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Authors

Inceoglu, Bora
Bettaieb, Ahmed
Haj, Fawaz G
et al.

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Modulation of mitochondrial dysfunction and endoplasmic reticulum stress are key mechanisms for the wide-ranging actions of epoxy fatty acids and soluble epoxide hydrolase inhibitors

Bora Inceoglu[a,*,‡], **Ahmed Bettaieb**[b,c,*,‡], **Fawaz G. Haj**[d,e], **Aldrin V. Gomes**[f,g], and **Bruce D. Hammock**[a]

[a]Department of Entomology and Nematology, UC Davis Comprehensive Cancer Center, University of California Davis, Davis, CA 95616

[b]Department of Nutrition, The University of Tennessee, Knoxville, TN 37996-0840

[c]Graduate School of Genome Science and Technology, The University of Tennessee, Knoxville, TN 37996-0840

[d]Department of Nutrition, University of California Davis, CA 95616

[e]Division of Endocrinology, Diabetes and Metabolism, Department of Internal Medicine, University of California Davis, Sacramento, CA 95817

[f]Department of Neurobiology, Physiology, and Behavior, University of California Davis, Davis, CA 95616

[g]Department of Physiology and Membrane biology, University of California Davis, Davis, CA 95616

Abstract

The arachidonic acid cascade is arguably the most widely known biologic regulatory pathway. Decades after the seminal discoveries involving its cyclooxygenase and lipoxygenase branches, studies of this cascade remain an active area of research. The third and less widely known branch, the cytochrome P450 pathway leads to highly active oxygenated lipid mediators, epoxy fatty acids (EpFAs) and hydroxyeicosatetraenoic acids (HETEs), which are of similar potency to prostanoids

[‡]Corresponding: Bora Inceoglu, Department of Entomology, University of California Davis, One Shields Ave. Davis, CA 95616, Tel: 011 1 530-752-7519 Fax: 011 1 530-752-1537, abinceoglu@ucdavis.edu. Ahmed Bettaieb, Department of Nutrition, University of Tennessee-Knoxville, Knoxville, TN 37996, abettaie@utk.edu.

[*]Co-first authors

Conflicts of Interest

BI, AB, FH and BH are all inventors on University of California Patents related to this work. BI and BH are founders of EicOsis which is developing sEHI as therapeutics.

Contributors

BI, AB, FGH, AVG, BDH all contributed to writing the manuscript.

and leukotrienes. Unlike the COX and LOX branches, no pharmaceuticals currently are marketed targeting the P450 branch. However, data support therapeutic benefits from modulating these regulatory lipid mediators. This is being approached by stabilizing or mimicking the EpFAs or even by altering the diet. These approaches lead to predominantly beneficial effects on a wide range of apparently unrelated states resulting in an enigma of how this small group of natural chemical mediators can have such diverse effects. EpFAs are degraded by soluble epoxide hydrolase (sEH) and stabilized by inhibiting this enzyme. In this review, we focus on interconnected aspects of reported mechanisms of action of EpFAs and inhibitors of soluble epoxide hydrolase (sEHI). The sEHI and EpFAs are commonly reported to maintain homeostasis under pathological conditions while remaining neutral under normal physiological conditions. Here we provide a conceptual framework for the unique and broad range of biological activities ascribed to epoxy fatty acids. We argue that their mechanism of action pivots on their ability to prevent mitochondrial dysfunction, to reduce subsequent ROS formation and to block resulting cellular signaling cascades, primarily the endoplasmic reticulum stress. By stabilizing the mitochondrial – ROS – ER stress axis, the range of activity of EpFAs and sEHI display an overlap with the disease conditions including diabetes, fibrosis, chronic pain, cardiovascular and neurodegenerative diseases, for which the above outlined mechanisms play key roles.

Keywords

arachidonic acid; epoxydocosapentaenoic acid; epoxy fatty acids; soluble epoxide hydrolase; mitochondria; endoplasmic reticulum stress

1 Introduction

Eicosanoids generated from cyclooxygenase (COX) and lipoxygenase (LOX) mediated metabolism of arachidonic acid (ARA) are well-known bioactive lipids that promote and maintain inflammatory signaling cascades. Efforts towards understanding the fundamental characteristics of the potent and profound actions of these lipid metabolites in addition yielded a multitude of therapeutic approaches to quell inflammation and pain [1, 2]. There is continued interest in therapeutically targeting the enzymes, receptors and metabolites within these two predominantly pro-inflammatory pathways, while the latest identified branch of the ARA cascade, known as the cytochrome P450 branch offers underexploited features and novel therapeutic targets [3].

The cytochrome P450 branch, yields potent ARA metabolites including hydroxyeicosatetraenoic acids (HETEs) and epoxyeicosatrienoic acids (EETs) [4]. Progress attained within the last decade strongly suggests that EETs and other EpFA are opposing counterparts to the largely pro-inflammatory prostanoids, leukotrienes and HETEs [5, 6]. This P450 branch of the ARA cascade is attracting increasing attention both in fundamental regulatory biology and as a clinical target to treat a variety of diseases [7–9]. It is now clear that unsaturated free fatty acids in general are substrates for the cytochrome P450s generating EpFA. There is considerable structural diversity among bioactive lipids bearing an epoxide functionality (Figure 1). Together with the hepoxilins these molecules are classified as epoxy fatty acids (EpFAs).

1.1 Epoxide hydrolase mediated degradation rapidly regulates the titers of EpFAs

The epoxide moiety on an EpFA is dissimilar to the reactive epoxide toxins such as those from aflatoxin or polycyclic aromatic hydrocarbons in that it does not react with nucleophiles in biological milieu. In addition, the lipid backbones of EpFA do not intercalate into nucleic acids which would be critical for genotoxicity. The EpFAs are chemically stable at neutral pH although, consistent with their roles in cellular signaling, exhibit short *in vivo* half-lives due to rapid hydrolysis by epoxide hydration [10]. This property has been a stumbling block that impeded early progress in the field. The breakthrough was contingent on identification of epoxide hydrolase (EH) activity and recognition of the soluble epoxide hydrolase (sEH, *EPHX2,* E.C.3.3.2.10) as an enzyme that rapidly degrades EpFAs followed by tools to chemically and genetically knock out or induce the expression of this enzyme [8]. While mammals express several different epoxide hydrolases, sEH seems to be the common denominator for the inactivation of most, if not all EpFA species in most tissues. In tissue extracts, if sEH activity is blocked with selective inhibitors, much of EpFA degrading activity is eliminated [11]. Spector estimated that most of the degradation of EpFAs was due to sEH explaining why its inhibition increases titers of EpFAs *in vivo* [10]. However, these higher levels of EpFAs seem to be cleared from the system by degradation via alternate pathways limiting the EpFA titers achieved by sEHI. Therefore, degradation by sEH seems to be the major regulatory step that, with biosynthesis and release from phospholipid stores, adjusts the EpFA titers. In at least one exceptional case, brain microsomal EH (mEH, *EPHX1*, E.C.3.3.2.9) may contribute to degradation of EpFAs [12]. The mEH and sEH share a common structure and catalytic mechanism involving the formation and hydrolysis of a covalent intermediate [11]. Even though EpFAs are not among the preferred substrates for mEH, its expression in the brain is much higher than that of sEH suggesting that brain titers of EpFAs are co-regulated by both EHs. In several cases, the efficacy of sEH inhibitors have been enhanced by maintaining animals on a diet enriched with ω-3 and depleted in ω-6, suggesting that epoxides of DHA and EPA are more potent mediators in some systems than the corresponding EETs [13]. Additionally, the ω-terminal epoxides of EPA and DHA are turned over by sEH at much slower rate than most of the other regioisomers [14]. This *in vivo* stability may contribute to the biological activity of these EpFA and to the broad biological benefits of dietary ω-3 fatty acids.

