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Cytochrome P450-derived linoleic acid metabolites EpOMEs and DiHOMEs: a review of recent studies

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Abstract

Linoleic acid (LA) is the most abundant polyunsaturated fatty acid found in the Western diet. Cytochrome P450-derived LA metabolites 9,10-epoxyoctadecenoic acid (9,10-EpOME), 12,13-epoxyoctadecenoic acid (12,13-EpOME), 9,10-dihydroxy-12Z-octadecenoic acid (9,10-DiHOME) and 12,13-dihydroxy-9Z-octadecenoic acid (12,13-DiHOME) have been studied for their association with various disease states and biological functions. Previous studies of the EpOMEs and DiHOMEs have focused on their roles in cytotoxic processes, primarily in the inhibition of the neutrophil respiratory burst. More recent research has suggested the DiHOMEs may be important lipid mediators in pain perception, altered immune response and brown adipose tissue activation by cold and exercise. The purpose of this review is to summarize the current understanding of the physiological and pathophysiological roles and modes of action of the EpOMEs and DiHOMEs in health and disease.

Keywords: 12,13-DiHOME; Soluble epoxide hydrolase; Leukotoxin; Isoleukotoxin; Cytochrome P450; Linoleic acid

Contents

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1. Introduction

Polyunsaturated fatty acids (PUFAs) are the backbone of numerous lipid signaling molecules that broadly serve as homeostatic regulators for inflammation, vasotension and other physiologic processes [34,52,59,69,93,103]. In addition to undergoing common reactions of all fatty acids, such as chain elongation and fatty acid beta-oxidation, PUFA metabolism is known to involve cyclooxygenase, lipoxygenase and cytochrome P450 (CYP) enzymes, leading to the production of eicosanoids and numerous other lipid metabolites [8]. Linoleic acid (LA) is the most abundantly consumed PUFA in the human diet, which is mostly derived from vegetable oils, nuts, seeds, meats and eggs [98]. Through a CYP-dependent metabolism, LA is converted to linoleic epoxides 9,10-epoxyoctadecenoic acid (9,10-EpOME) and 12,13-epoxyoctadecenoic acid (12,13-EpOME), also known as leukotoxin and isoleukotoxin, respectively [68].

The primary CYP isoforms responsible for this conversion are CYP2J2, CYP2C8 and CYP2C9; however, other inducible CYP isoforms, including CYP1A1, can generate epoxy-fatty acids and may be relevant when pharmacologically induced [15,24,100]. These epoxides are then metabolized principally by soluble epoxide hydrolase (sEH) to 9,10-dihydroxyoctadecenoic acid (9,10-DiHOME) and 12,13-dihydroxyoctadecenoic acid (12,13-DiHOME), also named leukotoxin diol and isoleukotoxin diol, respectively (Fig. 1) [68]. These diols can be further metabolized by oxidation, generating THF diols [58,62] and DiHOME-glucuronides [47]. sEH is widely expressed throughout the human body and has been reported to be expressed in the liver, kidney, adrenals, pancreatic islets, pituitary gland, lymphoid tissues, muscles, specific vascular smooth muscles, epithelial cells, prostatic ducts and gastrointestinal tract [18]. Interestingly, CYP2C9 expression is closely associated with sEH distribution, showing a coincidence of epoxide production and hydrolysis within the tissue [18]. sEH mRNA and/or protein expression can also be induced by various stimulations, including pharmacological agents such as the peroxisome proliferator clofibrate and PPARγ agonist rosiglitazone [12,29].

Physiologic concentrations of EpOMEs and DiHOMEs may be dependent on both the regulation of biosynthetic pathways (CYP450 and sEH) and dietary intake of their parent fatty acid, LA. Mice studies with soybean oil- or margarine-containing high-fat diets have demonstrated that increased LA consumption leads to increased concentrations of EpOMEs and DiHOMEs in the livers [14,20] and plasma [20]. Interestingly, high LA consumption in these studies was generally associated with increased weight gain and a worse metabolic phenotype [14] [20]. Treating volunteers with Intralipid, an intravenous fat emulsion that is high in linoleate-rich soybean oil, has also been shown to increase concentrations of 12,13-DiHOME [17]. Since 12,13-DiHOME is not present in Intralipid, this physiologic increase is likely due to endogenous production.

