generation of action potentials, is decreased in sciatic nerves in modeled diabetic neuropathy [68, 69]. Omega-3 supplementation partially corrects the activity of Na,K-ATPase and nerve conduction velocity (NCV) as well as prevents endoneurial edema and axonal degeneration in this chronic condition [70]. When tested alone DHA significantly reduces the decrease in diabetic sciatic NCV and corrects nerve blood flow (NBF) in diabetic rats [71]. However DHA alone did not have an effect in sciatic nerve Na,K-ATPase activity in these rats. Thus, there is direct effect of DHA distinct from changes in membrane lipid composition or modulation of Na,K-ATPases that improves NCV and NBF.

Recently omega-3 dietary supplementation has been investigated in the diabetic model for effects specifically on nociceptive behavioral outcomes (Fig. 2). A feeding study using custom diets of 1% DHA (omega-3) with a corresponding control of 1% oleic acid (omega-6) was conducted in the diabetic neuropathy model. Rats were fed the respective diets for 1 week prior to the induction of diabetes. Following diabetes induction, the withdrawal thresholds of the animals on the DHA diet were on average higher than controls while they were kept on the custom diets. The diets were administered for a total of 25 days before both groups were returned to a conventional chow diet. The pain thresholds of the DHA fed rats remained higher for an additional week after the switch to conventional diet Interestingly, a lasting effect of an increased omega-3 to omega-6 epoxyeicosanoid index post cessation of diet supplementation was previously observed in humans[21]. Thus, it is possible the omega-3 metabolite levels remain elevated in rat as well and would correlate to the nociceptive assay results, though this remains speculation. After this one week period the withdrawal thresholds of the two experimental groups normalized on the regular chow diet demonstrating the improved scores were related to the DHA supplementation. Thus, a high DHA background is able to improve nociception as well as improve on microvascular function such as nerve blood flow. The ability to dose omega-3 PUFAs long term would be a benefit in chronic pain disorders where prolonged treatment with many analgesic therapies such as steroids, opioids, and non-steroidal anti-inflammatory drugs cause severe adverse side effects.

Other biologies of parent PUFAs

The role of omega-3 PUFAs in the central nervous system, especially DHA, is well known in development of the pre and post natal brain. Astrocytes and also hippocampal neurons have demonstrated synthesis of DHA in primary culture from prenatal rat [72]. There is growing evidence that this supportive function extends to adult brain as well [73]. Experimental and clinical results indicate that omega-3 supplementation seems beneficial in Alzheimer's [74] as well as major psychiatric disorders such as schizophrenia [46, 75] and select forms of depression [76, 77]. Omega-3 PUFA also have demonstrated effects in neuroprotection and the delaying of seizures [78]. Evidence in spinal cord nerve injury

suggests both acute bolus and dietary omega-3 supplementation are able to improve survival of motor neurons as well as oligodendrocytes after compression injury [73]. The pleiotropic effects of omega-3 PUFAs in the nervous system underscore their potential for improving CNS structure and function in addition to the specific antinociceptive activity.

EpFA metabolites

The fate of long chain fatty acids includes oxidation of the PUFAs into epoxidized bioactive metabolites. Each of the PUFAs gives rise to multiple possible epoxidized regioisomers at the double bonds but also optical isomers (*R* or *S*) of each regioisomer resulting in a plethora of potentially bioactive metabolites. One significant difference of omega-3 and omega-6 metabolites is that there are also major proinflammatory metabolites formed from the omega-6 ARA. However, the CYP450 generated EETs derived from ARA have consistently demonstrated anti-inflammatory activity. This is in contrast to ARA metabolites generated by other enzymes such as the prostaglandins (PGE_2, PGD_2) and leukotrienes (LTB_4) that are predominately proinflammatory. Thus, although the EETs are omega-6 products, so far similar biology is observed when omega-6 versus omega-3 derived EpFAs are examined. In general the omega-3 derived EpFAs appear to be more potent. One notable exception is that the EETs from ARA are moderately proangiogenic in the presence of VEG-F while the EDPs derived from DHA are strongly antiangiogenic thus slowing tumor growth and metastasis [79].

The EpFAs, specifically EETs, have well described effects on cardiovascular function but have only recently been investigated for their role in nociceptive signaling. Most of the COX and LOX produced metabolites of ARA have been found to act on G protein coupled receptors, many of which have several isozymes [80, 81]. However, despite decades of research describing the biological effects of EpFAs and in particular EETs, there is currently no known receptor for these CYP450 derived metabolites [37]. There have been candidates suggested for the receptor and given the diverse and tissue dependent biological outcomes it is expected to be one or several GPCRs with multiple isozymes [23]. Despite the lack of an identified receptor, multiple EETs from ARA and increasingly EDPs from DHA and other EpFA research are demonstrating their involvement in the modulation of multiple biological processes. Notably EETs have been shown to be antihyperalgesic in several pain models.

Antinociceptive activity of EpFA metabolites

Potential mechanisms of action of the EET regioisomers in the context of pain have been investigated using direct microinjection into the ventrolateral periaqueductal gray (vlPAG) [82]. Of the individual EET regioisomers 14,15 EET demonstrated efficacy peripherally as well as the ability to act centrally. Similar to the parent PUFA, the 14,15 EET metabolite does not bind opioid receptors directly but alters levels of endogenous opioid peptides. The conditional knock out of the CYP450 reductase in neurons halts opioid analgesia and indicates multiple CYP450 metabolites are possibly responsible for this activity [83]. Thus, while there is evidence that EETs activate TRP channels [84–86], and their application to naïve animals transiently sensitizes neurons [87], the analgesic activity of EpFAs, specifically EETs, in pain states has been demonstrated repeatedly.