The discovery of potent and selective inhibitors of sEH beginning early 2000s resulted in broader interest towards EpFAs and the sEH [15]. Consequently, simultaneous progress was attained both in fundamental understanding of these bioactive lipid mediators and in therapeutic applications by way of inhibiting sEH. An increasing number of functions and activities of EpFAs and sEHI continue to be reported. With the use of potent sEH inhibitors, numerous groups published key observations that support clinical development of sEH inhibitors [7, 16].

2 Novel paths to positively modulating EpFA titers

The dynamics of EpFA generation is much less clear with the numerous cytochrome P450 isozymes involved in EpFA synthesis [4]. Cytochrome P450 2C and 2Js appear to dominate the synthesis of EpFAs, under normal conditions, but they carry out multiple oxidative

reactions in addition to epoxidation [17]. It appears that synthesis of EpFAs occurs on a continuous basal level and a portion of the *de novo* EpFA mass is diverted to lipid bilayer membrane fractions for storage. However, the rate of EpFAs synthesis is not constant and seems to be acutely responsive to multiple stimuli, particularly under pathological conditions. A recent report demonstrated a biphasic change in EpFA levels over the course of 0–24h of inflammation [6]. Clearly, there are pools of quantifiable intra- and extra-cellular, free acid EpFAs both of which are stabilized and augmented by sEH inhibitors. Tetrachlorodioxin and other xenobiotics induce cytochrome P450 enzymes usually studied for their role in xenobiotic metabolism, sufficiently that they contribute to titers of EpFAs [18]. Since the 2C and 2J as well as a variety of other cytochrome p450s will oxidize multiple unsaturated fatty acids, it is not surprising that the EpFA produced depends in part on the diet as well as the circulating and stored lipids. The dramatic increase in dietary linoleic acid resulting in formation of bioactive 18 carbon metabolites, often termed leukotoxin and iso-leukotoxin, is an example of deleterious effects of dietary lipids [19]. In contrast, the epoxides of ω-3 unsaturated fatty acids such as DHA and EPA in some fish oil appear to be more powerful and generally more beneficial chemical mediators than the corresponding EETs from ARA [13, 14].

A recent report further illustrated the feasibility of enhancing the synthesis of EpFAs while impeding their degradation using omeprazole, a strong inducer of several cytochrome P450s, combined with sEH inhibitor [20]. Individually applied, omeprazole and sub-therapeutic dose of sEHI were devoid of activity whereas the combination was sufficient to modulate the EpFA titers to display an anti-nociceptive effect. The dramatic changes in the levels of EpFAs and the resulting biological effects of altering them illustrate how environmental chemicals and pharmaceuticals can alter this regulatory system. An additional approach to regulate the titers of EpFAs appears to be co-administration of inhibitors of phosphodiesterase with sEHI [21]. Elevated levels of cAMP or cGMP are obtained by blocking their degradation by PDE isozymes. This results in release of free fatty acids from membrane stores [22]. Such treatments resulted in a 10 to 30-fold increase in the ratio of circulating EpFAs to their dihydroxy- metabolites and displayed anti-nociceptive effects. Consequently, the use of sEHI enabled significant progress in understanding the conditions under which EpFAs are synthesized, released, stored and metabolized. The use of sEHI along with other probes offers further advantages in gaining mechanistic insight into the EpFA system and as possible synergists for therapeutic applications [5].

2.1 Converging towards an overarching mechanism of action

Among the remaining scientific challenges is the need for a more comprehensive understanding of how EpFAs function. A myriad of biological functions is attributed to EETs and other EpFAs and these are covered in several recent reviews [3, 7–9, 23, 24]. In many cases, these characteristics were either discovered or substantiated with the use of sEH inhibitors. On the other hand, results from the broader community illustrate the functional equivalency of using individual EpFAs, mixtures of EpFA regioisomers, EpFA mimics, sEH inhibitors, sEH−/− mice, cytochrome P450 inducers and inhibitors, P450 −/− mice and combinations thereof.

Like numerous other signaling molecules, the function of EpFAs and sEHI strongly depend on the biological context. This context dependent activity of EpFAs and sEHI increase the overall complexity but could be key for elucidating their mechanism of action. Specifically, the analgesic efficacy of EpFAs and sEHI appear to occur through activity dependent modulation of pain signaling [25]. In the absence of an underlying painful condition, neither EpFAs nor sEHI display quantifiable effects [26]. The absence of measurable effect on normal physiology is consistently observed across different laboratories and studies involving rodent and non-rodent experimental models. This is in stark contrast to their strong efficacy under pathological conditions [27]. Therefore, EpFAs and sEHI seem to function in a way to maintain homeostasis. This argument is best illustrated by the ability of sEHI to shift both hypertension and hypotension towards normotension in rodent models [28, 29].

A receptor molecule that mediates the multifarious effects of EpFAs is yet to be identified. However, a growing body of evidence demonstrate that differences in structure yield changes in functionality. Different regio- and optical isomers can yield different effects K+ channel function [30, 31]. The parent fatty acid of EpFA is also critical. For example, selective effects of DHA vs. ARA derived EpFAs on angiogenesis have been described, while epoxides and particularly diol metabolites of LA have been observed to be pro-inflammatory and toxic [19, 32–34]. EpFA and their corresponding diols can be further metabolized to bioactive tetrahydrofuran diols by P450s and some are substrates for COX and LOX enzymes [35]. Such findings support the argument that EpFA may function through multiple receptors or transduction systems and the possibility that EpFAs are biased ligands of a G-protein coupled receptor.