In addition to their production from endogenous metabolism, the EpOMEs may be available from food sources. 9,10-EpOME and 12,13-EpOME have been identified in seed oil as well as the rice plant *Oryza sativa L.* [1,48,78]. In the rice plant, the EpOMEs were characterized as self-defense substances produced by the rice plant against rice blast disease [48]. The oils high in EpOME, also known as vernolates, were used in varnish and, in theory, could be valuable synthetically since each vernolate carbon could be used in nylon production.

Recently, one of these linoleate metabolites, 12,13-DiHOME, has been demonstrated as a novel lipokine that regulates brown adipocyte biology in response to cold [57] and exercise [86]. Given the emerging

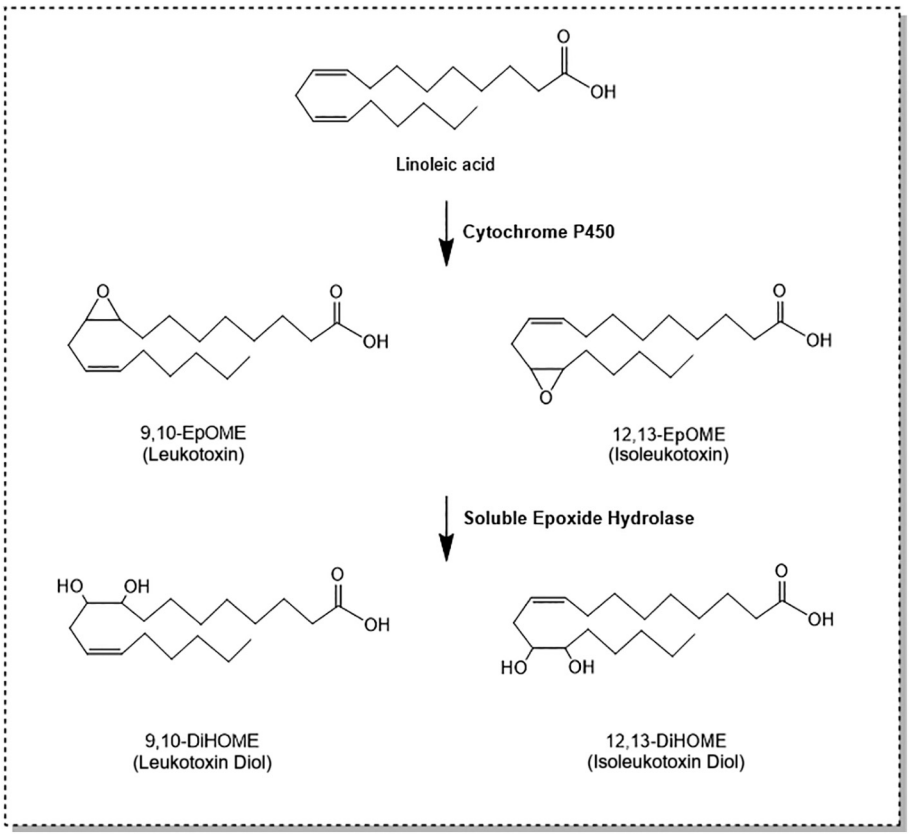

Fig. 1. Linoleic acid is metabolized by cytochrome P450s to produce 9,10- and 12,13-EpOME. Subsequent hydrolysis by soluble epoxide hydrolase yields 9,10- and 12,13-DiHOME, respectively.