Inflammatory pain

The origins of investigating the EpFAs for antinociceptive effects stemmed from early lipomic analyses revealing one effect of elevating the EETs was a reduction in plasma concentrations of PGE_2 [35]. Schmelzer *et al*. followed this observation and found a correlation between lowered plasma PGE_2 levels and inflammatory pain [88]. A regioisomeric mixture of exogenous EETs also blocked inflammatory pain in the rat model [87]. Later binding studies provided a potential mechanism of action for this analgesia by determining that EETs bind the peripheral benzodiazepine receptor also known as the translocator protein (TSPO) [89]. All four specific regioisomers of EETs were evaluated revealing 14,15 EET and 5,6 EET isomers are active. However, TSPO binding has not been determined for EPA or DHA epoxide containing metabolites. The TSPO receptor contributes to the translocation of cholesterol from the outer to inner mitochondrial membrane by cooperating with the steroidogenic acute regulatory protein (StarD1). The StarD1 is a carrier protein that is the rate limiting step in cholesterol delivery to mitochondria for subsequent steroidogenesis. EETs have previously been shown to upregulate StarD1 expression [90]. Thus, EpFAs are hypothesized to modulate neurosteroid synthesis. As such their antinociceptive effects are reversed by inhibitors of steroid synthesis but not by antagonists of 5 major steroid receptors.

The role of EpFAs in mediating antihyperalgesia has also been supported by topical [91] and intraplantar administration of individual regioisomers of both EETs and omega-3 derived EpFAs. Both classes of chemical mediators significantly improve withdrawal thresholds in models of inflammatory pain [32]. The EpFAs of ARA, EPA and DHA are more effective than their respective parent PUFAs. Additionally, EDPs are the more potent antihyperalgesic mediators of the EpFA metabolites. As mentioned earlier omega-3,4 epoxides are turned over more slowly than other epoxide metabolites of EPA and DHA and seem to be more stable *in vivo*. For example, the slower metabolism of the 19,20 EDP may contribute to the relative efficacy of EDP regioisomeric mixtures over other EpFAs. Furthermore, the effectiveness of different regioisomers within the EDPs has been determined. The omega-3 diols tested in the inflammatory pain model were inactive against nociception further supporting the assignment of the EpFAs as the major biologically active molecules [32].

Neuropathic pain

To test the effect of the EpFAs in chronic pain, well characterized regioisomer mixtures of EETs and EDPs were administered in a murine model of type I diabetic neuropathy. The diabetic mice were assessed for allodynia, a painful response to a normally innocuous stimulus (Fig. 3). While both EpFAs block pain, the EDPs are more effective than the EETs in this model. This observation is consistent with previous evidence demonstrating EDPs have better efficacy *in vivo* against inflammatory pain in the rat [32]. The antihyperalgesia is rapid but not long lasting because these EpFAs are substrates of the sEH and therefore are subject to rapid degradation *in vivo*. The acute doses of EpFAs in the chronic pain model define the antihyperalgesic effects as distinct from the benefits of longer term omega-3 dietary supplementation on other comorbidities of diabetes such as hypertension. Moreover, this evidence supports the hypothesis that the EpFAs are directly mediating antihyperalgesia and are responsible for the antinociceptive activity of PUFAs. Table 1 summarizes the major

outcomes of the nociceptive experiments outlined in these sections including both the parent PUFAs and the metabolites EpFAs.

Other biologies of EpFA metabolites

Much of the information on the biochemistry of EpFAs in the brain relates to EETs. One source of EETs in the CNS are astrocytes and the excitatory neurotransmitter glutamate can cause release of EETs from these cells [92, 93]. Astrocytes also have demonstrated epoxide hydrolase activity indicating the tight regulation of these metabolites [94]. EETs are thought to be involved in K+ signaling by opening large-conductance, calcium activated K^+ channels (BK) in astrocytes [95]. Relevant to nociception and action in the central nervous system, EETs modulate COX-2 mRNA expression, regulate cerebral blood flow, and control neurohormone release [16, 36, 96]. Electrical or chemical stimulated cerebral blood flow and tactile sensory stimulated sensory cortex blood flow are all blocked by the EET antagonist 14,15-EEZE *in vivo* suggesting that the effects are mediated by the epoxidized metabolites [97, 98]. EETs also increase axonal outgrowth in dorsal root ganglia (DRG) neurons in a concentration dependent manner [99]. Importantly, these results demonstrate that EETs have a direct action in non-vascular associated cells in the central nervous system. The EETs have demonstrated activity on centrally mediated pain and seizure via intrathecal and i.c.v. injection [36, 100]. Picrotoxin, an antagonist of GABA signaling, blocks antinociception mediated by elevated EpFAs and EpFAs demonstrate efficacy altering GABA signaling in chemically but not electrically induced seizure models [100, 101]. Interestingly, EpFA effectiveness differs in these seizure models. Notably, the ARA derived EETs are active against seizure but not the EPA or DHA derived EpFAs. This result is unexpected because the EDPs have demonstrated better potency and efficacy in previous *in vitro* biochemical endpoints and *in vivo* nociceptive assays. Despite this, the EpFAs directly affect neuronal and vascular cell populations and mediate beneficial outcomes in multiple types of CNS pathology.

Soluble epoxide hydrolase inhibitors (sEHI)

The exogenous administration of EpFAs has demonstrated the direct activity of these metabolites. Another approach is to inhibit the degradation of endogenous EpFAs with small molecule inhibitors of the sEH enzyme. The inhibitors bind the sEH enzyme as transition state mimics and this mechanism of action effectively inhibits the enzyme thereby elevating levels of endogenous EpFAs. Inhibiting the sEH has been used as an approach for renal driven hypertension and many other diseases [102]. More recently sEHI have been used to test the role of EpFAs in pain modulation and for eliciting analgesia mediated by EpFAs [34, 103, 104]. EpFA levels can be therapeutically increased by sEHI. However, the EpFAs are metabolized by other routes including but not limited to beta-oxidation and are reincorporated into cellular membranes. This may contribute to the therapeutic index of sEHI which do not have substantial behavioral effects in naïve animals even at high doses [102, 105].