Here we discuss two recently described molecular mechanisms mediating EpFAs functions (Figure 1). These appear to unify a framework for the diverse actions of EpFAs in that they are far-reaching, fundamental mechanisms. The caveat is that this contemporary view is an evolving concept and may illuminate how EpFAs act under different disease conditions.

3. The enigma of broad efficacy

Potent and metabolically stable sEH inhibitors (sEHI) allowed by inference a variety of biologists to query the action of EpFA on many pathological conditions. In most cases, these observations were followed up by extensive complementary information. sEHI were first observed to reduce blood pressure [36] and then vascular inflammation and downstream effects such as atherosclerosis. A study showing initial resolution of systemic inflammation [37] was followed by a variety of studies demonstrating dramatic reduction of inflammation in a variety of systems. As these inflammatory pathologies were examined unexpected and usually beneficial effects were observed. For example, sEHI not only reduce periodontal inflammation but also enhanced the regrowth of bone through alteration of the RANK - RANKL system [38]. The reduction of inflammation was observed in multiple models of intestinal, renal, pancreatic, brain and even cardiac inflammation, so it was not a surprise that they reduced atrial fibrillation and fibrosis that lead to cardiac hypertrophy [39]. Since inflammation drives a number of disease states it was not a surprise that sEHI reduce hepatic, renal, pancreatic, and asthma-associated pulmonary fibrosis [40–45]. The compounds were even active in a bleomycin model of idiopathic pulmonary fibrosis [40].

Since sEHI were so effective in resolving inflammation it was no surprise that the sEHI also reduce inflammatory pain [5, 26]. However, it was unexpected to observe a reduction in neuropathic pain since these pain states are often refractory to control with NSAIDs and steroids [25]. This indication will be covered in more detail. The broad activity of EpFA supports the hypothesis that the EpFA act as homeostatic modulators returning pathological conditions such as hypertension, hypotension, or inflammation back toward normal physiology. Broadly the compounds reduce the comorbidities associated with diabetes and in the presence of elevated ω-3 lipids seem to ameliorate the actual disease state. However, a biochemical pathway to explain efficacy on these disparate biological processes remains elusive.

The observation that reduction of the unfolded protein response (UPR) and endoplasmic reticulum stress (ER stress) pathways was associated with the beneficial action of sEHI in a variety of pathological conditions in rodent systems raised the possibility of a universal mechanism of action of EpFA [27, 45–47]. It is well established that high blood carbohydrate can alter biology through the ER stress and in severe states can result in diabetes. Thus, the ER stress pathway can explain how sEHI alleviates many of the pathologies associated with diabetes. Another input into the regulation of ER stress is the increase in reactive oxygen species (ROS) production. ROS themselves can be considered an endogenous chemical mediator and high ROS can be formed by a number of conditions. However, a major source of ROS occurs under conditions that cause mitochondrial dysfunction [48]. Thus, in this review we present the hypothesis that the beneficial effects of EpFA in many disease states can be explained, at least in part, through modulation of the ER stress pathway and stabilization of mitochondria.

4. EpFAs, sEHI, ROS and mitochondrial function

4.1. Mitochondrial function and ROS

Since the mitochondria are the primary source of intracellular energy, efficient mitochondrial function is critical. The mitochondria are also important regulators of cell survival and death [49]. ROS can be generated from many other intracellular sources including xanthine oxidase (XO), lipoxygenases, nitric oxide (NO) synthases, cytochrome P450 enzymes, and nicotinamide adenine dinucleotide phosphate (NAD(P)H) oxidases [50]. However, mitochondria have been suggested to be the major intracellular site of ROS production [51, 52]. ROS can act as a signaling molecule important in the regulation of cellular events such as cell growth. However, high ROS levels were shown to be associated with cell dysfunction and death [53]. To prevent ROS from causing cellular dysfunction, cells have several systems to reduce ROS levels. However, overproduction of ROS leads to oxidative stress and cellular dysfunction when the antioxidant systems are unable to reduce ROS levels. The mitochondria themselves are susceptible to ROS produced in the mitochondria and elsewhere, resulting in damages to electron transport chain components and mitochondrial DNA [54, 55].

ROS has been shown to cause ARA peroxidation, resulting in the formation of isoprostanes and isofurans [56, 57]. Isoprostanes and isofurans show biological properties that may account for some of the pathophysiological effects associated with oxidative injury [58]. In

an animal model of mitochondrial dysfunction (mice treated with doxorubicin), the ratio of kidney isofurans to F2-isoprostanes increased relative to control mice [59]. The ratio of isofurans to F2-isoprostanes was proposed as an indicator of mitochondrial dysfunction since isofurans are preferentially formed under high oxygen tension conditions such as when less oxygen is required by the mitochondria for oxidative phosphorylation [60]. It is possible that the lower ARA levels, due to the formation of isofurans in the presence of high ROS levels, is the result of less EETs being produced leading to mitochondrial dysfunction.

4.2 Ischemia reperfusion (IR), mitochondrial damage, and EETs

Ischemia reperfusion (IR) injury has been shown to cause significant mitochondrial damages including the opening of the mitochondrial permeability transition pore (mPTP) allowing free passage of molecules < 1.5 kDa [49, 61]. The opening of the mPTP is affected by the mitochondrial membrane potential ($\Delta\Psi_m$), which is a measure of the electrochemical gradient formed to generate ATP, with lower $\Delta\Psi_m$ enhancing mPTP opening. A few reports suggest that EETs and other EpFAs may have protective intracellular effects by reducing mitochondria damage that occurs during IR [62]. The loss of $\Delta\Psi_m$ and mPTP opening caused by laser-induced oxidative stress was significantly reduced in in H9c2 cardiac cells treated with 14, 15-EET (1μM) [62]. The protective effect of EET was prevented by the EET antagonist 14,15-epoxyeicosa-5(Z)-enoic acid (1μM, 14, 15-EEZE) [62]. Improved post ischemic recovery following IR was observed in hearts from wild-type mice treated with 11,12-EET, sEH null mice, and mice overexpressing cardiac myocyte-specific human cytochrome P450 2J2 [62]. Co-administration of 14,15- EET and K^+ channel blockers 5-HD (mitochondrial ATP-sensitive K^+ channel inhibitor) or paxilline (high-conductance Ca^{2+}-activated K^+ channel inhibitor) diminished EET-mediated loss of ΔΨm. These results suggest that EET-mediated protection involved limiting mPTP opening and that EET-mediated effects might involve activating mitochondrial K^+ channels that are important for mitochondrial membrane potential [62]. Similar to the studies by Katragadda et al., sarcolemmal ATP-sensitive K^+ channels were found to be important for the EET-mediated effects in starved HL-1 cells [63]. Other studies have also suggested that EETs can activate cardiac mitochondrial K_{ATP} channels [64]. Additionally, Samokhvalov et al. found that AMP-activated protein kinase (AMPK) was important in conferring the positive effects of EETs on starved HL-1 cells [63]. A summary of how mitochondrial ROS affects other intracellular signaling systems is shown in Figure 2.