Table 1
Biological functions of EpOMEs and DiHOMEs

Function	Model	Lipid	Key findings	Mechanism	Reference
Inflammation and immune response	*In vitro* — endothelial cells from porcine pulmonary arteries	9,10-EpOME and 9,10-DiHOME (60 and 90 μM)	Induced oxidative stress	Activated NF-κB and AP-1 transcription factors	[94]
	In vitro — human neutrophils	9,10-EpOME (0.1–1 μM) and 9,10-DiHOME (1 nM–0.1 μM)	Induced chemotaxis of human neutrophils: 9,10-DiHOME with 100× greater potency	Undetermined: chemotaxis activation pathway different from that of fMLP that does not include the induction of expression of adhesion molecules or peroxides	[91]
	In vitro — HL-60 cells	Methylated EpOMEs (10–200 μM) and DiHOMEs (200 μM)	DiHOMEs inhibited the respiratory burst, while the EpOMEs were weak stimulators of the burst	Undetermined: inhibition of the respiratory burst by a mechanism different from that of cyclosporin H or lipoxin A4 that involves modulation of NADPH oxidase activity	[90]
	In vivo — adult men	12,13-DiHOME	Plasma levels were elevated during Intralipid infusion	Undetermined: proposed to lead to impaired neutrophil function and thereby immunosuppression	[17]
	In vivo and *in vitro* — neonates	12,13-DiHOME (75, 130, and 200 μM)	Fecal concentrations were elevated in individuals with NGM3, and its application to dendritic cells and autologously purified naive CD4+ cells resulted in a reduced percentage of $CD4^{+}CD25^{+}Foxp3^{+}$ T cells		[22]
	In vivo and *in vitro* — neonates, mice, human dendritic cells	12,13-DiHOME (30 mg/kg (mice) and 75, 130 and 200 μM (cells))	Increased neonate fecal concentrations were associated with risk of asthma at age 4, treatment of mice exacerbated pulmonary inflammation and decreased lung regulatory T cells, and treatment of cells decreased immune tolerance	Undetermined: proposed immune dysfunction *via* PPARγ signaling in dendritic cells	[52]
Endocrine disruption	*In vivo* — adult rats	9,10- and 12,13-DiHOME (2 μg/ml)	A 1:1 mixture of the DiHOMEs disrupted the estrous cycle of female rats but did not affect the sexual behavior of male rats	Undetermined: proposed modulation of prolactin release similar to that of the arachidonic and steric acid epoxy-derivatives	[60]
	In vitro — U-33/γ2 cells	9,10-DiHOME (120 μM)	Stimulated adipogenesis but inhibited osteoblastogenesis	Acted as a PPARγ2 ligand	[51]
Mitogenesis	*In vitro* — MCF-7 cells	9,10- and 12,13-DiHOME (0.32–1.6 μM)	Stimulated cell proliferation	Undetermined: mechanisms not involving estrogen receptor or nuclear type II binding sites	[60]
	In vivo — zebrafish and mice	12,13-DiHOME (10 μM)	Modulated proliferation and mobilization of HPC in zebrafish embryos and the spleen of mice for the vascular development and repair process	Activated the canonical Wnt signaling	[21]
Pain	*In vitro* — mice	12,13-DiHOME	Increased calcium flux in sensory neurons through TRPV1	Activated PKC	[105]
	In vivo — rats	12,13-EpOME, 9,10- and 12,13-DiHOME	Endogenous localized levels increased with painful stimuli	Undetermined: proposed to act as an endogenous TRPV1 agonist	[19]
	In vivo and *in vitro* — mice and cultured murine dorsal root ganglions	9,10-EpOME (250 nM–10 μM)	Induced mechanical and thermal pain in wild-type but not TRPV1-deficient mice; Increased calcium influx in DRGs which was blocked by the TRPV1 antagonist	Sensitization of TRPV *via* PKA pathway	[85]
BAT activation	*In vivo* — humans	12,13-DiHOME	Plasma levels were increased by acute cold exposure and were negatively correlated with BMI, insulin resistance, fasting plasma insulin and glucose concentrations, and circulating triglyceride and leptin levels		[57]
	In vivo — mice	12,13-DiHOME (1 and 10 μg/kg)	Increased BAT activity and BAT-specific fatty acid and glucose uptake in mice	Promoted translocation of fatty acid transporters to the membrane	[57]
Exercise and skeletal muscle regulation	*In vivo* — humans	12,13-DiHOME	Plasma levels were increased by acute exercise and were positively correlated with cardiorespiratory fitness		[86]
	In vivo — mice	12,13-DiHOME (1 μg/kg)	Decreased respiratory exchange ratio and increased fatty acid uptake in skeletal muscle and removal of interscapular BAT fully blunted the exercise-induced increase of 12,13-DiHOME	Induced expression of genes involved in mitochondrial activity and biogenesis and fatty acid uptake in the muscle	[86]
	In vitro — C2C12 muscle cells and 3T3-L1 adipocytes	12,13-DiHOME (1.5 μM)	Increased fatty acid uptake, oxidation and mitochondria respiration in the C2C12 but not 3T3-L1 adipocytes		[86]

roles of this metabolite, it is important to understand how these metabolites relate to health and disease. In this review, we summarize early findings on the cytotoxicity of the LA-derived EpOMEs and DiHOMEs and recent studies elucidating their diverse roles with a focus on immune response, pain perception and brown adipose tissue (BAT) activation by cold and exercise (Table 1).