Antinociceptive activity of sEHI

Many of the results from experiments investigating the antinociceptive effects of EpFAs using exogenous applications of the metabolites have been recapitulated with sEHI administration. This strategy has been shown repeatedly to correlate with elevated EpFA levels in plasma and tissues including brain and spinal cord as well as *in vivo* antinociceptive efficacy [32, 35, 36, 100, 105]. An advantage to using sEHI over direct exogenous application of EpFAs or their mimics is that the inhibitors elevate several of the naturally produced EpFAs concurrently. In addition, the EpFAs are rapidly degraded by the sEH, so blocking this enzymatic activity is still imperative to yielding the maximal beneficial effects of exogenous or endogenous EpFAs. Of course a limitation in the therapeutic use of sEHI is that they only stabilize the EpFAs but do not influence their *de novo* synthesis. This limitation could be overcome by the use of stable mimics of EpFA. The mimics, however, may lack the massive therapeutic index associated with sEHI.

Inflammatory Pain

The first demonstration of sEHI administration in a pain model revealed potent antihyperalgesic activity against lipopolysaccharide induced inflammatory pain [87]. These experiments demonstrated sEHI mediated decreases in COX-2 protein concomitant with inflammatory prostaglandin decreases [35]. Coadministration of sEHI with COX-2 selective inhibitors also leads to synergistic increases in EETs and pain thresholds while decreasing COX-2 protein and prostaglandins E_2 and D_2 levels [88]. In the inflammatory model sEHI demonstrate down regulation of COX-2 induction in the spinal cords of rats [36]. The potential for synergistic activity has been further exploited by synthesis of a designed multiple ligand inhibitor of sEH and COX-2 enzymes resulting in better efficacy in nociceptive assays [106]. Related to this, but an important distinction, sEH inhibition attenuates PGE_2 induced pain [101]. Blocking pain induced by a direct acting algogen which is also a metabolite of the COX enzyme demonstrates that the pain relief is independent of anti-inflammatory properties and also of COX regulation.

The inhibitors have a demonstrated effect on changing the levels of spinal somatostatin expression in induced inflammatory pain supporting central effects on nociception [107]. Picrotoxin, a GABA antagonist, blocks sEHI mediated antinociception and the inhibitors are active against seizure induced by this antagonist suggesting the modulation of GABAergic signaling [100, 101]. The sEHI do not elicit nociceptive change in the absence of a pain state [36, 105]. Nevertheless, sEH inhibition modulates COX-2 expression, but it can also block PGE_2 induced pain and possibly affect neurosteroid production [101]. It is clear the activity is not limited to anti-inflammatory mechanisms as demonstrated by the several experiments outlined above. However, the positive correlation in all of these indications underscores the idea that the sEHI engage their target and are mediating analgesic activity.

Neuropathic pain

More recently the use of sEH inhibition has extended to neuropathic pain models. Similar to the activity of exogenous EpFAs in chronic pain, the sEHI dose dependently relieves allodynia in a model of diabetic neuropathy [36]. The acute administration of sEHI and the

elevation of the EpFAs has no effect on the glycemic status of diabetic rats per glucose tolerance, insulin tolerance and glucose stimulated insulin secretion tests [108]. Thus, inhibiting sEH improves nociceptive outcomes in the diabetic model (also demonstrated in Fig. 4), but not by altering metabolomic parameters. Correlated with improved pain scores, plasma EpFAs levels from ARA and DHA but not EPA are significantly increased with sEH inhibition. However, only EDP to diol metabolite ratios are increased in spinal cord of animals in this model.

The use of sEHI has been explored at high doses to determine the limits of this approach to analgesia in both inflammatory and chronic pain models [105]. The sEHI potently block pain and are not motor impairing even at high doses. However, using low dose sEHI combined with omega-3 dietary supplementation is another approach for optimizing pain relief with the inhibitors. This approach was tested in the type I diabetic neuropathy model. In this model DHA diet supplemented neuropathic rats had higher pain thresholds which further improved with single administration of the sEHI TPPU (Fig. 4). Rats fed an omega-6 control diet displayed modest changes at a low dose of the inhibitor but the DHA diet fed rats scored up to 24% higher (insert Fig. 4) at the same dose. Thus, with omega-3 dietary supplementation there is a potential to improve pain relief or potentially dose limit sEHI for long term use in relieving chronic painful conditions.

The sEHI also induce a conditioned place preference (CPP) in diabetic mice [109]. The CPP assay uses conditioning with drug paired environmental cues to assess a preference for context associated pain relief. The CPP assay is believed to assess the non-evoked tonic pain typical of chronic pain conditions [110, 111]. sEHI induce a robust CPP in neuropathic but not naïve mice which is expected because inhibitors do not typically show activity in the absence of a painful state. The CPP assay also demonstrates the sEHI do not have rewarding side effects common of addictive analgesics despite their efficacy against pain. These results argue that in man sEHI could be powerful and non-addictive pain therapeutics.

Inhibition of sEH is also effective against equine laminitis (Fig. 5), a chronic pain condition that often leads to euthanasia of afflicted horses [112]. The result in a clinical patient demonstrates the activity of the sEHI in a complex setting and above the standard of care treatment. Importantly, the efficacy of sEH inhibition extends beyond rodents to multiple species including canine and feline work in preparation for publication. Table 2 summarizes the major outcomes of the nociceptive experiments using the sEHI to elevate multiple classes of EpFA outlined in these sections.

