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Authors

Wagner, Karen M
Gomes, Aldrin
McReynolds, Cindy B
et al.

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REVIEW

Soluble Epoxide Hydrolase Regulation of Lipid Mediators Limits Pain

Karen M. Wagner[1] · Aldrin Gomes[2] · Cindy B. McReynolds[1] · Bruce D. Hammock[1]

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Abstract
The role of lipids in pain signaling is well established and built on decades of knowledge about the pain and inflammation produced by prostaglandin and leukotriene metabolites of cyclooxygenase and lipoxygenase metabolism, respectively. The analgesic properties of other lipid metabolites are more recently coming to light. Lipid metabolites have been observed to act directly at ion channels and G protein–coupled receptors on nociceptive neurons as well as act indirectly at cellular membranes. Cytochrome P450 metabolism of specifically long-chain fatty acids forms epoxide metabolites, the epoxy-fatty acids (EpFA). The biological role of these metabolites has been found to mediate analgesia in several types of pain pathology. EpFA act through a variety of direct and indirect mechanisms to limit pain and inflammation including nuclear receptor agonism, limiting endoplasmic reticulum stress and blocking mitochondrial dysfunction. Small molecule inhibitors of the soluble epoxide hydrolase can stabilize the EpFA *in vivo*, and this approach has demonstrated relief in preclinical modeled pain pathology. Moreover, the ability to block neuroinflammation extends the potential benefit of targeting soluble epoxide hydrolase to maintain EpFA for neuroprotection in neurodegenerative disease.

Key Words Soluble epoxide hydrolase (sEH) · epoxy-fatty acids (EpFA) · inflammatory pain · neuropathic pain · analgesia.

Introduction

In the last several decades, the understanding of the role of lipids in pain sensation has increased substantially, including the potential to target them as strategies to relieve pain. This understanding of targeting lipids through regulatory enzymes lagged behind its practice. An example is the long history of using salicin from willow bark as an analgesic well before the elucidation of the mechanism of action of aspirin inhibiting cyclooxygenase enzymes to modulate prostaglandin production [1]. The more recent example of endocannabinoid signaling and elucidation of endogenous lipids that serve as ligands at cannabinoid receptors also has a long history of engagement prior this mechanistic insight (for a review, see Pertwee [2]).

✉ Bruce D. Hammock
bdhammock@ucdavis.edu

[1] Department of Entomology and Nematology and University of California Davis Comprehensive Cancer Center, University of California Davis, One Shields Avenue, Davis, California 95616, USA

[2] Department of Neurobiology, Physiology, and Behavior, University of California, Davis, California, USA

With increased knowledge of both the biological systems in which lipids play a role, as well as far more sensitive and quantitative technologies for assaying these systems, knowledge regarding the biological activity of endogenous lipids has greatly advanced. Here we will describe the role of lipids in pain sensation with special attention to epoxy-fatty acid (EpFA) bioactive metabolites that have been more recently described given new tools to investigate their biology.

Pain Sensation and Lipids

Nociception, the sensing of pain, is mediated by the action of a specialized subset of sensory afferent neurons called nociceptors. Nociceptors are activated in response to thermal, mechanical, and chemical stimuli through a variety of mechanisms. Although not an exhaustive list, ion channel modulation including transient receptor potential (TRP) channels, G protein–coupled receptor (GPCR) activation, and alteration of cellular membranes are all described mechanisms in which lipids act to impact signaling in nociception [3].

Pain has been described alongside inflammation since the Roman Aulus Celsus in the first century AD, and it is now well known from the work of Sune Bergstrom, Bengt

Samuelsson, John Vane, and others to result in part from lipid metabolites of cyclooxygenase isozymes, the prostaglandins acting at GPCRs (in this case, the EP receptors named for prostaglandin E agonism) [4]. Raphael Mechoulam and collaborators were able to identify another type of bioactive lipid arachidonoylethanolamide (AEA) named anandamide that agonized the GPCR cannabinoid receptors [5]. Sensitization of neurons to painful stimuli can also occur via lipid agonism of TRP channels in thermal sensitivity. David Julius demonstrated the activity of TRP channels in the 1990s, first with exogenous ligands like capsaicin; however, they are now known to be agonized by endogenous lipids as well [6, 7]. Although this review will focus on monoepoxide lipid signaling mediators formed from fatty acids, there is evidence of lipids such as phosphatidylinositol 4,5-bisphosphate (PIP_2) acting directly on ion channels [8]. Interestingly, there is also a growing body of literature on the effects of lysophosphatidic acid (LPA) which signals via a GPCR with 6 subtypes and is indicated in the generation of pain [9, 10]. In addition to these actions, there is also accumulating evidence about lipid–protein interactions that modulate signaling by indirect mechanisms affecting the properties of cellular membranes [11, 12].

The lipid ligands including the EpFA are unlike peptide neurotransmitters. They are released or synthesized on demand and are not stored in vesicles for release into synaptic terminals. They have putative GPCR mechanisms although they may act indirectly to influence ion channel proteins or the membranes in which they reside. Typically, as is the case with EpFA, they are tightly regulated by the activity of regulating enzymes that degrade them or reincorporate them into membranes [13, 14]. Some lipids such as AEA and a few EpFA that conform to the structural requirements of TRP lipid agonism also have been demonstrated to additionally activate TRP channels [10, 15]. Given the pleiotropic functions of these lipid classes, the question remains what role the TRP currents they produce serve in comparison to their more potent and contrasting analgesic actions *in vivo*.

The confounds of researching the role of analgesic lipids *in vivo* include the measurement and quantification of the individual lipids from tissue matrices and their presence and oxidation state being, in part, dependent on sample handling, storage, and extraction methods that can greatly alter their measured concentrations, and the sensitivity and separation capacity of the analytical methods available [16]. Therefore, a focused metabolomics approach for regulatory lipids remains challenging, requiring expertise and equipment not commonly available. However, it is still a useful tool to describe the greater breadth of lipid metabolites present in samples and provide the context for the relative role these metabolites play in contrast to single analyte analysis. This approach has been exploited to assess metabolites broadly across the arachidonic acid cascade as well as the metabolism of other long-chain polyunsaturated acids (PUFA). This strategy has proven valuable because there is now the knowledge that not only PUFA of several different classes are substrates for several metabolic enzymes, but also that the product lipid metabolites of these can be further substrates of these same enzymes.

Arachidonic acid (ARA), a 20-carbon length PUFA, is converted by CYP450 enzymes into epoxide metabolites at any or several of the 4 double bonds in the molecule. The metabolites formed from ARA maintain their 20-carbon length structure and are generalized with all eicosanoids (*eicosa* Greek for 20). However, the omega-3 long-chain fatty acids, DHA in particular, are also substrates of the major metabolizing enzymes of what is classically known as the ARA cascade and have demonstrated competition with ARA in enzyme assays *in vitro* [17, 18]. The ARA cascade generates oxidation metabolites by the cyclooxygenase and lipoxygenase enzyme families. These metabolites have been well described for ARA including their synthesis and bioactivity and are mostly, but not exclusively, pro-inflammatory. Some of these metabolites such as prostaglandin E_2 have a more complex role in inflammation being initially pro-inflammatory and later regulating resolution in at least some models [19]. The monoepoxygenated EpFA generated by cytochrome P450 enzymes, on the other hand, are anti-inflammatory and balance the action of other pro-inflammatory eicosanoids (Fig. 1).