2. Cytotoxicity of EpOMEs and DiHOMEs

2.1. Identification of 9,10-EpOME (leukotoxin) as a major factor in burn and ARDS patients

The first studies on CYP metabolites of LA came from investigations on mitochondrial toxic factors from burn victims. This work stemmed from the observation that many burn victims still had a "late death" after surviving the initial shock of the burn injury. This late death and later development to acute respiratory distress syndrome (ARDS) and sepsis were thought to be due to a toxic substance produced in the skin that entered general circulation [2]. Rodent skin samples from experimentally burned animals were extracted, and a single lipid was isolated [88] that was eventually identified as 9,10-EpOME by gas chromatography/mass spectrometry and nuclear magnetic resonance analysis [102]. This metabolite was named "leukotoxin" since it was presumed that these metabolites were produced in the leukocytes [31,32,71]. The same metabolites were found to be produced by neutrophils in the lung after hyperoxic exposure [71]. This production in neutrophils was enhanced by a Ca^{2+} ionophore, inhibited by carbon monoxide and was enhanced by epoxide hydrolase inhibition in liver microsomes, demonstrating leukotoxin was produced by consecutive reactions with phospholipase A_2 and CYP but could be metabolized by epoxide hydrolase [73].

2.2. Pulmonary toxicity of the EpOMEs and DiHOMEs

After identification of leukotoxin as the toxic substance produced in the skin of the burn patients, leukotoxin was found in the lung lavages from patients with ARDS [72]. Subacute doses of leukotoxin administered to rats intravenously caused severe pulmonary edema in as little as 10 min that lasted at least 12 h, demonstrating a causal effect *in vivo* [46]. It should be noted that although the doses were high in these experiments (50–150 μmol/kg, which corresponds to approximately 15–45 mg/kg), the blood concentrations of leukotoxin detected in patients with sepsis or severe burns were found to be as high as 100 μM [50] to 580 μM [30,50] (Hammock, B.D. et al., unpublished data).

Several perfusion studies have been conducted in isolated lungs to investigate the mechanisms responsible for the leukotoxin-induced pulmonary edema [40–43]. In this isolated system, relatively high doses of leukotoxin (200 μM) were required to elicit edematous injury [42]. This injury was associated with the release of lactate dehydrogenase (a measure of cellular damage) and increases in effluent nitrite, both of which could be blocked by a nitric oxide synthase (NOS) inhibitor or superoxide dismutase [41]. This edematous injury in the isolated lung can be synergized by cotreatment of the vasoconstrictor endothelin-1 and can be blocked by treatment with an endothelin receptor antagonist [40]. At lower doses (2–20 μM) where edema is not observed, leukotoxin caused changes in pulmonary vasotension and capillary filtration. During hypoxic vasoconstriction, leukotoxin but not linoleate was able to elicit a transient increase in vasoconstriction followed by vasodilation. This relaxation was observed from a variety of vasoconstrictors including angiotensin II, phenylephrine and KCl and could be blocked by a methylene blue (a soluble guanylate cyclase inhibitor) and NG-monomethyl-L-arginine (a NOS inhibitor) [43]. These studies demonstrate that high doses of leukotoxin induce pulmonary cell damage, whereas low doses induce vasoconstriction followed by vasodilation in the lung, both of which are mediated by nitric oxide.

The hydration of the leukotoxins to leukotoxin diols by sEH was thought to be a means of detoxification [50] until it was revealed that this conversion to DiHOMEs enables toxicity of the EpOMEs [26]. To study this, insect-derived Sf-21 cells with low endogenous epoxide hydrolase activity were transfected with sEH or microsomal epoxide hydrolase. DiHOMEs but not EpOMEs were toxic in the nontransfected naive cells, while both DiHOMEs and EpOMEs were toxic in the sEH transfected cells [7,63]. To support this conclusion, administration of 9,10-DiHOME (35 mg/kg) to rats by cardiac puncture initiated respiratory stress and death within 2 h, while no symptoms or mortality occurred with 9,10-EpOME at doses up to 100 mg/kg [63]. Consistently, treatment of mice with 300 mg/kg of a 1:1 mixture of methyl leukotoxin/isoleukotoxin esters was not lethal, while the same dose of the corresponding diols resulted in the death of all mice [104]. Histopathologic analysis showed that the lungs of the DiHOME-treated mice had massive alveolar edema and hemorrhage with interstitial edema around blood vessels in the lungs, while EpOME-treated mice had only perivascular edema and a small change in alveolar spaces [104]. Moreover, treatment with an sEH inhibitor, 4-phenylchalcone oxide, decreased mortality induced by EpOME but not DiHOME [104].