Other biologies of sEHI

The sEH enzyme is present in multiple tissues and cell types including neurons. In the mouse brain sEH immunoreactivity has been found localized to neurons in the cerebral cortex [113], trigeminal and sphenopalatine ganglia [98]. sEH, which modulates EpFAs, also has a role in axonal growth in neurons. Inhibition of sEH increases axonal outgrowth alone or in combination with EETs [114]. This includes the use of multiple structurally unrelated inhibitors which repeat the results observed with direct application of EETs. The ability to reproduce the effects of the EETs supports targeting the sEH to induce the effects

of the EpFAs. Additionally, the increased axonal outgrowth ranges from DRG to diverse neuronal subtypes including cortical neurons. Inhibiting sEH also effectively delays the onset and reduces the lethality of GABA antagonist induced seizures in mice [100]. The sEHI mediated delay in seizure response suggests the brain penetration of sEHI barring blood flow changes. These effects are partially blocked by the steroid synthesis inhibitor finasteride and are enhanced by the neurosteroid allopregnanolone which supports earlier evidence that the action of EpFAs may be related to neurosteroid genesis. Thus, inhibition of sEH is a novel strategy to increase levels of both omega-6 and omega-3 derived EpFAs to mediate both peripheral and CNS mediated analgesia and address additional CNS pathologies.

Translational pathways

The ultimate purpose of this basic research in nociception and lipid mediators of this biology is to find ways to improve human and animal health. There is now a substantial amount of evidence that long chain fatty acids and their metabolites are potent mediators of analgesia. The path forward to translate this evidence into improving human health is determining the best strategy of intervention. Several strategies have been outlined here. Dietary supplementation of omega-3 fatty acids is a safe alternative to improving nociceptive outcomes. However, there are issues with patient compliance in taking daily capsules as well as an aversion to the odor of the typically fish sourced material and gastrointestinal effects in some patients. Currently there is also very little standardization of nutritional supplements of omega-3 fatty acids and storage and handling are very important to the stability of the product. Another strategy is the dosing of the EpFAs themselves as they have been shown to be active metabolites mediating analgesia. While the administration of EpFAs elicits rapid antihyperalgesia, their use may be limited to clinical settings. EpFAs are rapidly hydrolyzed at the pH of stomach acid but they can be administered readily by simple enteric coating. They would likely be metabolized while crossing the intestinal epithelium by the sEH so EpFAs are less suited to enteral administration and would most often need to be injected. They are also natural products which makes proprietary coverage difficult. Synthetic mimics of EpFAs may provide an alternative solution to the lability of EpFAs, however, their improved stability and resistance to metabolic degradation may in fact limit their safety for use *in vivo*. Future investigations will determine whether these novel molecules have potential for use in animals or man. The strategy that perhaps holds the most promise at least in the short term is inhibition of the soluble epoxide hydrolase which elevates the endogenous EpFAs. The EpFAs are normally turned over very rapidly by sEH which is the major path of their degradation. Small molecule inhibitors of sEH elicit potent analgesia at low doses in both acute and chronic painful conditions. They have demonstrated antihyperalgesic activity in experimental species as outlined above, they have favorable pharmacokinetics in dogs [115] and non-human primates [116] and they have successfully treated clinical cases of equine laminitis [112]. The efficacy of sEH inhibition in these species is attractive given expansion of the animal health market from industrial production to high value companion animals and the greater attention paid to chronic painful conditions such as osteoarthritis in dogs and cats. Given the toxicity associated with cyclooxygenase inhibitors in companion animals, the use of sEHI alone or in combination with NSAIDs and

coxibs is attractive. Moreover, the success of sEHI in multiple species suggests they may have success in humans as well. There is one additional strategy demonstrated here (Fig 4) which is to combine omega-3 supplementation with administration of sEHI. The omega-3 derived EDPs are the most potent of the EpFAs and they are also the best substrates for sEH [32]. Therefore combining dietary supplementation with sEH inhibition is a logical approach to eliciting the greatest benefit while limiting the risk of pharmaceutical intervention in treating chronic pain.

Conclusion

Early research regarding the antinociceptive effects of EpFAs focused on the EETs as the major bioactive metabolites stabilized by sEH inhibition. It is now becoming apparent that the omega-3 epoxy fatty acid metabolites and specifically EDPs are important mediators of this activity. The ability to supplement diet to affect nociception is a promising and safe alternative to current pharmaceutical therapeutics. Moreover, the ability to improve on dietary supplementation with inhibitors of sEH is a new strategy to intervene in painful conditions with demonstrated effects in both acute and chronic pain settings. In the future dietary supplementation and sEHI or their combination may be novel therapeutics for multiple CNS disorders including pain, and possibly seizures, depression and schizophrenia in man.

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Highlights

- Long chain fatty acids mediate analgesia in acute and neuropathic pain models.
- Omega-3 and omega-6 epoxy fatty acids demonstrate potent analgesia.
- PUFA elicited analgesia may be mediated by epoxidized fatty acid metabolites.
- Soluble epoxide hydrolase inhibition elevates epoxide metabolites and is analgesic.