Treatment of starved HL-1 cells or neonatal cardiomyocytes with UA-8 (13-(3-propylureido)tridec-8-enoic acid), a dual-acting synthetic analog with both sEHI and EET-mimetic properties, improved enzymatic activities of key proteins involved in mitochondrial respiratory chain (citrate synthase, succinate dehydrogenase, and cytochrome c oxidase) [63]. In another study using HL-1 cells, co-treatment of starved cells with UA-8 or 14,15-EET improved mitochondrial function [65]. Aconitase activity, often used to access mitochondrial oxidative stress [66], was decreased after 6 or more hours of starvation, while treatment of cells with UA-8 and 14,15-EET prevented the reduced activity [65]. In the latter study, the beneficial effect of UA-8 and 14,15-EET was prevented by 14,15-EEZE. Mitochondrial respiration, measured as the ratio between the basal mitochondrial oxygen consumption and ADP-stimulated states is used to calculate the respiratory ratio control

(RCR). The mitochondrial RCR, measured as the ratio between the basal mitochondrial oxygen consumption and ADP-stimulated states, is an important indicator of oxidative phosphorylation efficiency [65]. Starvation of HL-1 cells reduced RCR while increasing the ADP/ATP ratio, which were both prevented by UA-8 [65]. In this study, El-Sikhry et al. further demonstrated that EETs were important in preserving mitochondrial function.

Furthermore, Batchu et al. showed that a nicotinamide-based sEHI, BI00611953, provided cardioprotection against IR injury in mice [67]. This cardioprotection was suggested to be partly due to the sEHI maintaining mitochondrial function. BI00611953 reduced the infarct (damaged area) size following IR when compared to control hearts, similar to what was observed in hearts from mice lacking sEH, or hearts perfused with 1μM 11,12-EETs. In H9c2 cells, BI0611953 also delayed the loss of $\Delta\Psi_m$ caused by anoxia-reoxygenation and increased hypoxia-inducible factor (HIF)-1α DNA binding [67]. Use of the plasma membrane K(ATP) channel inhibitor (glibenclamide), or the putative EET receptor antagonist 14,15-EEZE, eliminated the cardioprotection conferred by BI00611953[67].

4.3 Mitochondrial damage and EETs in non-cardiovascular cell types

In addition to the cardioprotective effects of EETs on mitochondrial function discussed above, EETs are also protective against pathological conditions induced by non-cardiovascular mitochondrial dysfunction, such as in hippocampal astrocytes [68]. Exposure of cultured neonatal hippocampal astrocytes to amyloid-β (Aβ) induces mitochondrial dysfunction as judged by reduced $\Delta\Psi_m$ and fragmentation. Pre-incubation of the cells with 11,12-EET or 14,15-EET prevented the mitochondrial depolarization and fragmentation. Reducing the endogenous EET levels with a P450 inhibitor, MS-PPOH, further reduced the $\Delta\Psi_m$ caused by Aβ. Pretreatment with EETs also reduced the decrease in mitochondrial oxygen consumption observed when Aβ is added. A well-established mechanism for Aβ-induced toxicity is through increasing ROS generation [69] which was prevented by EETs [68].

4.4 Potential mechanisms for attenuation of mitochondrial dysfunction by EETs or sEH inhibition

An important aspect of the HL-1 study was that EETs reduced caspase 3 and proteasome activities in starved HL-1 and neonatal cardiomyocytes cells [63]. Another cell-based study using H9c2 cardiac cells showed that BI0611953 decreased ROS generation, proteasome activity and caspase 3 activity [67]. Changes in caspase 3 and proteasome activities were shown to be associated with the intracellular stress response. Proteasomes are proteolytic complexes that are responsible for the degradation of most damaged, misfolded, or unneeded intracellular proteins. Proteasome dysfunction is associated with several major diseases including Parkinson’s disease, Huntington’s disease, cardiac hypertrophy, heart failure, cancer, and autoimmune diseases [70–72]. A recent report suggests that proteasome inhibition is an important contributor to mitochondrial dysfunction and the subsequent increased cytosolic oxidative stress and cell death [51]. How EETs can cause changes in proteasome activity remains to be resolved.

Caspase 3 activation plays an important role in apoptosis [73] and increased or decreased levels of apoptosis occur in diseased cells [74]. Since the release of certain proteins from mitochondria into the cytoplasm is known to activate caspase proteases and cause apoptosis [75], the reduction in caspase 3 is likely to be a consequence of EETs improving mitochondrial function.

One important question is how do EET-mediated effects reach the mitochondria. One possibility is that caveolin-1, and possibly other caveolins, may be important for cardioprotection mediated by EETs [76]. Cardiomyocyte plasma membrane and mitochondria isolated from hearts of wild-type mice subjected to IR showed caveolin-1 loss and lack of caveolae. Plasma membrane and mitochondria isolated from sEH null hearts or hearts from 11,12-EET-treated mice showed increased caveolin-1 and the presence of caveolae when compared to wild-type mice [76].

5. The unfolded protein response

The endoplasmic reticulum (ER) is a dynamic, multifunctional organelle with specialized domains and structures spanning the cell from the nuclear envelope to the plasma membrane [77]. ER functions encompass protein biosynthesis, folding and trafficking as well as calcium homeostasis among others [78]. Hence, the ER plays a critical and elegant role in maintaining cellular and organismic homeostasis [79]. Numerous and fluctuating stressors impact ER function including excessive accumulation of unfolded proteins, hypoxia, oxidative stress, ROS and calcium depletion of ER stores that can lead to ER stress. Cells use potent adaptive mechanisms to counter the deleterious effects of ER stress and to maintain the functional integrity of the ER, collectively known as the unfolded protein response (UPR) [80]. In eukaryotic cells UPR is triggered by transmembrane sensors that detect unfolded proteins in the ER through their luminal domain and convey information through their cytosolic domain. Three canonical branches of the UPR have been well characterized and include the ER transmembrane proteins PKR-like ER-regulated kinase (PERK), inositol requiring enzyme 1α (IRE1α), and activating transcription factor 6 (ATF6) [81, 82]. These initiator proteins respond to changes in protein-folding status and activate distinct and sometimes overlapping pathways. In the absence of stress, these sensors are rendered inactive through binding of their intraluminal domains to the chaperone 78-kDa glucose regulated protein (Grp78, also termed BiP) [83]. Accumulation of unfolded proteins in the ER lumen dislodges BiP from these sensors leading to the oligomerization and activation of IRE1α and PERK, and translocation of ATF6 to the Golgi resulting in a cascade of downstream signaling events [84]. IRE1α activation leads to the unconventional splicing of X-box binding protein 1 (XBP1) mRNA leading to the synthesis of a nuclear XBP1 form which induces the transcription of genes encoding ER chaperones, proteins involved in ER biogenesis and in secretion [85–87]. The second canonical branch includes PERK, which is a serine/threonine kinase whose autophosphorylation at threonine (Thr980) within its activation loop is essential for its activation [88]. PERK phosphorylates the α-subunit of the eukaryotic translation initiation factor 2 (eIF2α) at serine51, a modification that blocks initiation of mRNA translation in response to ER stress [88–90]. Of note, three other kinases can phosphorylate eIF2α at serine 51: double stranded RNA-dependent kinases (PKR), heme-regulated inhibitor kinase and general control nonderepressible protein