Structure–activity relationship studies using the insect-derived Sf-21 cell line have shown cytotoxicity can be observed in a number of analogous chemical structures with altered hydrocarbon length and functional groups [27]. Although not tested, these experiments suggest that a large variety of dihydroxy-fatty acids, including dihydroxyeicosatrienoic acids and dihydroxyoctadecadienoic acids, may be similarly cytotoxic. Interestingly, some of the reported epoxides were toxic independent of sEH hydrolysis. It is unknown whether the sEH-independent cytotoxicity of these epoxides is mechanistically related to the cytotoxicity of DiHOME. The doses reported in these cytotoxicity experiments were generally high (~100 μM) but may still represent relevant concentrations during fatal sepsis where concentrations of DiHOMEs are massively elevated [28].

Moreover, EpOMEs and DiHOMEs also seem to be implicated in chronic lung conditions caused by environmental insults. One study investigating responses to subway air exposure found decreased levels of both regioisomers of DiHOME in bronchoalveolar lavage fluid after a subway air exposure in asthmatic individuals in comparison to increased levels in healthy individuals [55]. Healthy volunteers exposed to biodiesel exhaust exposure showed increased levels of plasma 9,10-DiHOME compared to filtered air controls [25]. Further, both EpOMEs and DiHOMEs were increased in bronchoalveolar lavage fluid of female, but not male, smokers with chronic obstructive pulmonary disease relative to smokers with normal lung function [3]. Together, these studies suggest the EpOMEs and DiHOMEs may be part of the inflammatory response to environmental insults in the lung.

A likely underlying mechanism of 9,10-DiHOME toxicity is its ability to disrupt mitochondrial function [84]. Treatment of human HeLa cells with methylated 9,10-DiHOME at concentrations corresponding to those seen in ARDS patients (180 to 210 μM) was shown to cause mitochondrial swelling (i.e., increase of mitochondrial volume), cytochrome c release and leakage of mitochondria-specific dye Mitotracker Green, all of which are indicative of 9,10-DiHOME's ability to compromise mitochondrial inner membrane permeability and, consequently, disrupt mitochondrial function [84]. The release of cytochrome c in these cells then triggers cell death [101]. In contrast, the treatment of these cells with methylated 9,10-EpOME, linoleic acid and structurally similar compounds did not result in mitochondrial swelling. Moreover, exposure to the *threo* configuration of 9,10-DiHOME induced swelling to a greater extent than the *erythro* configuration, suggesting 9,10-DiHOME's effects are moiety- and regio-specific in the Hela cells [84].

Relative toxicity and mechanisms of LA, EpOMEs, and DiHOMEs were directly compared in the rabbit renal proximal tubule. It was shown that both methyl LA (1 mM) and an equimolar mixture of the methyl EpOMEs (1 mM) were not toxic, while an equimolar mixture of the methyl DiHOMEs (1 mM) induced mitochondrial dysfunction and cell death [66]. Conversely, 500 μM of the free acid forms of LA, 9,10-EpOME, 9,10-DiHOME, 12,13-EpOME and

12,13-DiHOME all induced mitochondrial dysfunction and cell death in the renal proximal tubules [64]. The free acid forms of LA, 9,10-EpOME and 12,13-EpOME were most toxic. Moreover, the free acid forms of 9,10-DiHOME and 12,13-DiHOME were more toxic than their methyl ester derivatives [64,66]. The toxicity of LA and the EpOMEs was attributed to their ability to induce the uncoupling of oxidative phosphorylation [64].

Furthermore, in rabbit renal cortical mitochondria, treatment with the free acid form of 12,13-EpOME at 50 μM resulted in reduced ADP-stimulated respiration (State 3 respiration) and increased ADP-depleted respiration (State 4 respiration) [65]. Additionally, respiration sensitive to the ATP synthase inhibitor oligomycin was decreased by 12,13-EpOME, while oligomycin-insensitive respiration was increased. Oligomycin-sensitive oxygen consumption can serve as a marker of oxidative phosphorylation, and oligomycin-insensitive respiration represents the oxygen being consumed that is not associated with ATP synthesis in mitochondria, i.e., mitochondrial uncoupling [80,81]. Compared to 12,13-EpOME, 12,13-DiHOME did not affect these variables, suggesting that hydrolysis to 12,13-DiHOME is a mechanism of detoxification of the linoleic acid metabolite for the prevention of mitochondrial dysfunction in renal cortical mitochondria [65]. Divergent effects of the EpOMEs and DiHOMEs demonstrate species- and tissue-specific toxic effects of these metabolites.