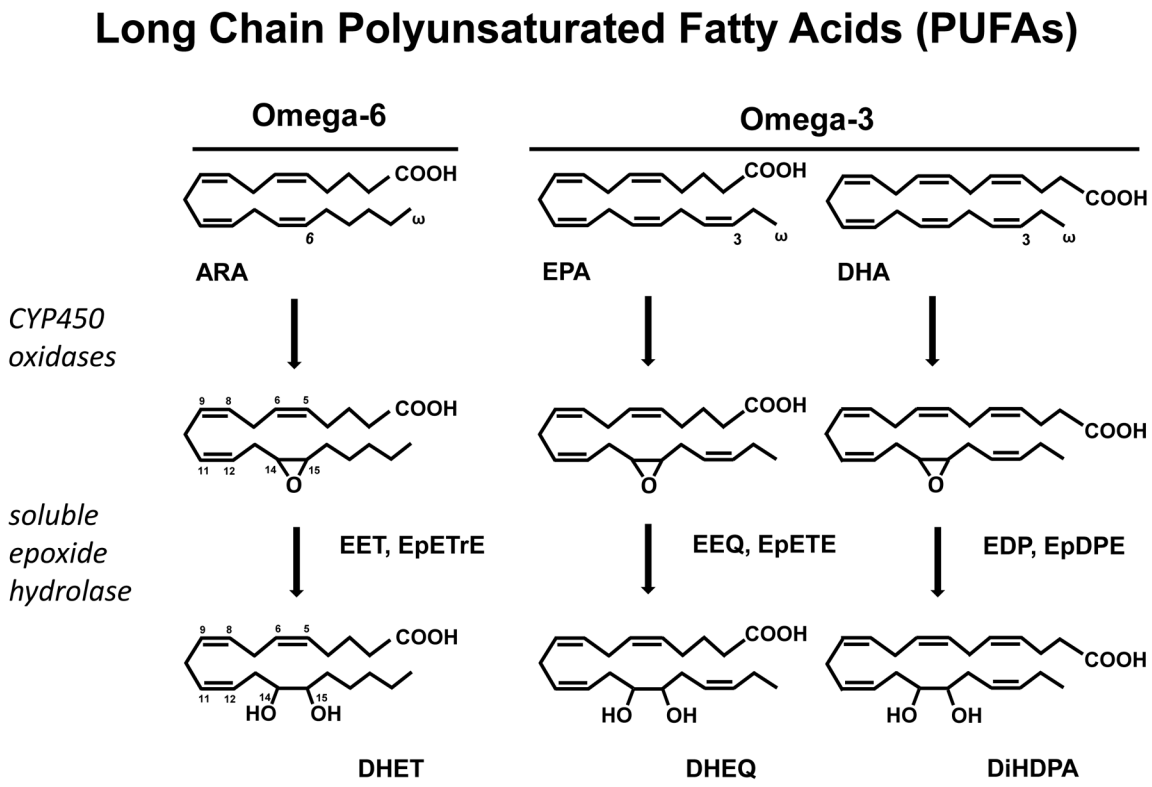

Figure 1.

Omega-6 and omega-3 long chain PUFAs share a similar metabolic fate. The fatty acids are designated based on the position (3 or 6) of the first double bond from the methyl (omega, ω) end of the molecule. Both omega-6 arachidonic acid (ARA) and omega-3 eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are metabolized by cytochrome P450 oxidases. Several epoxy fatty acid regioisomer metabolites the epoxyeicosatrienoic acids (EETs), epoxyeicosatetraenoic acids (EEQs) and epoxydocosapentaenoic acids (EDPs) respectively, and their enantiomers are possible for each of the substrates based on which double bond is epoxidized. Arachidonic acid, for example, may form the 5–6, 7–8, 11–12, or 14–15 EET (pictured). The epoxidized fatty acids are subject to further metabolism by the soluble epoxide hydrolase which converts them to their vicinal diols termed the dihydroxyeicosatrienoic acids (DHETs), dihydroxyeicosatetraenoic acids (DHEQs) and dihydroxydocosapentaenoic acids (DiDPAs) respectively. The EpFAs have multiple beneficial biological effects including mediating analgesia while the diols are thought largely to lack these effects.