2 [91]. Activation of the third canonical branch of the UPR requires ATF6 translocation to the Golgi body and processing to generate an active transcription factor [92, 93]. Active ATF6 translocates to the nucleus and induces the expression of genes that facilitate the degradation and clearance of misfolded proteins [94]. These UPR arms synergize to attenuate stress by increasing the relative folding capacity by attenuating translation, modulating ER biogenesis and ER-associated protein degradation [95]. However, if the compensatory mechanisms fail to facilitate the adaptation of cells to ER stress, induction of UPR can lead to elimination of stressed cells by apoptosis [96, 97].

6 Modulation of ER stress by soluble epoxide hydrolase

Several studies utilizing genetic and pharmacological approaches establish sEH as a physiologically relevant regulator of UPR and a potential therapeutic target for mitigating complications associated with ER stress. Indeed, sEH genetic deficiency or pharmacological inhibition attenuates ER stress and favorably impacts metabolic homeostasis, fibrosis/tissue remodeling and neuropathic pain [41, 42, 45, 46, 98].

6.1 Metabolic homeostasis

The ER is highly responsive to nutrient status of the cell and impacts responses such as inflammation and stress-signaling pathways that are linked to metabolic regulation and diseases such as obesity and type 2 diabetes [99, 100]. Accordingly, the UPR is a critical determinant of metabolic homeostasis and ER dysfunction is a contributor to the pathogenesis of metabolic diseases. Chronic ER stress in adipose and liver, which significantly impacts their functions, has been described in obese rodents and humans [101, 102]. In addition, up-regulation of ER stress markers such as BiP, phosphorylated PERK/eIF2α in adipose and liver of Ob/Ob mice are linked to insulin resistance and hepatic steatosis [103]. Moreover, activation of eIF2α/ATF4 pathway alters glucose metabolism by decreasing insulin sensitivity in peripheral insulin-responsive tissues [104]. Notably, attenuation of ER stress using chemical chaperones restores systemic insulin sensitivity and enhances insulin action in insulin-responsive tissues of obese and diabetic mice [105], prevents high fat diet (HFD)-induced body weight gain in mice [106], and improves hepatic and muscle insulin sensitivity in obese human subjects in mice while treatment of obese human subjects [107].

A growing body of evidence identifies sEH as a regulator of metabolic homeostasis, at least in large part, by modulating ER stress. sEH deficiency yields a favorable metabolic outcome, prevents HFD-induced insulin resistance and improves glucose tolerance in mice [98]. In addition, sEH deficiency and pharmacological inhibition *in vivo* associate with enhanced hepatic and adipose insulin signaling [98]. Bettaieb *et al* reported that sEH expression increases in liver and adipose tissue of mice fed HFD and in adipose tissue of overweight human subjects concomitant with increased ER stress [46]. In addition, they demonstrated that sEH deficiency and pharmacological inhibition *in vivo* attenuate HFD-induced ER stress in liver and adipose tissue. Moreover, pharmacological inhibition of sEH in HepG2 cells and 3T3-L1 adipocytes mitigates chemical-induced ER stress consistent with being cell autonomous. Importantly, sEH pharmacological inhibition enhances insulin signaling in

HepG2 cells [46]. In line with this observation, cells treated with epoxy fatty acids (sEH substrates) exhibit increased insulin signaling while cells treated with diols (sEH products) exhibit blunted insulin signaling that is mitigated by a chemical chaperone that attenuates ER stress [46]. These findings were confirmed and extended by Lopez-Vicario *et al* who demonstrate that sEH inhibition improves hepatic function through attenuation of ER stress and enhanced autophagy [47]. Together, these studies establish sEH as a regulator of metabolic homeostasis through modulation of ER stress.

sEH through its metabolism of EpFA is also emerging as a significant regulator of pancreas endocrine and exocrine functions. In a type 1 diabetes model (streptozotocin-treated mice), sEH deficiency and pharmacological inhibition attenuate hyperglycemia and promote insulin secretion [108, 109]. In addition, in a HFD-induced model of type 2 diabetes sEH deficiency and pharmacological inhibition increase pancreatic islet mass and vascularization [98]. The molecular mechanisms underlying sEH action in pancreatic β-cells and whether they encompass modulation of ER stress remain to be determined. However, it is worth noting that the UPR (in particular the PERK/eIF2α branch) is indispensable for proper functioning and survival of pancreatic β-cells in response to metabolic stresses such as increased demand for insulin [110] [80, 110, 111]. sEH is also a contributor to pancreas exocrine functions and is implicated in chronic and acute pancreatitis. Indeed, simultaneous pharmacological inhibition of sEH and RAF1 proto-oncogene serine/threonine kinase (c-RAF) reduces the severity of chronic pancreatitis [112]. Moreover, sEH deficient mice exhibit attenuated chemical-induced (cerulein- and arginine-induced) acute pancreatitis [42]. Importantly, sEH pharmacological inhibition, before and after induction of acute pancreatitis, mitigates the severity of disease in mice suggesting that sEH inhibition may be of therapeutic value in this disease [41]. Moreover, sEH pharmacological inhibition was associated with decreased cerulein- and arginine-induced pancreatic inflammatory response and ER stress. Of note, activation of ER stress is associated with acute pancreatitis [113] and treatment with ER chaperones mitigates the disease severity in animal models [114, 115]. Collectively, these studies open the possibility that modulation of EETs and other EpFA to reduce ER stress may be a potential approach to prevent perturbations of pancreatic exocrine functions and treat pancreatitis. Yet, more studies are required to determine whether this is indeed a possibility.