2.3. Cardiotoxicity of the EpOMEs and DiHOMEs

One component of the toxic response to leukotoxin is the ability to disrupt cardiovascular function. In dogs, leukotoxins were more cardiodepressive than linoleates by reducing aortic flow and blood pressure [23,87]. Treating leukotoxin or isoleukotoxin to isolated papillary muscles from cats decreased developed force, an index of myocardial contractility. In addition, both leukotoxin and isoleukotoxin caused vasoconstriction in isolated perfused carotid arteries from cats [82].

Arachidonic acid-derived epoxides or epoxyeicosanoids (EETs), and sEH are known to be important factors in regulating cardiovascular function [11,13,35,36]. Thus, most of the cardioprotective effects of sEH inhibitors, including protection against cardiac hypertrophy [99], ischemia–reperfusion injury [44] and fibrosis post-myocardial infarction [83], have been attributed to their ability to increased epoxy-fatty acids (EpFAs) and EETs in particular. However, there is evidence of an alternative hypothesis in which dihydroxy-fatty acids (DiHFAs) partially contribute to these effects and that limiting the production of DiHFAs by sEH inhibitors may be beneficial. Mice that had endothelial cell-specific overexpression of CYP2C8 (CYP2C8-Tie2), but not CYP2J2 or sEH, had reduced recovery of left ventricular developed pressure and increased infarct size after ischemia–reperfusion injury [16]. This reduced recovery was associated with increased concentrations of 9,10- and 12,13-DiHOMEs in the heart perfusates. Dosing Langendorff-perfused WT hearts with either 9,10-DiHOME or 12,13-DiHOME was able to recapitulate the reduced recovery and increased coronary resistance found in CYP2C8-Tie2 mice [4,16]. In addition, in a comparison of young and aged sEH-null and cardiac myocyte specific CYP2J2 overexpressing mouse hearts, sEH-null mice had postischemic protection in both young and aged hearts, while CYP2J2 overexpression was only protective in young but not the aged hearts [10]. The amount of protective EETs present in both genetic backgrounds was comparable, and no increase was found in sEH expression and activities in aged CYP2J2-overexpressing hearts; however, the authors found CYP2J2 overexpression resulted in increased 12,13-DiHOME, which correlated with increased reactive oxygen species markers. Consistently, the authors found that sEH inhibition improved cardioprotection in the aged CYP2J2-overexpressing hearts. Thus, in addition to enhancing the cardioprotective role of EETs, sEH inhibition may also block the harmful effects of DiHOMEs.

In contrast to the postischemic toxic effects of 12,13-DiHOME observed in the hearts with ischemia/reperfusion, other studies have found that modest increases in contractile function within 10–20 min were observed in LA, 12,13-EpOME and 12,13-DiHOME perfused rat hearts, with 12,13-DiHOME's positive effects lasting until washout. No arrhythmias and negative inotropic effects were observed [61]. Moreover, the administration of a mixture of 9,10- and 12,13-EpOMEs to rats *in vivo* showed only a small decrease in blood pressure with no significant effect on heart rate or pulse [61]. It seems that the effects of EpOMEs and DiHOMEs on cardiovascular function are complex and may be dose and species dependent.

3. Biological functions of the EpOMEs and DiHOMEs

3.1. Inflammation and immune response

It has been reported that LA-induced endothelial cell activation or dysfunction in atherosclerosis may be mediated through oxidative stress [33]. Consistently, in endothelial cells isolated from porcine pulmonary arteries, 90 μM LA induced oxidative stress (as measured by DCF fluorescence), while both 9,10-EpOME and 9,10-DiHOME induced oxidative stress at high concentrations (90 μM) but not at low concentrations (up to 30 μM) [94]. Moreover, as measured by electrophoretic mobility shift assays, LA (90 μM) and high concentrations of 9,10-EpOME (90 μM) and 9,10-DiHOME (60 μM) activated NF-κB and AP-1 transcription factors, both of which mediate inflammation [94].