Thus, the ARA-derived metabolites are known as eicosanoids and epoxyeicosatrienoic acids (EETs) are classical eicosanoids as EpFA. However, other long-chain PUFA such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) are also substrates of the CYP450 enzymes and form several regioisomers of epoxide metabolites based on the number of double bonds available for epoxidation [20]. The metabolites are the epoxydocosapentanoic acids (EDPs) and the epoxyeicosatetraenoic acids (EEQ, so named quaternary to avoid confusion with trienoic acids) of DHA and EPA, respectively. All of these classes of long-chain PUFA-derived EpFA have shown anti-nociceptive action and all of the EpFA have been shown to be metabolized to diols rapidly by the soluble epoxide hydrolase (sEH) [21]. We will briefly outline common lipid signaling systems and then focus on long-chain EpFA to better describe their role in nociception.

Classical Eicosanoids

Cyclooxygenase Metabolites

It is over 80 years since the identification of lipids that elicited uterine contractions and over 35 years since the Noble prize for the discovery of prostaglandins was awarded. In more recent times, the role of prostaglandins in physiology and pathophysiology is well known and well exploited pharmacologically to limit inflammation and pain. Cyclooxygenase (COX) enzymes present in 2 major isoforms: COX-1 and

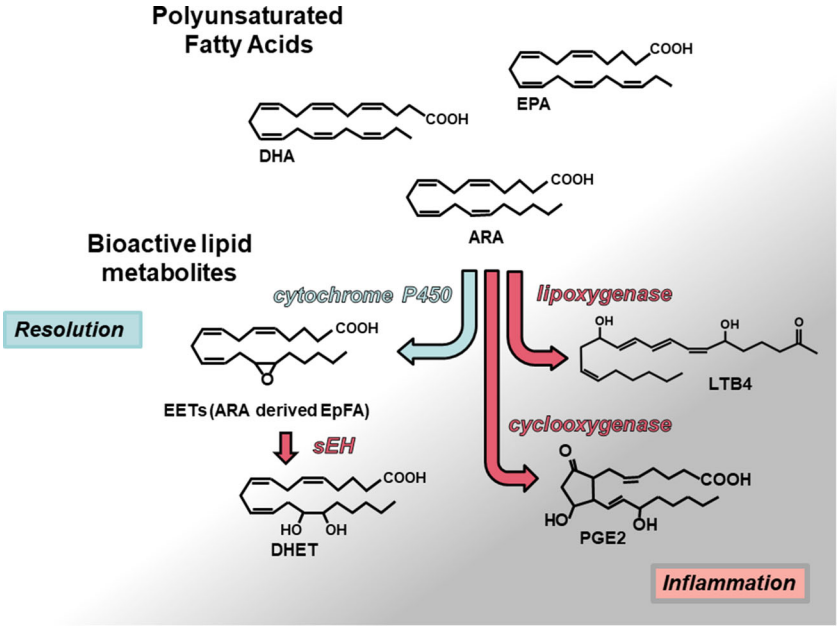

Fig. 1 Metabolism of long-chain polyunsaturated fatty acids (PUFA). PUFA both omega-3 such as epoxyeicosatetraenoic acid (EPA) and docosahexaenoic acid (DHA) and omega-6 arachidonic acid (ARA) are metabolized by several families of enzymes including lipoxygenase (LOX), cyclooxygenase (COX), and cytochrome P450 enzymes into bioactive lipid metabolites. The leukotrienes from LOX metabolism (leukotriene B_4, LTB_4 pictured) and prostaglandins from COX metabolism (prostaglandin E_2, PGE_2 pictured) are known to be primarily inflammatory mediators, whereas the epoxy fatty acids (EpFA) formed from cytochrome P450 metabolism (epoxyeicosatrienoic acids (EETs) pictured) are known to be anti-inflammatory and proresolving. However, EpFA are rapidly metabolized by the soluble epoxide hydrolase (sEH) to their corresponding inactive or even inflammatory diols (dihydroxyeicosatrienoic acids, DHET). The activity of this enzyme is often increased during inflammation and disease, further limiting the efficacy of EpFA and increasing inflammation

COX-2. COX-1 is constitutively expressed, and COX-2 is upregulated by inflammation [22]. Both isoforms convert ARA into prostaglandin H_2, a precursor of metabolites including prostacyclin, prostaglandins, and thromboxane all of which have their own diverse biologic activity [23]. ARA is the canonical COX substrate; however, COX enzymes metabolize other long-chain PUFA such as the omega-3 DHA [24]. In addition, COX-2 has broad substrate specificity that includes other parent PUFA and their lipid metabolites. As an example, COX-2 converts AEA into prostamides [25] and EpFA into further hydroxy-epoxide metabolites termed EHETs [26].

The prostanoid metabolite subclasses are formed by several different synthases and bind different GPCR receptors. For example, the prostaglandin E synthase which has 3 isozymes forms prostaglandin E_2 (PGE_2) that binds at receptors which are 4 isomers of a GPCR termed EP receptors named for their prostaglandin ligand [27]. Similarly, thromboxane binds TP receptors and prostacyclin (PGI) binds IP receptors and the other prostaglandins follow in the same fashion. PGE_2 is the most widely produced prostanoid in the body and has a well-described pronociceptive function. Other prostanoid receptors have been found in nociceptive neurons as well and investigation into their biological role in this cell type is ongoing [28].

Lipoxygenase Metabolites

The lipoxygenase enzymes (LOX) transform ARA into leukotrienes, a name which indicates a cell type where they are predominantly but not exclusively located. Hydroperoxyeicosatetraenoic acid (HPETE) metabolites are formed by the 5-, 12- and 15-LOX enzymes which are further reduced to monohydroxyeicosatetraenoic acids (HETEs). 5-LOX transforms them into first leukotriene A_4 which can then be formed into B_4 and C_4 leukotrienes (LTB_4 and LTC_4) and other downstream cysteinyl-leukotrienes [29]. Present in the leukocytes, the metabolites that 5-LOX produces play a role in the anaphylaxis response [30]. In addition, 12- and 15-LOX enzymes appear in platelets and endothelial cells [31]. LOX enzymes can also metabolize linoleic acid (LA) into 9- or 13-hydroxyoctadecadienoic acid which have demonstrated some bioactivity [32]. In addition, inhibiting 15-LOX has demonstrated anti-hyperalgesic effects in an intrathecal LPS pain model and prevented formalin flinching and tactile allodynia [33].

Canonical leukotriene receptors are GPCRs and present in 4 subtypes, the BLT 1 and 2 and CysLT 1 and 2 [34, 35]. There are other recently identified receptors from the orphan receptor classification which are bound by leukotriene [36]. The action of hydroxyoctadecadienoic acids

formed from LA has also been observed in sensitizing TRP channels [37].

Nonclassical Eicosanoids

Specialized Proresolving Mediators

Specialized proresolving mediators (SPMs) are a group of metabolites from several lipid precursors involved in the resolution of inflammation. SPMs include resolvins, protectins, and maresins derived from omega-3 long-chain fatty acid DHA and EPA and the ARA-derived lipoxins [38]. Although considered endogenous lipid mediators of resolution, several of the metabolites are "aspirin triggered" adding to the importance of this pharmacological agent. SPMs in the series D and E resolvins have demonstrated a role in inflammatory pain pathology [39].

There are newly identified GPR binding relationships for some lipoxins and resolvins at FPR2/ALX and GPR32 [40]. These receptors have a number of widely ranging ligands and structural elucidation may be required to confirm the mechanisms.