6.2 Fibrosis and tissue remodeling

Activation of the canonical branches of the UPR is established in models of fibrotic diseases including heart, lung, hepatic and renal fibrosis [116–119]. For example, activation of IRE1α or ATF6 promotes TGF-β-induced myofibroblastic differentiation of lung fibroblasts and inflammation of epithelial cells and their mesenchymal transition [120]. On the other hand, the chemical chaperone 4-phenylbutyric acid (4-PBA) attenuates cardiac fibrosis [121] as well as TGF-β1 expression and Smad signaling in the lung [122] and liver [123].

Several studies establish that sEH deficiency/inhibition attenuates the development of fibrosis in multiple organs. sEH deficiency and pharmacological inhibition (using 1-trifluoromethoxy-phenyl-3-(1-propionylpiperidin-4-yl) urea; TPPU) attenuate hepatic fibrosis and ER stress induced by carbon tetrachloride in mice [45]. Further,

pharmacological inhibition of sEH using two additional inhibitors (1- trifluoromethoxy-phenyl-3 (1-methylsulfonyl)piperidin-4-yl) urea; TUPS and *trans*-4-{4-[3-(4-trifluoromethoxyphenyl)ureido]cyclohexyloxy}benzoic acid; t-TUCB) yields comparable effects on ER stress and fibrosis indicating that these effects are not unique to a particular probe but a characteristic of inhibition of sEH [45]. In addition, EETs analogs reduce renal fibrosis in a mouse model of unilateral ureteral obstruction. These protective effects are mediated, at least in part, through downregulation of NF-κB, TGF-β1/Smad3 and inflammatory signaling pathways [124, 125]. Moreover, sEH pharmacological inhibition using TPPU decreases bleomycin-induced inflammation and deposition of collagen in lungs of mice [40]. Similarly, administration of 14,15-EET suppresses cigarette smoke extract-induced inflammation and apoptotic cell death of lung epithelial cells through attenuation of ER stress [126]. Collectively, these studies establish a role for sEH in fibrotic diseases and suggest that attenuation of ER stress through sEH inhibition may serve as a potential therapeutic target for these diseases either alone or with other inhibitors of mitochondrial dysfunction, ER stress or aspects of the UPR response.

6.3 ER stress is a key determinant of neuropathic pain

Although the role of ER stress is well established for a number of neurological disorders such as Alzheimer's, Parkinson's, amyotrophic lateral sclerosis and prion disease, these conditions are invariably chronic and degenerative diseases. In contrast, we demonstrated a key role for the ER stress pathway using models of both chronic and acute pain [27]. Studies investigating the *in vivo* involvement of ER stress pathways in various disorders consistently utilized models of chronic diseases since it was thought that ER stress occurs over a long period of time such as weeks to months. Therefore, it was surprising to observe rapid and robust pain responses when two structurally and mechanistically distinct ER stress inducers were administered to the glaborous skin of the hind paw in rats. Although injection of tunicamycin and dimethyl celecoxib both resulted in nocifensive behavior and drastically reduced withdrawal responses within minutes, the two inducers also had significant phenotypical differences. Both ER stress inducers increased sensitivity to tactile stimulation, while tunicamycin led to decreased sensitivity to thermal stimuli. In contrast, dimethyl celecoxib did not induce changes on thermal withdrawal threshold. Remarkably, a small molecule blocker of ER stress response, 4-PBA reversed the rapid pain-related behavior in these models and other models of chronic pain along with preventing the activation of the UPR. Since the publication of these key results several other studies corroborated the role of sub-arms of the UPR, in particular, phosphorylation and activation of eIF2α, in nociceptive physiology [127, 128].

Although the role of ER stress in regulating pain responses continues to emerge, we demonstrated the causal role of activated ER stress pathways in diabetic neuropathy [27]. The mechanistic understanding of diabetes induced neuropathy is still incomplete and multiple pathological mechanisms seem to be responsible for the progressive degeneration of nerve fibers, starting from distal sites. However, the activation and sustained presence of the ER stress response in peripheral nerves seem to be a uniting feature that could cooperate with and encompass other mechanisms and cause peripheral neuropathy. High levels of glucose have been demonstrated to lead to the activation of UPR branches [105]. In

peripheral nerves, ER components are distributed across and juxtaposed with mitochondria along the axons [129]. The protein synthetic machinery including ribosomes also travel across the axons to the nerve endings. Excess glucose and active ER stress signaling seems to predispose peripheral nerves to aberrant firing and activation of apoptotic signaling. These anatomic and physiologic constraints seem to be in line with earlier occurrence of neuropathic pain in type I vs type II diabetic patients. Furthermore, the importance of mitochondria generated reactive oxygen species and the contribution of numerous endglycation products to both ER stress and pain have been demonstrated independently. Therefore, in peripheral nerves of diabetic patients the unique spatial disposition of mitochondria and ER components may underlie the susceptibility of these neurons to ROS generation and simultaneous activation of ER stress. These events could then maintain the conditions which ultimately lead to neuropathy. In summary, work on sEH and EpFA resulted in identification of the key role of ER stress responses in chronic pain. Even though this concept and related findings are new and limited in scope, they argue that therapeutic targeting of the ER stress pathway or its components is a potential approach for the treatment of diverse painful conditions.

6.4 Other modes of action of EETs

In addition to the mode of actions mentioned above, other cellular events may mediate the beneficial effects of EpFAs. Exogenous EET supplementation *in vitro* attenuates the rise in intracellular Ca^{2+} and increased SERCA2a protein levels and activity. These protective effects are thought to be mediated through the role of EETs in increasing the expression of antioxidant enzymes resulting in reduced levels of reactive oxygen species, thus preventing SERCA oxidation [130]. Likewise, the overexpression of the CYP2J2, a predominant epoxygenases responsible for the biosynthesis of EETs in heart and endothelial cells suppressed ER stress in mice cardiomyocytes and protected against cardiac failure by maintaining intracellular Ca^{2+} homeostasis and SERCA2a expression and activity [131]. In line with these findings, overexpression of the rat homologue CYP2J3 prevented high fat diet-induced ER stress in adipose tissue of rats and inhibited thapsigargin-induced ER stress in differentiated 3T3-L1 mouse adipocytes [132]. In agreement with these observations, exogenous administration of EETs to differentiated 3T3-L1 adipocytes was protective against ER stress and resulted in increased adiponectin expression and secretion [132]. Moreover, pretreatment of lung epithelial cells with 14,15-EET decreased cigarette smoke extracts-induced expression of ER stress markers and was protective against apoptotic cell death [126]. The protective effects of epoxy fatty acids against ER stress seem to be accompanied with a reduction in mitochondrial dysfunction, inflammation and production of ROS, which are closely linked with ER stress [133]. In agreement with these observations, exogenous EETs, or CYP2J2 overexpression, inhibited mitochondrial dysfunction and decreased the levels of oxidative stress and cell apoptosis in lung tissues induced by ischemia/reperfusion in a human pulmonary artery endothelial cells [134]. Additionally, exposure to cigarette smoke extracts increased ROS generation and the phosphorylation of p38 and JNK, which were all attenuated by pretreatment with 14,15-EET. Collectively, these findings establish EpFAs as a significant contributor to ER stress and mitochondrial dysfunction and identify sEH as a novel promising target to prevent ER/mitochondria stress and their associated diseases.