DiHOMEs are synthesized by activated neutrophils and induce chemotaxis of other neutrophils at relatively low doses (~10 nM) [39,91]. This induction was not through the expression of adhesion molecules or peroxide production, as is the case of the well-known chemoattractant fMLP, but rather through an independent pathway [91]. At relatively higher doses (20–200 μM), both DiHOME isomers inhibited the neutrophil respiratory burst in HL-60 cells, which are neutrophil-like cells derived from human promyelocytic leukemia [90]. Given the relatively significant difference in doses required for the chemotactic and inhibitory effects, DiHOMEs may serve as a type of negative feedback that limits the inflammation. DiHOME-mediated inhibition occurs by a mechanism different from that of cyclosporin H or lipoxin A4, both of which are respiratory burst inhibitors that prevent both superoxide production and degranulation. It is thought that the DiHOMEs stimulate the use of NADPH oxidase substrates or induce physiochemical alterations in the membrane microenvironment of NADPH oxidase, thereby modulating the activity of NADPH oxidase, which is responsible for the production of the respiratory burst [90]. Because of its ability to inhibit the respiratory burst, 12,13-DiHOME may inhibit the immune response. Plasma levels of 12,13-DiHOME were found to be significantly elevated in healthy adult men with normal BMIs during Intralipid infusion; therefore, it is thought that 12,13-DiHOME may contribute to immunosuppression seen in patients receiving Intralipid infusion for parenteral nutrition [17]. This may be particularly true in the case of total parenteral nutrition.

12,13-DiHOME has recently been found to be associated with the gut microbiome of young children who develop asthma [22]. This study showed a particular community of gut bacteria, referred to as the neonatal gut microbiome 3 (NGM3), was associated with an elevated relative risk of developing atopy, a heightened immune response to allergens, by 2 years of age and asthma by 4 years of age. 12,13-DiHOME was found to be the major metabolite identified from these samples that could shift the regulatory T cell populations to cause adaptive immune cell dysfunction and was found to be associated with an increased relative risk of developing asthma [22,52]. Exploration of the sources of 12,13-DiHOME in the neonate gut microbiome identified three bacterial epoxide hydrolase genes

that were thought to be responsible for mediating the effects on atopy and asthma [52]. Each of these genes was able to convert 12,13-EpOME to 12,13-DiHOME, and the combination of the three genes was associated with atopy and asthma. Furthermore, treating mice with 12,13-DiHOME before a challenge with cockroach antigen increased the allergic response, including an increase in peribronchial and perivascular inflammatory infiltrates and increased expression of inflammatory cytokines, compared to vehicle controls [52]. Activation of PPARγ was presumed to be the primary mechanism responsible for these biological effects; however, other receptors that are activated by 12,13-DiHOME, such as transient receptor potential vanilloid 1 (TRPV1), could not be ruled out [52].

3.2. Endocrine disruption

The endocrine-disrupting effects of DiHOMEs were initially investigated from the observation that corncob bedding disrupted normal mating behavior and reproductive capabilities in rats [58]. A few years prior, Moghaddam et al. reported that, when hydrolyzed, the diepoxides of linoleate yielded trivial amounts of the expected tetra hydroxy products and that the major products were cis- and trans-tetrahydrofuran diols (THF-diols) [62]. DiHOMEs and their corresponding THF-diols byproducts were later identified as major components of corncob bedding responsible for these effects [59,60]. Interestingly, dosing rats with a 1:1 mixture of 9,10- and 12,13-DiHOME was able to cause disruption of the estrous cycle in female rats but was unable to cause changes in male reproductive function or mating behavior in either sex [60]. Based on the disruption of the estrous cycle in female rats, it is presumed that both components of corncob bedding could dysregulate normal estrogen signaling pathways. These endocrine-disrupting effects could be particularly relevant to the growth of hormone-sensitive cancers, including breast and ovarian cancers. When tested *in vitro,* 12,13-DiHOME was able to stimulate the proliferation of MCF-7 cells (estrogen-receptor-positive breast cancer cells) but was unable to compete [^{3}H]estradiol binding to the estrogen receptor [58]. It was not tested whether 12,13-DiHOME could enhance tumor growth *in vivo*. It is possible that these reproductive effects may be relevant to human health due to the ubiquity of corn oil in Western diets; however, to the best of our knowledge, no studies have followed the relationship between 12,13-DiHOME in the blood and reproductive function.