Endocannabinoids

N-arachidonylthanolamine (AEA) and 2-arachidonoyl glycerol (2-AG) are endogenous lipid ligands at cannabinoid receptors and thus named endocannabinoids [41]. The endocannabinoids are synthesized on demand with AEA forming from N-arachidonoyl phosphatidylethanolamine upon release from cell membranes because of phospholipase D action [42, 43]. Endocannabinoids are degraded by hydrolyzing enzymes, fatty acid amide hydrolase (FAAH) for AEA and monoacylglycerol (MAG) lipase for 2-AG, respectively [44, 45]. Of note, the endocannabinoid AEA can also be metabolized by cytochrome P450 (CYP450) enzymes into the epoxyeicosatrienoic acid ethanolamines, EET-EAs [46, 47], and these epoxy-AEA metabolites are further substrate for the sEH enzyme [48]. Interestingly, the ester-linked constitutional isomer AEA (O-AEA, virodhamine) has been identified as an endogenous inhibitor of CYP2J2 [49], and this inhibition stimulated the migration of endothelial cells and inhibited wound healing. Because of the abundance of CYP2J2 in the heart, and based on the above findings, caution must be taken when considering targeting CYP2J2 systemically with repurposed drugs [50].

Endocannabinoid receptors are also GPCRs with cannabinoid receptors 1 and 2 being the most well studied; however, there are additional GPCRs such as GPR18 and GPR55 which can interact with endocannabinoids as well as other lipids including lysophospholipid [36]. Agonism of the cannabinoid receptors elicits pain relief in addition to other effects. Attempts to target this system pharmacologically have involved several strategies including different exogenous cannabinoid ligands and inhibiting the enzymes that degrade 1 or more of the endocannabinoids all to limited success (for a review, see Lau and Vaughan [51]).

AEA can also agonize TRP channels, for instance at the TRPV1 channel [7, 15]. Importantly, assessing TRPV1 activity in cells includes stimulating with capsaicin to measure the effects of the incubated ligands. This adds a layer of complexity in determining agonism because of the demonstrated effects of capsaicin altering the elasticity of lipid membranes influencing voltage-gated ion channels, changing membrane fluidity and phase separation [52]. Just as there are identified actions of AEA on additional receptor types such as TRP receptors [53], AEA is also metabolized by COX and LOX enzymes which therefore adds to the complexity of defining the *in vivo* mechanisms in these signaling pathways. Although lipid ligand binding can demonstrate high specificity, there are also other examples such as FPR2/ALX that bind both lipids and proteins. It is possible that these receptors heterodimerize with other receptors or that the signaling pathways converge. Additionally, these binding assays are done in *in vitro* conditions that may be substantially altered from the *in vivo* presentation [36]. Thus, there is much to be done to accurately determine the effects of lipid ligands and how they affect protein receptors as well as the lipid membranes in which the receptors reside.

Epoxy-Fatty Acids: Classical and Nonclassical Eicosanoids

The EpFA, both 20- and 22-carbon chain length epoxy-fatty acids, are synthesized by the cytochrome P450 enzyme family upon the release of the parent fatty acid from cellular membranes. These fatty acids are cleaved off of membrane phospholipids by calcium-independent or inducible phospholipase A2 (iPLA2 or cPLA2, respectively) [54]. The epoxide metabolites are relatively stable against chemical hydrolysis and nucleophilic substitution and, therefore, are mostly metabolized sEH activity [21].

Although not a long-chain polyunsaturated acid, LA is an 18-carbon length fatty acid with 2 double bonds and is also a substrate for epoxide metabolite formation as are oleate and other unsaturated fatty acids. Notably, these epoxides are not included when speaking about EpFA, but interestingly, LA is the most prevalent fatty acid in the Western diet and bioactivity has been demonstrated for the epoxyoctadecenoic acid (EpOME) metabolites of this fatty acid. The EpOMEs are also known as leukotoxin for their increased production in acute respiratory distress syndrome [55]. However, the more toxic effects in this pathology were observed to be due to the diols produced from sEH metabolism of the epoxides [56]. The dihydroxylated products (DiHOMEs) of this metabolism are also increased in inflammatory pain states [57] and have been

demonstrated to increase thermal hypersensitivity [58]. DiHOME administration was able to cause thermal sensitivity [59], and EpOMEs combined with this treatment were unable to abate the thermal sensitivity, though there was no inhibition of sEH which rapidly converts the epoxides to diols. Additionally, the 100-μM application of metabolites needed to elicit TRPV1 currents in these experiments questions the physiological relevance of this hypothesized mechanism of action.

EpFA Bioactivity

EpFA have well-described roles in limiting both inflammatory and neuropathic pain *in vivo* [13, 21, 60]. In addition to its role in modulating pain conditions, epoxide metabolites have been shown to be protective in vascular neointima formation [61] and macrophage recruitment [62]. CYP450-derived metabolites have been shown to regulate the M1 and M2 phenotype and limit inflammation in monocyte and macrophage [63]. This relates the *in vivo* action of EpFA released at the site of inflammation in nerve injury [64] to their ability to limit inflammation and pain. The anti-inflammatory action of EpFA has been well documented [65] and is thought to result from multiple additional mechanisms such as blocking NF-κβ activation [66, 67].

EpFA and sEH inhibition prevent the phosphorylation of the NF-κβ regulator, IκBα, inhibiting the translocation of NF-κβ into the nucleus which therefore cannot activate transcription factors for further downstream inflammatory cytokines [66, 67]. By preventing the translocation of NF-κβ into the nucleus, EpFA indirectly block the subsequent transcription of inflammatory cytokines and regulate monocyte chemotaxis [63, 66]. Blocking NF-κβ–induced transcription of TNFα prevents recruitment of pro-inflammatory cells and TNFα induced vascular cell adhesion molecule-1 (VCAM), thereby reducing inflammation [66, 67]. This was observed in a recent study simulating arthritis *in vitro* by activating chondrocytes with IL-1β where incubation of EETs with IL-1β treatment was able to block the increase of TNFα (which signals chemotaxis of monocytes and lymphocytes) and protect the chondrocytes from cytotoxicity [68]. Kundu et al. observed that MCP-1 chemotaxis is dependent upon sEH-derived DHET and inhibiting the sEH activity blocked the MCP-1–driven chemotaxis [69]. This indicates importantly that blocking the function of the sEH enzyme has a dual purpose of increasing natural anti-inflammatory lipid mediators as well as preventing the formation of chemotactic diols.

Although a confirmed GPCR is not identified for EpFA, the ARA-derived EETs are known to directly activate the nuclear receptor PPAR-γ, and this direct action at the receptor is anti-inflammatory [70]. Notably, the anti-inflammatory action extends to inflammatory pain [57]. A majority of lipid-sensitive GPCRs are in the class A rhodopsin family [36]. It has been determined that short-chain fatty acids (C2-8) and medium-chain fatty acids (C12-18) have GPCR receptors identified from formerly orphan receptors [36]. Unsaturated long-chain fatty acids (C16-22) are ligands for GPR120/FFAR4 and EET, metabolites have been suggested to bind GRP40 but at low affinity [71]. The determination of specific lipid ligands at GPCRs is complicated by the physical and chemical properties of lipids. These properties can lead to nonspecific ligand binding or require adjuvant to deliver the ligands in *in vitro* assays which then require long incubations with radioligand binding assays because of their poor dissociation. These complications support the use of X-ray crystallography as another strategy to provide information; however, obtaining this information is labor intensive and time consuming (3-4 years for a new receptor elucidation) [72]. Thus, determining a unique GPCR for EpFA may lag behind modulating their concentrations for beneficial biological action.