6.5 Potential mechanisms for attenuation of ER stress by sEH deficiency

The molecular mechanisms that underlie attenuation of ER stress by sEH deficiency and pharmacological inhibition remain to be determined. A potential mechanism involves regulation of calcium homeostasis by EETs. Often, elevation of intracellular free calcium results from inhibition of sarco/endoplasmic reticulum calcium ATPase (SERCA) activity [135]. Exogenous supplementation with EETs *in vitro* attenuates the rise in intracellular calcium and increases SERCA2a expression and activity [136]. Likewise, overexpression of the CYP2J2, a predominant oxygenase responsible for EETs biosynthesis suppresses ER stress in mice cardiomyocytes and protects against cardiac failure by maintaining intracellular calcium homeostasis and SERCA2a expression and activity [130]. These mechanisms would reduce the response of ER stress to agents such as glucose, xenobiotics, and ROS. Another potential mechanism through which EETs can alleviate ER stress is decreased ROS production. EETs reduce ROS production and preserve mitochondrial function [137] and recent studies suggest that mitochondrial ROS production and oxidative stress can contribute to ER stress [138, 139]. This suggests that increased expression of antioxidant enzymes and reduction in ROS production mediated by EETs [137, 140] may lead to decreased ER stress. Thus, additional studies are needed to decipher the precise molecular mechanisms underlying regulation of ER stress by sEH and the contribution of ER stress to the beneficial effects of sEH deficiency and inhibition [141].

7 Conclusions

Overall, the data strongly suggest that significant reductions in EETs and other EpFA are likely to result in mitochondrial dysfunction, endoplasmic reticulum dysfunction, and subsequent metabolic impairment while increasing EpFA may ameliorate these pathologies. Although experimental data are lacking, it is plausible that EETs could directly prevent the activation of the signaling cascade governed by the three major ER resident sensors IRE-1α, *protein* kinase RNA-like endoplasmic reticulum kinase (PERK), and ATF6. ROS seems to be important contributor to mitochondrial and endoplasmic reticulum dysfunction. However, the source of the ROS production under most conditions is unclear. While the mitochondria have been suggested to be an important intracellular ROS producer [48], several other sources of ROS exist. It is also possible that some cell types produce the ROS which then acts on other cell types (Figure 3). Yet it increasingly appears that modulation of the mitochondria – ROS – ER stress axis by increasing key EpFAs provides a unifying hypothetical mechanism of action for sEHI and EpFA mimics that explain their positive biological effects on what at first appear to be quite diverse diseases.

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Abbreviations

4-PBA	4-phenylbutyric acid
ARA	arachidonic acid
ATF6	activating transcription factor 6
BiP or Grp78	78-kDa glucose regulated protein
COX	cyclooxygenase
CYP	cytochrome P450
DHA	docosahexaenoic acid
eIF2α	eukaryotic translation initiation factor 2 alpha
EPA	eicosapentaenoic acid
DHET	dihydroxyeicosatrienoic acid
EpDPE	epoxydocosapentaenoic acid
EET	epoxyeicosatrienoic acid
EpFA	epoxy fatty acid
ER	endoplasmic reticulum
HEPE	hydroxyeicosapentaenoic acid
HETE	hydroxyeicosatetraenoic acid
HFD	high fat diet
IRE1α	inositol-requiring enzyme 1 alpha
LOX	lipoxygenase
mEH	microsomal epoxide hydrolase
mPTP	mitochondrial permeability transition pore
$\Delta\Psi_m$	mitochondrial membrane potential
NSAID	non-steroidal anti-inflammatory drug
PERK	PKR-like ER-regulated kinase
RANK and RANKL	Receptor Activator of Nuclear Factor κ B and RANK ligand
ROS	reactive oxygen species
sEH	soluble epoxide hydrolase

sEHI	soluble epoxide hydrolase inhibitor
TPPU	1-trifluoromethoxy-phenyl-3-(1-propionylpiperidin-4-yl) urea
***t*-TUCB**	*trans*-4-{4-[3-(4-trifluoromethoxyphenyl)ureido]cyclohexyloxy}benzoic acid
TUPS	1- trifluoromethoxy-phenyl-3 (1-methylsulfonyl)piperidin-4-yl) urea
UA-8	13-(3-propylureido)tridec-8-enoic acid
UPR	unfolded protein response
XBP1	X-box binding protein 1

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Highlights

- The mechanism of action of epoxy fatty acids and inhibitors of the soluble epoxide hydrolase pivots on their ability to prevent mitochondrial dysfunction, to reduce subsequent ROS formation and to block resulting cellular signaling cascades, primarily the endoplasmic reticulum stress. By stabilizing the mitochondrial – ROS – ER stress axis, the range of activity of EpFAs and sEHI display an overlap with the disease conditions including diabetes, fibrosis, chronic pain, cardiovascular and neurodegenerative diseases, for which the above outlined mechanisms play key roles.

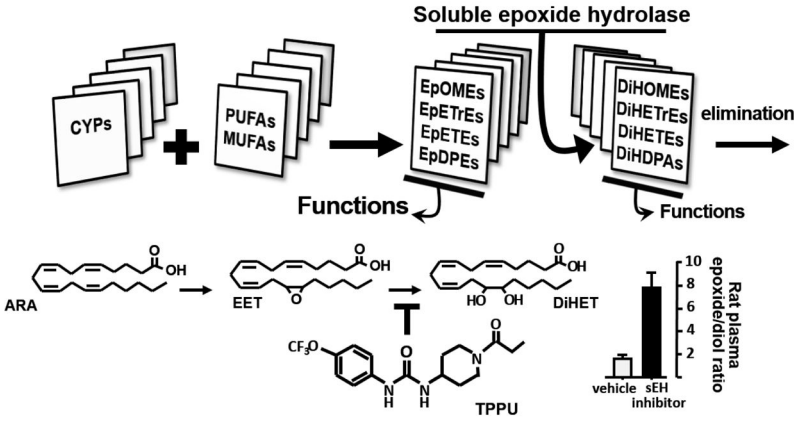

Figure 1. Epoxides of unsaturated and largely polyunsaturated fatty acids (MUFAs and PUFAs) are the major anti-inflammatory and analgesic mediators of the P450 branch of the arachidonic acid cascade

These epoxides are made largely by cytochrome P450 enzymes and may be stored as phospholipids. The best studied are the EETs (EpETrEs), but epoxides of LA (EpOMEs), EPA (EpETEs), and DHA (EpDPEs) also are chemical mediators with epoxides of the ω-3 DHA or EDPs being particularly more? active. These epoxides are converted at varying rates but generally with high Vmax and low Km to the corresponding diols, for example EETs are converted by sEH to DHETs (DiHETrEs). These diols are generally less bioactive, tend to move out of cells, and are rapidly conjugated and excreted. DHETs and particularly the diols of linoleic acid have been shown to be pro-inflammatory at the high concentrations present in sepsis. Inhibitors of sEH such at TPPU stabilize epoxy fatty acids (EpFA) such as the EETs shown, to increase the lipid epoxide to diol ratios in the blood which are associated with reduced inflammation and pain.