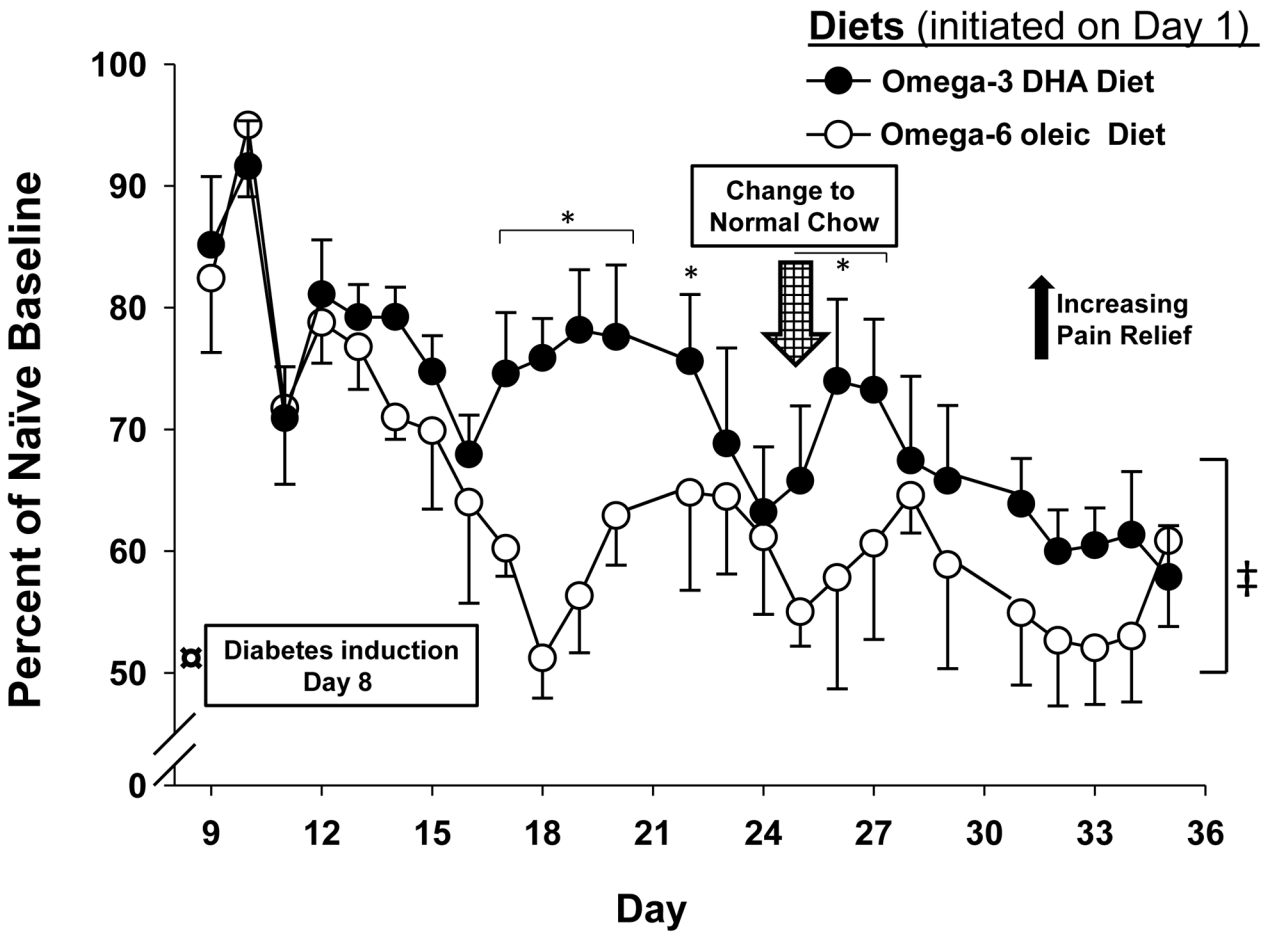

Figure 2.

DHA dietary supplementation improves pain thresholds in neuropathic rats.

In a type I diabetic neuropathy model, rats fed an enriched DHA diet (1% DHA custom made diet with Nu-Chek Prep Inc. 99% purity oil) one week prior to diabetes induction with streptozocin [117] and maintained on the diet for several weeks (pictured day 9–25) showed improved pain thresholds (von Frey assay) compared to rats on a omega-6 control diet (1% Oleic oil custom made diet with Nu-Chek Prep Inc. 99% purity oil) (Two Way Analysis of Variance, Holm Sidak post hoc, ‡ $p<0.001$ groups, *$p \leq 0.030$ time points). When both groups were switched to normal chow the improved scores were maintained for up to 10 days (pictured day 25–35) but then these effects dissipated. Thus, dietary supplementation with the omega-3 PUFA DHA improved nociceptive outcomes in a chronic pain model.

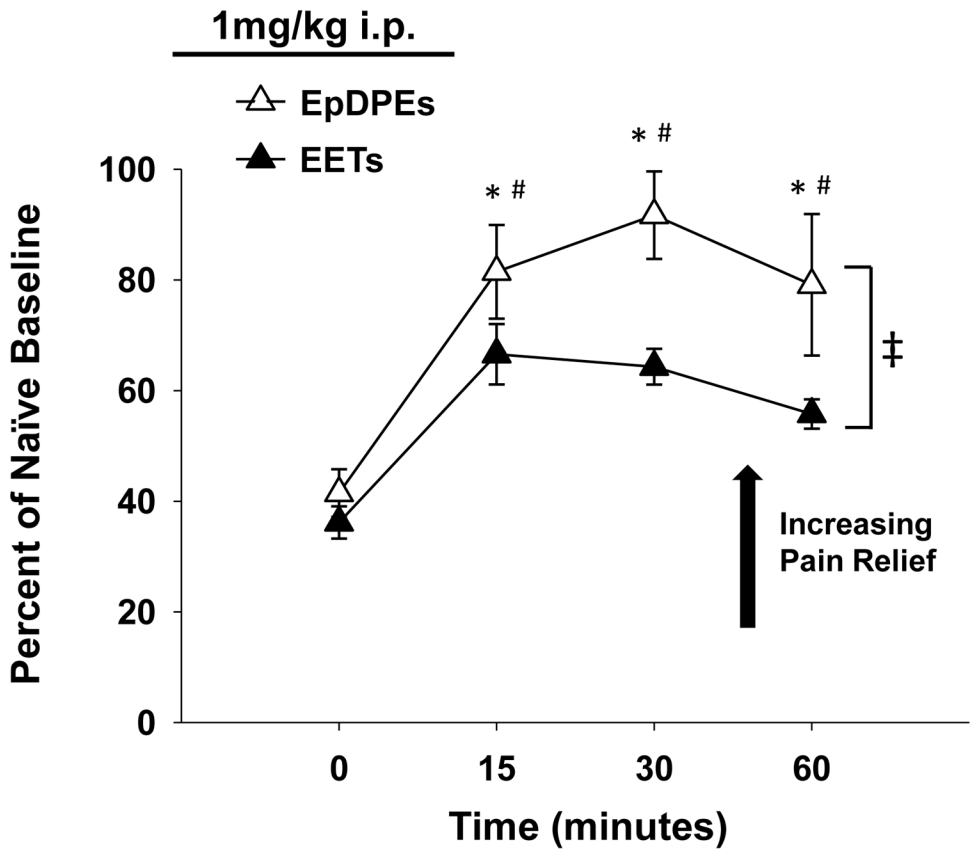

Figure 3.

EpFAs block chronic neuropathic pain in type I diabetic mice.