Electrophysiology results from some TRP channels indicated that currents are activated by a few single regioisomers of EpFA lipids [73–75] which might indicate they have a role in hyperalgesia; however, the analgesic action of several classes of EpFA is known [21, 60] as well as the protective role they play in several physiologies [76–78]. Understanding the role of this receptor activation is even more complex in states of chronically sustained inflammation and pain because these systems are further dysregulated with the presentation of sensitizing agents such as bradykinin, cytokines, temperature, pH, and other lipids. The TRPV1 sensor is activated by heat, protons, and other stimuli in addition to lipids which can include such diversity as LPA to EETs to AEA [15, 79]. The potency of LPA agonism at TRP channels is equal to capsaicin, and mutagenesis studies have shown in the case of LPA (and also AEA) that these are likely direct actions on the channel [79, 80]. An added caution when interpreting data particularly for the ARA-derived 5,6 EET is the knowledge that this molecule rapidly cyclizes at the carboxyl group and forms a δ-lactone [81, 82]. Therefore, any ascribed biological activity is difficult to attribute to a single molecular species without further workup. Another complication which is elucidated by previously documented research regarding the cardiovascular effects of EETs is that any lack of *in vivo* efficacy may be due to their rapid metabolism. The beneficial action of EpFA in active pain states and several other pathophysiologies in the CNS [83, 84] and the repeated observation that they lack activity in healthy animals [85–87] speak to a more complex relationship in the *in vivo* environment. Thus, the mechanism of action of these metabolites at several receptor types is not fully elucidated, and additional nonchannel-mediated mechanisms may also contribute to their sum effect observed *in vivo*.

EpFA and sEH as a Novel Target in the Arachidonate Cascade

Nonsteroidal anti-inflammatory drugs (NSAIDs) and selective COX-2 inhibitors (COXIBs) act by inhibiting the production by COX or receptor binding of prostaglandins and related molecules. Similarly, several pharmaceutical compounds act by blocking production with leukotriene synthesis inhibitors, such as zileuton, or blocking binding of inflammatory metabolites from the LOX pathway such as leukotriene receptor antagonists zafirlukast and montelukast. In contrast, the EpFA block inflammation and pain, and therefore, therapeutic approaches involve increasing the production of EpFA [88], mimicking EpFA with more biochemically stable molecules [89], or inhibiting the soluble epoxide hydrolase that rapidly degrades the EpFA [90].

The development of small molecule inhibitors of the sEH has enabled investigation of the *in vivo* effects of the EpFA which are substrates of this enzyme. The EpFA are potently analgesic but short-lived molecules that are eliminated within seconds *in vivo* by sEH. The development of sEH inhibitors (sEHI) in recent years has resulted in optimized compounds with low nanomolar to picomolar potency and favorable pharmacokinetic profiles. The safety of sEHI as a class and the value of sEH as a target have been demonstrated in early phase clinical trials in humans. Chen et al. [91] describe 1 sEHI planned as a therapy for hypertension and provide proof of safety in healthy humans. Separately, another sEHI GSK2256294A investigated in multiple phase I clinical trials by GlaxoSmithKline, which was developed for a potential pulmonary indication, was later abandoned but showed no signs of adverse events [92]. Furthermore, this compound has been made available for human testing. More recently, an optimized sEH inhibitor with picomolar potency has entered phase I clinical trials for neuropathic pain. Thus, sEHI have advanced to clinical trials in human with several independent molecules, all with the same therapeutic target of inhibiting the sEH. This means that all these inhibitors broadly performed with substantial efficacy in preclinical models as well as significant safety *in vivo* in preclinical species and *in vitro* toxicological studies with human enzymes, microsomes, and cells.

sEH Inhibition and EpFA in Pain

Investigation of the role EpFA play in pain pathophysiology began over a decade ago with the knowledge that altering inflammation could halt inflammatory pain as well [57, 93, 94]. After demonstrating that the ARA-derived EpFA (EETs) and inhibition of sEH resulted in analgesia in multiple inflammatory models [57, 85, 95], other EpFA formed from the omega-3 long-chain fatty acids were tested and found also to block inflammatory pain [21]. The DHA-derived EDPs have been found to be highly active and subject to the same regulatory enzymes as the omega-6–derived EETs. Direct application of EDPs in both inflammatory pain in rats [21] and in diabetic neuropathic pain in mice [60] has demonstrated robust analgesia by evoked stimulation and operant assay methods. The EEQ metabolites of EPA were also tested in an inflammatory pain model and found to be analgesic, however not to the same potency as the EDP from omega-3 DHA [21]. Importantly, these epoxide metabolites are additional substrates of the sEH, and the DHA metabolites have high relative potency among the EpFA; thus, inhibiting sEH to elicit analgesia is a unique strategy for intervention in pain states.

The possible mechanisms of this analgesic action have also been interrogated. One hypothesis was that the action depended on neurosteroid production which originated from a small set of receptor binding assays demonstrating the affinity of EETs for the translocator protein TSPO [95]. Testing this hypothesis revealed an upregulation of spinal steroidogenic acute regulatory protein StARD1 with inhibition of sEH [85]. This was coupled with testing a pain model of diabetic neuropathy which is not elicited with an acute inflammatory insult to investigate if the mechanism relied on the anti-inflammatory action known for EETs, the ARA-derived EpFA [85, 96]. In a streptozotocin-induced diabetic neuropathy model in rats, sEH inhibition limited neuropathic pain. This distinguished sEHI from NSAIDs because they are known to lack activity against neuropathic pain. This distinction was further defined by testing the efficacy of sEHI against the pain induced by PGE_2, the product of cyclooxygenase [97]. The pain of PGE_2 cannot be prevented using NSAIDs or anti-inflammatory steroids, both of which act upstream of the metabolite product by inhibiting cyclooxygenase enzymes. sEHI effectively blocked pain induced by PGE_2 administration which implicated a noninflammatory mechanism for the sEHI-mediated analgesia. It is also known that EET-mediated anti-inflammatory action is correlated with down-regulated *COX-2* at the transcriptional level as well as decreased COX metabolites [57, 93], and though relevant in inflammation, this is likely not the primary mechanism of the analgesic action of EpFA [97]. In fact, EpFA demonstrated activity against neuropathic pain that is recalcitrant to typical anti-inflammatory therapy [39]. There is more recent evidence that mitigating endoplasmic reticulum stress plays a prominent role in EpFA action against chronic pain [98].

One of the initial observations regarding the action of sEHI was the lack of activity in the absence of a pain state *in vivo* [57]. This has been recapitulated in several models and laboratory species over time [85–87], but earlier led to an investigation of the required factors for action in pain states. It has been hypothesized and data suggest that elevated cAMP plays a role in initiating analgesic action of the EpFA [97], and this

may add to the usefulness of sEHI to combat the pain of opioid withdrawal in which cAMP is increased [99] as seen in Fig. 2.

The evidence that sEH inhibition is effective both against inflammatory and neuropathic pain models was found in preclinical models; however, this translated to efficacy in an actual clinically presenting condition in yet another species. The sEHI *t*-TUCB was effective against equine laminitis when given in addition to the failed standard-of-care drugs and rescued a horse slated for euthanasia suffering from this neuropathic pain condition [100]. The efficacy of sEHI against this clinical presentation of a very complex and severe pain pathology demonstrated that the analgesia mediated by the EpFA extends beyond nociceptive withdrawal threshold assays. Additional evidence revealing the analgesic efficacy of sEHI against chronic pain was collected using operant testing methods to assess pain relief in preclinical models. Mice induced with diabetic neuropathy exhibited a dose-dependent response in the conditioned place preference (CPP) assay used to demonstrate pain relief as reward (negative reinforcement) [87]. The assay can also be used in its classical format to demonstrate reward potential of substances (positive reinforcement), and using this paradigm, the sEHI demonstrated no reward potential administered to both healthy wild-type and sEH null mice [87]. In further experiments in the CPP assay, there was no analgesic response using the sEHI in sEH null mice with diabetic neuropathy, but direct administration of DHA-derived EpFA (EDPs) elicited a CPP response indicating pain relief [60]. Inhibition of sEH was also active in Akita mice, a naturally occurring genetic type I diabetes with neuropathy [101].