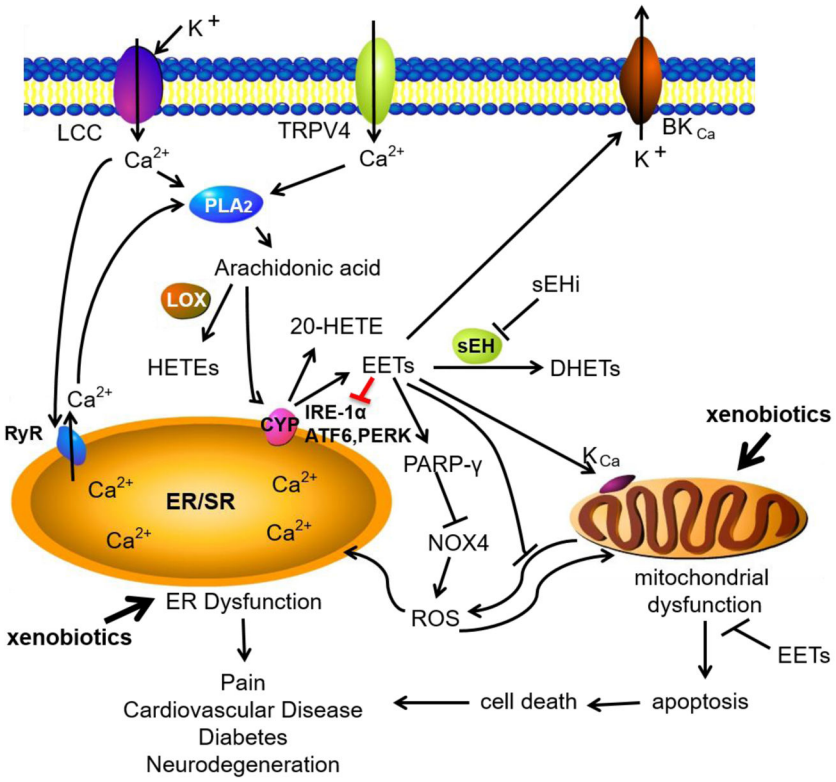

Figure 2. Signaling mechanisms involved in ROS induced ER dysfunction

In the presence of xenobiotic stress, ROS produced from the mitochondria may induce ER dysfunction. EETs and sEHI reduce the mitochondrial and NADPH oxidase (NOX4). EETs also activate important potassium channels on the mitochondria. Possible independent from these effects EETs and sEHI prevent the activation of the signaling cascade governed by the three major ER resident sensors inositol requiring protein 1α (IRE-1α), *protein* kinase RNA-like endoplasmic reticulum kinase (PERK) and activating transcription factor 6 (ATF6). RyR, ryanodine receptor; LCC, L-type Ca^{2+} channel; TRPV4, transient receptor potential cation channel subfamily V member 4; BK_{Ca}, calcium activated big potassium channel; K_{Ca}, calcium activated potassium channel; PARP-γ, poly(ADP-ribose) polymerase; K^+, potassium ions, Ca^{2+}, calcium ions.

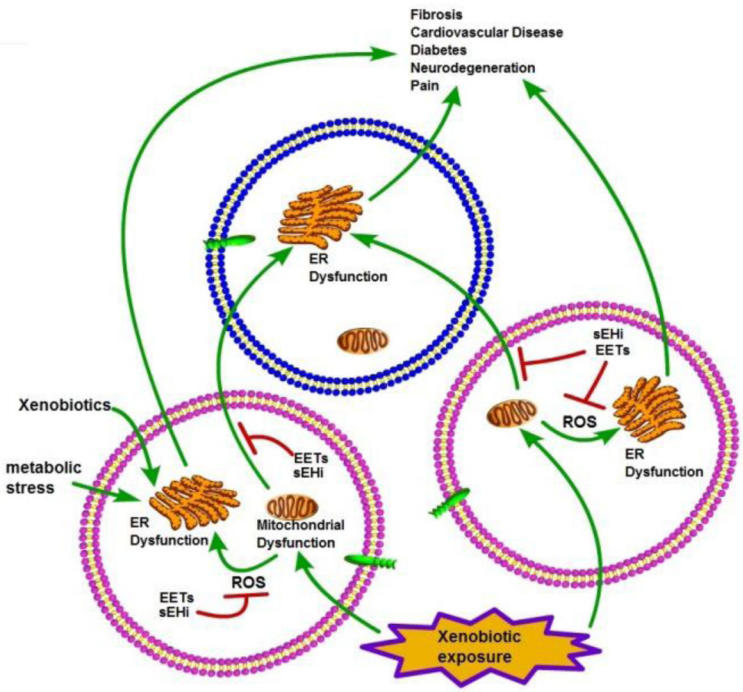

Figure 3. Schematic diagram showing the relationship among mitochondrial dysfunction, ER stress and EpFA

ER dysfunction is associated with numerous diseases including diabetes, cardiovascular, and neuronal diseases. sEH inhibitors (sEHI) and by implication the stabilized EpFAs are unique in improving symptoms in a variety of apparently unrelated disease states ranging from neuropathic pain and atrial fibrillation to pathological fibrosis and diabetes while sEHI have little if any effect on normal animals. This enigma is explained in part by EpFA stabilizing the mitochondrial →ROS → ER stress axis. The unfolded protein response and ER stress, working in part together with the cellular proteasome act to maintain cellular homeostasis. This homeostasis can be disrupted by a number of factors such as glucose associated with diabetes, xenobiotics such as paraquat or MPTP, and particularly reactive oxygen species (ROS) from many sources. ROS can be dramatically increased by mitochondrial dysfunction in turn associated both with disease states or exposure to xenobiotics including NSAIDs and triclosan. EpFA stabilized by sEHI protect the mitochondria, reduce the downstream activation of ER stress by ROS, hyperglycemia and other factors and thus ameliorate disease symptoms.