To test the hypothesis that EpFA metabolites exert analgesic effects the metabolites were administered to diabetic neuropathic mice. Type I diabetic neuropathy was induced with streptozocin [118] and mice were assessed for neuropathic pain (allodynia) before treatment using the von Frey assay. EDP and EET regioisomeric mixtures were both dosed at 1 mg/kg administered via intraperitoneal injection (i.p.) and pain thresholds were assessed over the time course. Both EpFA mixtures significantly increased pain thresholds indicating pain relief (One Way Analysis of Variance, Holm Sidak post hoc, *$p \leq 0.007$ EDPs, # $p \leq 0.003$ EETs). The EDP regioisomer mixture was more effective than the EETs (Mann-Whitney Rank Sum Test, $t=256.0$, ‡ $p \leq 0.048$) but the effects of both treatments were short lived due to their suspected rapid degradation by the soluble epoxide hydrolase enzyme. However, exogenous administration of the EpFA metabolites demonstrates their efficacy in chronic pain conditions.

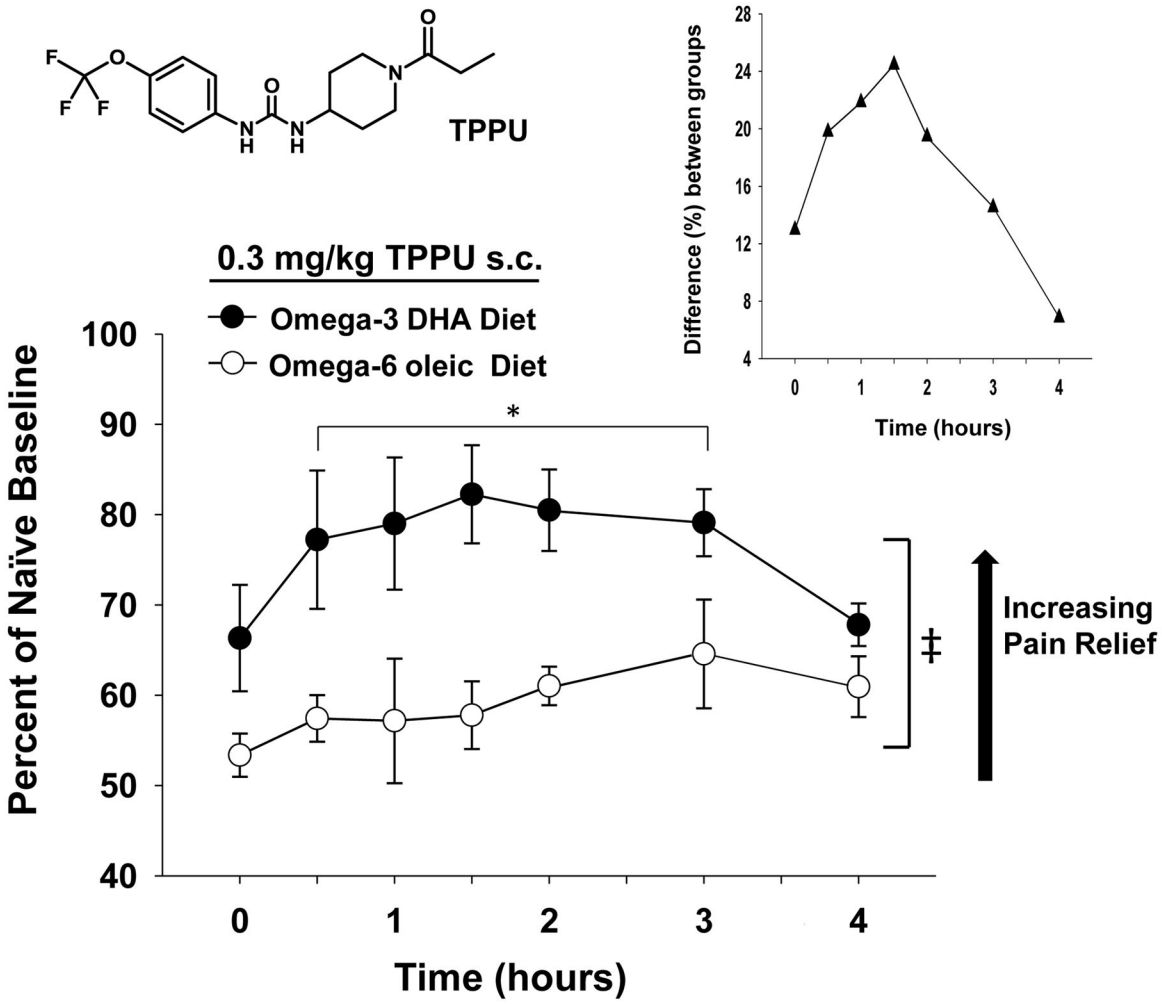

Figure 4.

The sEHI TPPU improves pain thresholds with enhanced efficacy in omega-3 supplemented neuropathic rats. Similar to the effects of parent PUFA and EpFA administration, sEHI mediate analgesia. A low 0.3 mg/kg dose of the sEH inhibitor TPPU (structure pictured) demonstrated increased efficacy when administered to rats fed a omega-3 DHA enriched diet compared to an omega-6 oleic control diet in the diabetic neuropathy model (Two Way Analysis of Variance, Holm Sidak post hoc, ‡$p \leq 0.001$ groups, *$p \leq 0.050$ time points). The DHA fed rats had higher baseline threshold before subcutaneous (s.c.) TPPU administration, nevertheless their thresholds improved over 24% of rats fed an omega-6 control diet (insert). Thus, the use of sEH inhibition with diet supplementation is potential strategy for increasing efficacy while dose limiting inhibitors for long term dosing in chronic pain conditions.