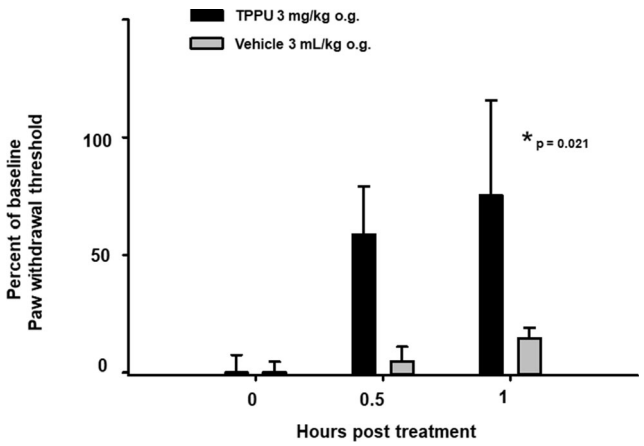

Fig. 2 The sEHI TPPU attenuates morphine withdrawal pain in tolerant rats. Male SD ($n = 8$) rats were administered morphine 10 mg/kg twice daily for 10 days to induce tolerance. The morphine was then withheld, and rats were tested at 18 h after their last morphine injection for their spontaneous withdrawal pain. The spontaneous withdrawal MWTs dropped from their naïve baseline of 75.3 ± 2.5 to 37.6 ± 4.2 g at 18 h of spontaneous withdrawal after their last injection on day 10. The painful 18 h MWT was normalized to 0 and used as the baseline for the calculation of analgesic measure (score/baseline × 100). TPPU or vehicle was given by oral gavage (o.g.) to each group ($n = 4$), and they were assessed at the listed time points in an electronic von Frey assay

The use of the CPP paradigm demonstrated sEHI did not have reward potential and were effective against diabetic neuropathic pain; however, these tests often relied on short duration trainings (maximum 3 days). Further studies investigated the effect of longer duration dosing which better approximates the potential use in human clinical settings for a chronic condition. These experiments demonstrated both that tolerance to sEHI-mediated analgesia did not develop and that repeated 2-week dosing lacked the CNS side effects of other standard-of-care drugs used to treat neuropathy [102].

EpFA Mechanisms of Action in Analgesia

Small molecule inhibitors of the sEH have allowed for the elucidation of the biological activity of EpFA and demonstrated the anti-inflammatory, anti-hypertensive, and analgesic properties of these lipids. Preclinical models of disease have identified multiple biological actions that contribute to the analgesic properties of EpFA (Fig. 3). These actions include reducing endoplasmic reticulum (ER) stress, preventing or reversing endothelial cell dysfunction (ECD), and stabilizing mitochondrial function. EpFA act to regulate cellular stress caused by reactive oxygen species and shift the ER stress response toward maintenance of homeostasis and away from activating inflammatory pathways, cell senescence, and cell death.

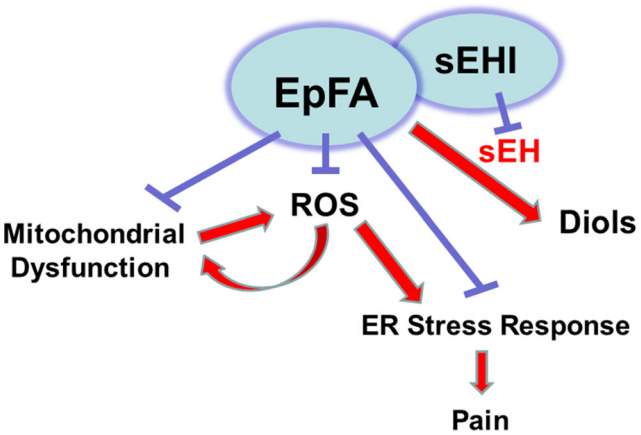

Fig. 3 Mechanisms of EpFA and sEH inhibition mediated analgesia. EpFA stabilized by inhibiting the sEH enzyme are analgesic and this occurs through multiple biological actions. EpFA reduce ER stress responses and limit reactive oxygen species (ROS) which leads indirectly to stabilization of mitochondrial function. EpFA also act directly to block mitochondrial dysfunction, and because the mitochondria are the major cellular organelles involved in ROS production, this helps in homeostatic regulation of ROS as well. Inhibiting the sEH enzyme stabilizes the EpFA and also limits the production of diol metabolites, some of which have demonstrated pro-inflammatory actions. EpFA mediate beneficial effects in all of these processes shifting the ER stress response to homeostasis and limiting pain

Regulation of ER Stress

The ER is the major cellular organ responsible for the production and transport of proteins and also lipids. The ER extends from the nuclear membrane into the cytosol of the cell near the plasma membrane and is in close proximity with mitochondrial membranes [103, 104]. The ER recognizes disturbances in homeostasis such as misfolded proteins in the ER lumen and responds with changes in metabolism and gene expression, or in prolonged situations, in activation of apoptosis. ER stress is transduced by several proteins (inositol-requiring enzyme 1α (IRE-1α), protein kinase RNA-like endoplasmic reticulum kinase (PERK), and activating transcription factor 6 (ATF6)) that activate both the survival and apoptotic pathways as regulatory mechanisms in the cell to combat or overcome cellular stress [105]. It is associated with several diseases such as diabetes in which glucose, reactive oxygen species, and other metabolites move ER stress away from a homeostatic function toward induction of inflammatory mediators and cell senescence pathways. Dysregulation of this ER stress pathway has been implicated in a variety of diseases, and ER stress is reduced in preclinical models, both in animals treated with sEHI and in human cellular models treated with EpFA (Fig. 4) [106, 107]. The efficacy of sEHI and EpFA against ER stress extends to inflammatory pathologies and peripheral neuropathy [98, 108, 109]. Recently, it was demonstrated that modulating the inositol-requiring enzyme 1α activation (one of the major ER stress pathways) in leukocytes results in compromised COX-2 and prostaglandin E synthase induction which are typically upregulated in inflammatory pain [110]. This relates to the previously mentioned LPS sepsis model where it was not surprising to find that EETs increased and diols decreased with sEH inhibition [93]. However, the dramatic decrease in PGE_2 and other inflammatory eicosanoids was unexpected in this study, where sEHI were shown to transcriptionally downregulate COX-2 expression after LPS challenge [93]. The accumulation of information over the last few years supports that EpFA reduce ER stress [111] suggesting a possible mechanism of reducing COX-2/mPGES-1–driven inflammatory pain through regulation of the ER stress pathway.