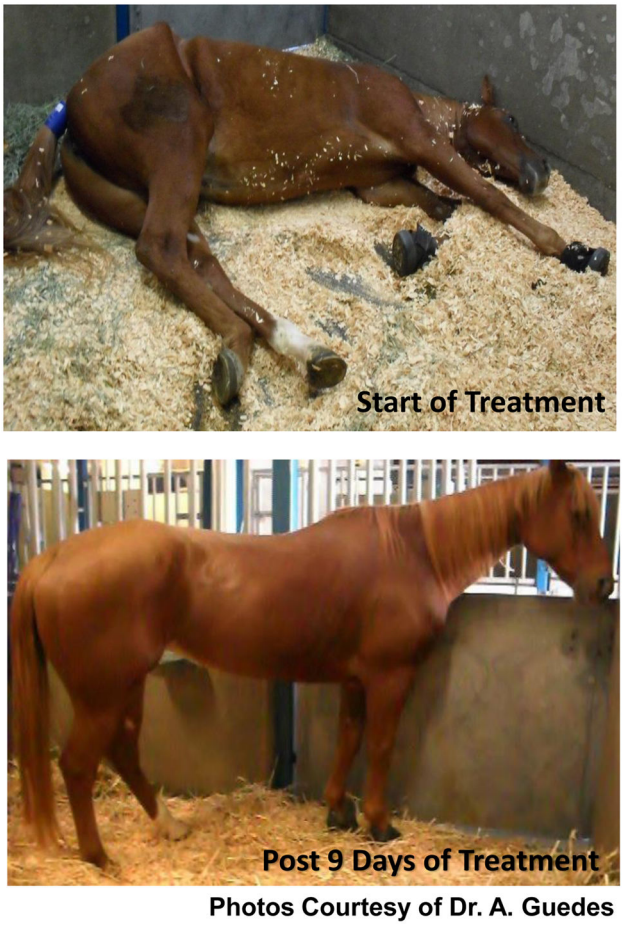

Figure 5.

sEHI block the chronic pain of equine laminitis. Laminitis is severe inflammation of the hoof leading to intense pain, tissue destruction, and morbid hypertension in horses. The condition can be crippling and often fatal. A horse presenting with laminitis in both front hooves was treated with NSAIDs but suffered refractory pain (Start of Treatment). Therefore the sEHI *t*-TUCB 0.1 mg/kg i.v. was administered once daily for 9 days in addition to the standard of care. After this treatment the horse was both standing well (Post 9 Days of Treatment) and able to walk around the stall. Treatment of equine laminitis has continued with success and no observed adverse reactions to date.

Table 1

Summary of outcomes of PUFA and EpFA administration in experimental models

Pain Model	Fatty acid	Route	Species	Major outcomes	Reference
PUFA					
Naïve (none)	n-6 (ARA)	int.pl.	Rat	Intraplantar ARA painful in Randall-Selitto assay	Gonzales et al. 1989
Inflammatory Pain	n-3(DHA)	oral	Mouse	Analgesic against acetic acid writhing, formalin, tail flick	Nakamoto et al. 2010
	n-3 (EPA+DHA)	oral	Mouse[1], Rat[2]	Analgesic against acetic acid writhing[1]and formalin[1], effective against CAR induced thermal withdrawal[2] paw edema[2] and peritonitis[2]	Nobre et al. 2013
	n-3(DHA)	oral/int.ar.	Mouse	Analgesic against CFA arthritis	Torres-Guzman et al. 2014
	n-6 (ARA) n-3 (EPA, DHA)	int.pl.	Rat	No significant change against mechanical allodynia	Morisseau et al. 2010
Neuropathic Pain	n-3 (DHA)	diet	Rat	Analgesic against mechanical allodynia	Figure 2
EpFA					
Naïve (none)	n-6 (EET)	microinj. vlPAG	Rat	Analgesic against thermal tail flick	Terashvili et al. 2008
Inflammatory Pain	n-6 (EET)	dermal	Rat	Analgesic against thermal withdrawal in LPS model	Inceoglu et al. 2006
	n-6 (EET) n-3 (EDP[1],EEQ)	int.pl./i.t.[1]	Rat	Analgesic against mechanical allodynia in CAR model	Morisseau et al. 2010
Neuropathic Pain	n-6 (EET) n-3 (EDP)	i.p.	Mouse	Analgesic against mechanical allodynia	Figure 3

Intraplantar int.pl., intraarticular int.ar., intraperitoneal i.p., microinjection of ventrolateral periaqueductal gray microinj. vlPAG, i.t. intrathecal, n-3 omega-3 fatty acids, n-6 omega 6 fatty acids LPS lipopolysaccharide, CAR carrageenan

Table 2

Major outcomes of sEHI administration in experimental models

Pain Model	Reference	sEHI Route	Species	Major outcomes
Inflammatory				
	Inceoglu et al. 2006	dermal	Rat	Analgesic against thermal withdrawal LPS model
	Inceoglu et al. 2008	s.c.[1] i.t.[2]	Rat	Analgesic against thermal withdrawal LPS model[1], mechanical allodynia assay CAR model[2], and mechanical allodynia diabetic neuropathy model[1]
	Hwang et al. 2011	s.c.	Rat	Analgesic against mechanical allodynia LPS model
	Inceoglu et al. 2011	s.c.	Rat	Analgesic against PGE_2 induced pain
Neuropathy				
	Inceoglu et al. 2012	s.c.	Rat	Analgesic against mechanical allodynia diabetic neuropathy model
	Wagner et al. 2013	s.c.	Rat	Analgesic in mechanical allodynia LPS model and diabetic neuropathy model
	Wagner et al. 2014	s.c.	Mouse	Analgesic in mechanical allodynia and conditioned place preference diabetic neuropathy model
	Guedes et al. 2013	i.v.	Horse	Analgesic in clinical chronic laminitis
	Figure 4	DHA diet +sEHI s.c.	Rat	Increased analgesia against mechanical allodynia diabetic neuropathy model compared to control supplemented rats

Subcutaneous s.c., intravenous i.v., prostaglandin E_2, PGE_2