ER stress is also implicated as a causative factor in driving ECD through both inhibition of nitric oxide and EDHF-driven vasodilation [112]. Activation of PERK stimulates macrophages to polarize toward a pro-inflammatory (M1) phenotype increasing cellular adhesion markers on endothelial cells [113]. When ER stress activating kinase PERK is inhibited, the macrophages shift to an anti-inflammatory, M2 phenotype [113]. These highly interdependent mechanisms for driving both inflammatory and neuropathic pain are particularly important in diabetic neuropathy in which ECD may drive neuronal degeneration [114, 115] and microangiopathy correlated with neuropathy in T2DM patients [116]. In addition, EETs as a class of EpFA are known to decrease with increased ECD in aging [117]. sEHI are known to block ER stress markers and neuropathic pain in modeled diabetes [98] indicating their ability to attenuate diabetic neuropathy in a pleiotropic manner. At this point, activated ER stress markers have been found in every pathological state in which sEH inhibitors show benefit, and sEHI have demonstrated beneficial effects in every pathology tested in which ER stress is involved, including both acute and chronic pain.

Mitochondria Function and Pain

A 2012 review summarized the role the mitochondria played in neuropathic and inflammatory pain [118]. This review showed that 5 different mitochondrial functions had important roles in pain: energy generation, intracellular calcium mobilization, ROS generation, apoptotic pathway, and the mitochondrial permeability transition pore (mPTP). This idea is bolstered by more recent studies which found mitochondrial dysfunction in animal models of chronic pain and patients with chronic pain [119]. A recent 2020 review summarized results which show that although chronic pain was not considered a core symptom in persons with mitochondrial disease, it is a commonly observed clinical symptom [120].

A significant side effect of the chemotherapy drug paclitaxel is the painful neuropathy that develops in patients. Using rats, it was found that paclitaxel causes mitochondrial dysfunction and reduced levels of adenosine triphosphate (ATP) in the dorsal root ganglia neurons [121]. Other studies also support that cancer chemotherapies trigger neuropathic pain by significantly reducing the amount of energy (ATP) available and by damaging the mitochondria in the peripheral nerves [122]. Neuropathy is 1 of the common symptoms observed with diabetes, and some evidence suggests that the mitochondria play an important role in diabetes-related neuropathy [123]. Dr. Chung's group [124] used mice to investigate the role of mitochondrial calcium uptake for synaptic plasticity in pain. They found that both calcium uptake and increased ROS production were needed for the pain-related synaptic changes. Increased Ca^{2+} uptake increases the amount of ATP produced by the electron transport chain, and with increased electron transport chain activity, ROS generation (in the form of superoxide ions) also increases. As such, the experimental data to support a major role for mitochondria in neuropathic pain is very strong. How lipid mediators and epoxy-fatty acids affect mitochondrial function are discussed below.

Lipids and Mitochondrial Function

Except in red blood cells, mitochondrial function is critical for cell survival in humans. The mitochondria are found in the cytoplasm of cells and are relatively large organelles that generate energy in the form of ATP. ATP provides cells essential

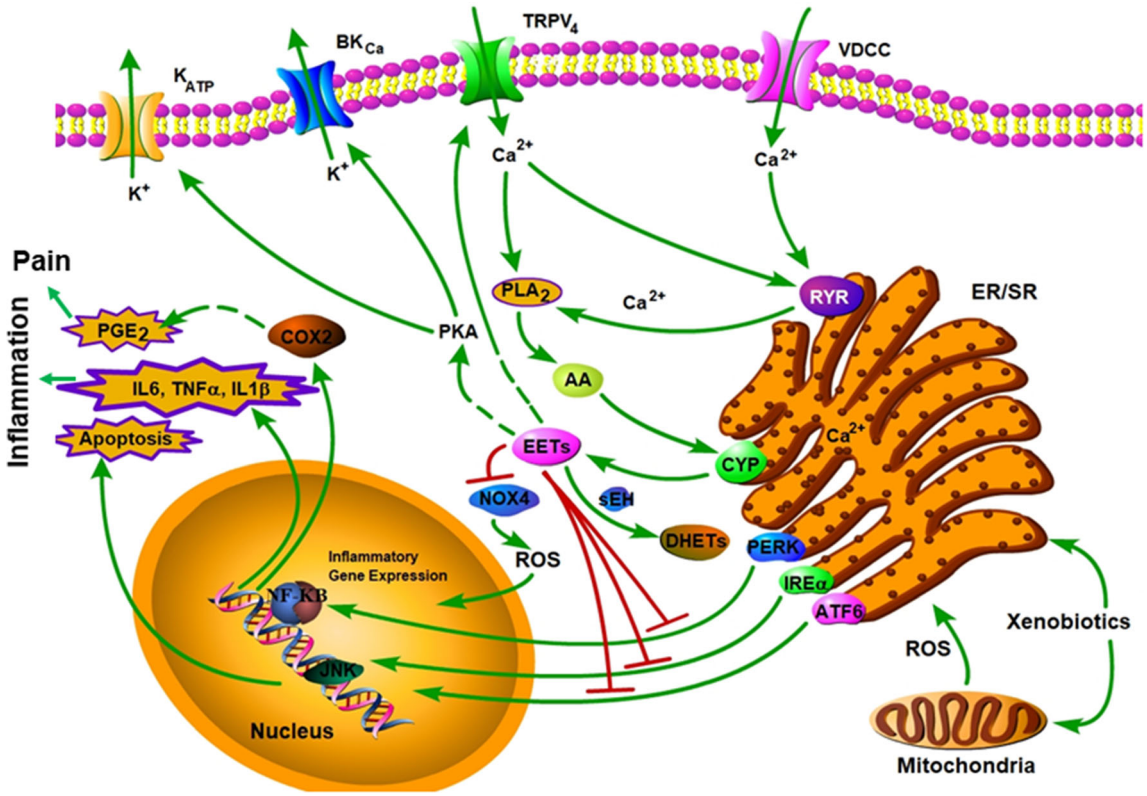

Fig. 4 Major pathways of ER stress-induced pain, inflammation, and apoptosis. Prolonged ER stress is associated with pain, inflammation, and apoptosis through various pathways. Calcium release from the ER/SR can induce activation of PLA_2 resulting in increased levels of arachidonic acid (ARA). ARA is metabolized by cytochrome P450 enzymes on the membrane of the ER/SR forming EETs which inhibit several pathways including the PERK, IREα, and ATF6 pathways associated with inflammation and apoptosis. Activation of COX-2 enzymes results in increased levels of PGE_2 from ARA. PGE_2 is a well-known pain mediator. Phosphorylated PERK can activate ATF4 and NF-κB binding to DNA, whereas phosphorylated IREα can activate JNK binding to DNA. CYP = cytochrome P450; PGE_2 = prostaglandin E_2; NOX4 = NADPH oxidase 4; PLA_2 = phospholipase A_2; PERK = protein kinase R (PKR)-like endoplasmic reticulum kinase; IREα = inositol-requiring enzyme 1α; ATF4 = activating transcription factor 4; ATF6 = activating transcription factor 4; K_{ATP} channels = ATP-sensitive potassium channel; BK_{Ca} channel = calcium-activated potassium channel; $TRPV_4$ channel = transient receptor potential vanilloid 4 channel; VDCC channel = voltage-gated calcium channel; RYR = ryanodine receptor; K^+ = potassium ions

energy to carry out many cellular processes such as muscle contraction and ATPase channel activities. However, the mitochondria also have several other functions including calcium homeostasis, the initial production site for steroid hormones, nerve conduction, oxidative free radical production, cell death, cell growth, and synthesis of biomolecules. As such, because of the importance of the mitochondria to cellular function, many diseases have been associated with mitochondrial dysfunction, ranging from cancer and heart disease to neurodegenerative diseases.

The proper functioning of the mitochondria requires appropriate lipid compositions in the mitochondrial membranes [125]. The stability and activity of the electron transport chain supercomplexes, as well as protein translocases, are affected by the phospholipids present in mitochondrial membranes. As such, it is not surprising that fatty acids are regulators of the structure and function of the mitochondria. Fatty acids can act as substrates for ATP production in the mitochondria [126], and lipids are also important regulators of mitochondrial ROS formation. Although ROS can be produced by several nonmitochondrial sources such as xanthine oxidase, experimental data suggest that the mitochondria are the major cellular organelles for ROS production [127]. In the mitochondria, the major site of ROS production is the electron transport chain [128]. Using bovine heart mitochondria and mitochondrial particles, ARA was found to selectively inhibit complexes I and III [129]. When respiring mitochondria provided succinate or pyruvate and malate as substrate were treated with ARA, the levels of hydrogen peroxide produced by the electron transport chain were significantly increased. These results suggest that ARA stimulates ROS generation by the mitochondrial electron transport chain. ARA and other PUFA also can act as propagators of reactive oxygen species because of their propensity to form reactive hydroperoxides. ARA also induces mitochondrial permeability transition in isolated rat liver mitochondria and in rat hepatoma MH1C1 cells [130] resulting in increased cytochrome c release from the mitochondria and induced apoptosis (as judged by the appearance

of annexin V-positive cells). Another report provided evidence using isolated myocytes that LOX products of ARA induced mitochondrial depolarization and contributed to arrhythmogenesis following ischemia–reperfusion injury [131]. Another common lipid, linoleic acid, has been found to promote mitochondrial biogenesis as well as reduce damage to the mitochondria caused by streptozotocin (which induces diabetes) in RIN-m5F cells [132].

In healthy cells, ROS produced by the electron transport chain is needed to activate some cellular process and to regulate other processes such as cell growth. As ROS levels rise, intracellular antioxidant systems are present which reduce the levels of ROS. If the levels of ROS present significantly exceed the capacity of the endogenous antioxidant systems, several deleterious effects are observed in cells, including cell death [133]. Although the mitochondria produce ROS, they are also susceptible to the ROS they produce as well as the ROS produced outside the mitochondria, with some effects including mitochondrial DNA damage and damage to electron chain complexes [134, 135]. Cells with higher levels of ROS increased the levels of oxidized lipids and proteins, which over time results in oxidative injury. Oxidized proteins can be degraded by the proteasome, which is an abundant proteolytic enzyme complex in the cytoplasm and nucleus of cells [136].

Mitochondrial Dysfunction and EpFA

ARA peroxidation can occur when sufficient levels of ROS are present, causing the formation of isofurans and isoprostanes [137, 138]. Some of the pathophysiological effects associated to oxidative injury have been linked to isofurans and isoprostanes [139]. In mice treated with doxorubicin, which induces mitochondrial dysfunction and ER stress, the ratio of isofurans to F2-isoprostanes in kidney was increased as compared with that in control mice [140]. Because increased levels of isofurans will result in lower levels of ARA, less ARA will be available for producing the protective EETs, increasing the chance of mitochondrial dysfunction.

Prolonged opening of the mPTP has been associated with cytochrome c release, mitochondrial swelling, and necrotic cell death [137]. Several reports suggest a role for the mitochondrial phospholipase isoform iPLA2γ in mPTP function [141]. iPLA2γ cleaves membrane phospholipids to release ARA. ARA can then be metabolized into many different eicosanoids. A few eicosanoids have now been found to affect mPTP function. 14,15-Epoxyeicosatrienoic acid (14,15-EET) reduced myocardial mPTP opening and was protective in an *in vivo* rat model of infarction [142]. Another metabolite of ARA, 20-hydroxyeicosatetraeonic acid (20-HETE), has been shown to play an essential role in the pathogenesis of hypertension [143]. 20-HETE was found to increase the levels of mitochondrial superoxide in pulmonary arteries. Interestingly, inhibition of glucose-6-phosphate dehydrogenase partially blocked 20-HETE–mediated superoxide formation by activating protein kinase G1α [143].

In cardiac tissue, ischemia–reperfusion (IR) injury is associated with the opening of the mPTP and subsequent mitochondrial damage [144, 145]. The mPTP opening is also affected by the mitochondrial membrane potential ($\Delta\Psi$m), with reduced mPTP opening at higher $\Delta\Psi$m. The level of $\Delta\Psi$m is vital as lower ATP formation is observed at lower $\Delta\Psi$m levels. EpFA and EETs have been shown to reduce mitochondrial damage that usually occurs during IR [146].

Using mitochondria purified from left ventricular tissues of human heart failure patients and control hearts, the addition of Ca^{2+} resulted in mPTP opening and increased production of 5-, 8-, 11-, 12-, and 15-HETEs and decreased 14,15-EET production relative to control hearts [147]. Baicalein, a lipoxygenase inhibitor, eliminated the Ca^{2+}-induced mPTP opening in failing hearts.

Effect of EpFA and sEH Inhibition on Mitochondrial Dysfunction

Mitochondrial dysfunction is associated with 2 key features: increased ROS and decreased ATP production. Another feature that is associated with mitochondrial dysfunction is the cytoplasmic proteasome complex showing some type of dysfunction. Increased ROS and proteasome dysfunction are observed in most, if not all, animal models that have upregulation of their intracellular stress response. Proteasome inhibition is known to be an important contributor to mitochondrial dysfunction [148].

H9c2 cardiac cells exposed to laser-induced oxidative stress showed increased mPTP opening and a reduction in $\Delta\Psi$m [146]. Treating these cells with 14,15-EET, an ARA-derived EpFA, significantly decreased the effects of the oxidative stress on mPTP and $\Delta\Psi$m. These results suggest that a major protective effect of EETs is to limit the opening of the mPTP (Fig. 5). This process is likely to involve mitochondrial K^+ channels, as the use of both 14,15-EET and paxilline (high-conductance Ca^{2+}-activated K^+ channel inhibitor) or K^+ channel blockers 5-HD (mitochondrial ATP-sensitive K^+ channel inhibitor) decreased the EET-mediated reduction of $\Delta\Psi$m [146]. Mitochondrial K^+ channels are important for maintaining $\Delta\Psi$m [146]. Other studies also support the involvement of mitochondrial K^+ channels in EET-mediated effects [149]. Inhibition of sEH (BI0611953) in H9c2 cells resulted in diminished proteasome and caspase activity and ROS generation [150].

Because inhibition of sEH results in increased levels of EETs [151], sEH inhibition should be cardioprotective for IR injury. Several labs have now shown that EH inhibitors

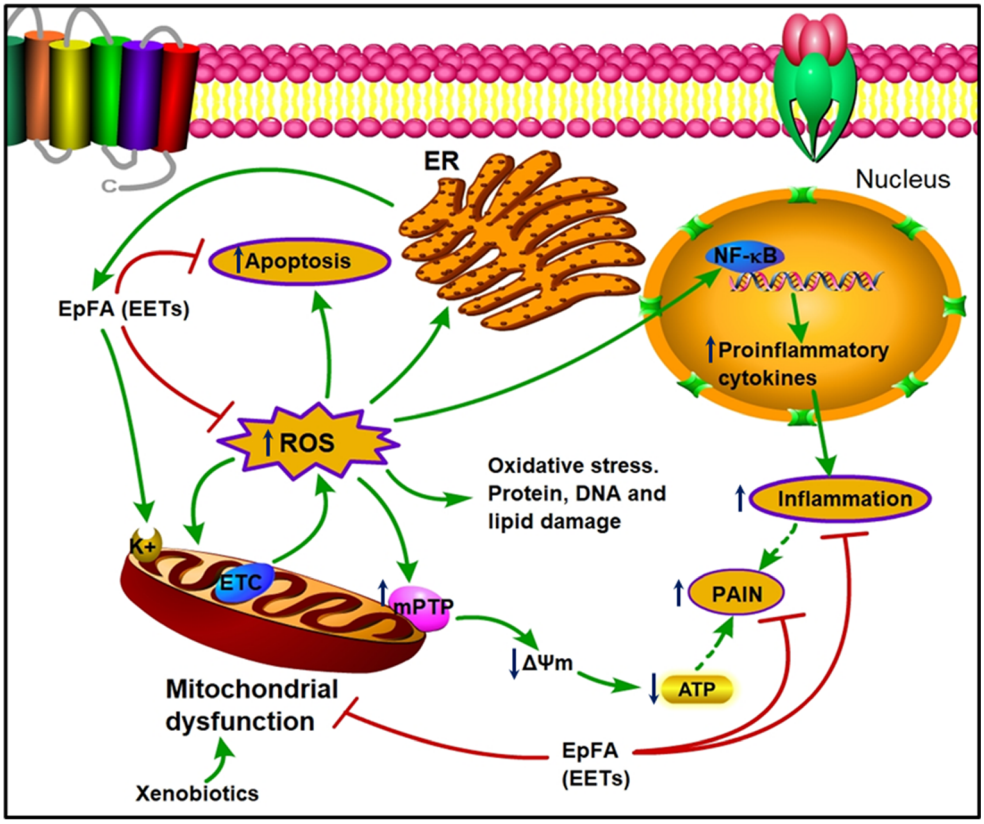

Fig. 5 EpFA block mitochondrial dysfunction. The continual function of mitochondrial to produce ATP generates reactive oxygen species (ROS), but when ROS are excessive, this can also result in oxidative stress and mitochondrial dysfunction. In mitochondrial dysfunction, the extended opening of the mitochondrial permeability transition pore (mPTP) is associated with cytochrome c release, mitochondrial swelling, and necrotic cell death. EpFA, specifically EETs, reduce mPTP opening and protect against damage. Opening of the mPTP decreases the mitochondrial membrane potential ($\Delta\Psi m$). EpFA additionally act to limit ER stress and apoptosis as a result of excessive ROS. By reducing mitochondrial dysfunction, oxidative and ER stress the EpFA limit downstream inflammation and pain

(BI00611953), sEH knockout mice, or 11,12-EETs are protective against IR injury [150]. In starved HL-1 cardiac cells, mitochondrial cell function was improved by 14,15-EET or UA8 (a synthetic compound with both EET-mimetic and sEH inhibitory properties) [152, 153]. Interestingly, EETs reduced proteasome activity in neonatal cardiomyocytes and starved HL-1 cells [152].

An important question is: How do EET and EpFA-mediated protective effects reach the mitochondria? Although this is somewhat unclear, several possibilities exist. Some ER CYP450 enzymes such as rat CYP1A1 and mouse CYP1A2 have been shown to be translocated into the mitochondria and are able to conduct CYP450-dependent ARA metabolism [154]. These P450 isozymes have been shown to be effective *in vivo* and *in vitro* at making EpFA. Caveolins, especially caveolin-1, could be a requirement for the cardioprotective effects of EETs [155]. There is also evidence that EETs may be increasing the levels of certain antioxidant enzymes which will result in decreased levels of ROS [156, 157]. So, although there remains much to be elucidated about the manner in which this occurs, evidence exists regarding its occurrence, including the end results of the beneficial actions of EpFA.

Beyond Pain

sEH inhibitors have been shown to work peripherally to control acute and chronic pain. However, the role of lipids in the brain is without question because the lipid composition of the brain is close to 50% of dry weight [158]. The brain contains little triglyceride which highlights the role of lipids as structural components and signaling messengers in cells *versus* energy storage in adipocytes. Of these structural and signaling lipids, 50% are phospholipids that incorporate long-chain fatty acids which are up to 30% of the total fatty acid content [158, 159]. Although sEH is present in the peripheral nervous system, it is also present in the brain along with the long-chain fatty acid substrates of CYP450 metabolism. Thus, the biosynthetic machinery for EpFA is present and inhibiting their degradation with sEHI has demonstrated improvements in modeled neurodegenerative pathologies such as Parkinson's disease (PD), epilepsy, and depression [160–162]. sEH inhibition has demonstrated activity in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine preclinical model of Parkinson's disease in mice [160, 163]. Importantly, in human dementia with Lewy body (DLB) patients, sEH expression and the ratio of phosphorylated α-synuclein to α-synuclein were increased in the striatum [160]. Thus, inhibiting sEH may provide a benefit in human PD pathology in addition to the induced PD models. Evidence is accumulating that increased sEH message and protein are markers as well as drivers of inflammation and other pathologies in both the peripheral tissues and central nervous system. In multiple diseases of the CNS, this hypothesis seems to hold in which the sEH enzyme is at higher levels in those parts of the brain thought to be

associated with the symptoms, and sEH inhibitors have been shown to attenuate the pathological effects [162, 164].

The presentation of bioactive lipids *in vivo* is highly complex [165]. They share many of the same metabolizing enzymes and undergo rapid *de novo* synthesis and degradation, plus the temporal and regional aspect of their appearance makes defining biological actions challenging. Adding another layer to this complexity is the question of whether the *in vivo* and *in situ* existence of these molecules is even represented by postmortem analysis of preclinical samples [166]. Additionally, much of the information surrounding the lipids as agonist or antagonists or both actions are determined *in vitro* or in reduced settings. However, in the organism, these bioactive lipids exist in a multifaceted environment with possible synergistic as well as antagonizing actions of other presenting metabolites and alterations of protein receptor expression depending on the time, tissue, or disease state. Fatty acids can also act both indirectly, affecting lipid protein interactions in cellular membranes, and also directly, at intracellular nuclear receptors such as PPAR-γ and at GPCRs as ligands [36]. Thus, there is still much to describe for the actual mechanistic action of these lipids and the challenge of determining this information *in vivo* with validation of the methods. These issues provide challenges but ultimately improved technical capacity, and repeated experimentation will elucidate the mechanisms at play.

Conclusions

There is still effort being put into identifying a specific receptor for the EETs and all long-chain–derived EpFA. The examples of other lipid ligands binding GPCRs and other channel types give insight that this likely may exist. Clear determination of ligand receptor interaction becomes even more complex when the ligands are highly lipophilic and biologically unstable. However, there is an even greater complexity to consider because these lipid ligands can bind to multiple receptor types and are also additionally metabolized by enzymes from multiple pathways, substantially increasing the number of bioactive metabolites to investigate. Thus, investigations of the effect of lipid ligands at individual receptors, though important, are still nascent and therefore give weight to the outcomes observable in whole animals. Overall, there is substantial evidence of the role of EpFA in nociception including blocking both inflammatory and neuropathic pain. sEHI have allowed a broader investigation of the action of EpFA *in vivo*, and the sEHI and EpFA mimics have great potential to alleviate pain in humans.

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Required Author Forms Disclosure forms provided by the authors are available with the online version of this article.

Compliance with Ethical Standards

Conflict of Interest The University of California holds patents on the sEH inhibitor used in this study as well as their use to treat inflammation, inflammatory pain, and neuropathic pain. KM Wagner, CB McReynolds, and BD Hammock are employees of EicOsis L.L.C., a startup company advancing sEH inhibitors into the clinic.

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