Both 9,10-DiHOME and 9,10-EpOME have been shown to act as PPARγ ligands, as determined by their displacement of the PPARγ ligand [^{3}H] T0900393 from the recombinant PPARγ ligand-binding domain [51]. Activation of PPARγ2 by the potent synthetic ligand rosiglitazone is known to stimulate adipogenesis but inhibit osteoblastogenesis [5,74]. Consistently, 9,10-DiHOME was shown to stimulate adipogenesis but inhibit osteoblastogenesis, measured by lipid accumulation, mineral deposition and gene expression in U-33/γ2 cells, a murine marrow-derived mesenchymal progenitor cell line with exogenous expression of PPARγ2 [51]. However, 9,10-EpOME prevented osteoblast differentiation but did not stimulate adipogenesis [51].

3.3. Mitogenesis

In zebrafish and mice, it was discovered that 12,13-DiHOME is a critical modulator of progenitor cell proliferation and mobilization for the vascular development and repair process, which is accomplished by activation of the canonical Wnt signaling cascade [21]. Knockdown or inhibition of sEH resulted in defects in the caudal vein plexus (CVP), a transient hematopoietic tissue, and decreased numbers of *cmyb*/*lmo2* double-positive cells, a subpopulation of hematopoietic cells in the CVP, in zebrafish embryos. 12,13-DiHOME, but not 12,13-EpOME, was able to restore numbers of *cmyb*/*lmo2* double-positive cells. In mice, sEH knockout led to decreased proliferation and colony formation of hematopoietic progenitor cells (HPCs) in the spleen of irradiated wild-type animals, decreased mobilization of HPCs into circulation from the bone marrow in response to G-CSF and decreased vascular repair after hindlimb ischemia. 12,13-DiHOME, but not 12,13-EpOME, restored the recovery of blood flow in sEH knockout mice, but not wild-type mice, with hind limb ischemia [21].

3.4. Pain

Many eicosanoids are implicated in the regulation of pain. Prostaglandins are positive regulators of pain that mediate thermal and mechanical hyperalgesia during inflammation [38]. In contrast, many EpFAs are negative regulators of pain by blocking inflammation-induced hyperalgesia and other forms of pain, such as diabetes caused peripheral neuropathy [67,96,97]. To increase the endogenous levels of pain-relieving EpFAs *in vivo*, sEH inhibitors have been used, which appeared to be highly effective in multiple types of pain [79,89]. sEH inhibitors appear to reduce pain through a number of mechanisms, including cannabinoid signaling [95] and ER stress [37]. These small molecules are currently being taken into the clinic for the treatment of pain in man and in companion animals [49].

Care should be taken not to equate sEH inhibition with the activity of EpFAs. Although EpFAs alone have established pain-relieving effects, these studies do not rule out the possibility of alternative hypotheses. One alternative hypothesis is that diol metabolites of sEH may be pronociceptive, and sEH inhibition blocks the formation of these painful metabolites. In support of this hypothesis, Zimmer et al. [105] recently showed in sensory neurons that 12,13-DiHOME increased calcium flux by TRPV1 through a PKC-mediated mechanism, leading to increased thermal hyperalgesia *in vivo*, which is not evident in TRPV1 knockout mice. Additionally, treatment with painful stimuli such as complete Freund's adjuvant or nerve growth factor increased endogenous concentrations of 12,13-DiHOME [19,105]. This work indicates that chronic pain increases endogenous concentrations of DiHOMEs, and sEH inhibition blocks the production of these proalgesic metabolites while increasing concentrations of analgesic and possibly other EpFAs.

However, the EpFAs may not be analgesic in all scenarios; it has been reported that 9,10-EpOME may mediate paclitaxel-induced neuropathic pain by sensitizing TRPV1 and increasing activities of nociceptive neurons [85]. Also, other types of oxidized linoleic metabolites that are not regulated by sEH have also been implicated in pain progression [75,76]; thus, it seems that EpOMEs and DiHOMEs represent only part of concerted oxidation of LA to mediate pain.

3.5. BAT activation by cold

BAT has recently emerged as a novel target for obesity treatment and prevention [9,53]. In contrast to white adipose tissue (WAT), BAT is responsible for nonshivering thermogenesis by uncoupling ATP synthesis from respiration, leading to heat production [9].

Studies investigating the role of 12,13-DiHOME in regulating BAT function started with a lipidomics screen to identify bioactive lipids that were increased in humans subjected to acute cold exposure [57]. Moreover, plasma levels of 12,13-DiHOME were found to be positively correlated with BAT activities but negatively correlated with BMI, insulin resistance, fasting plasma insulin and glucose concentrations, and circulating triglyceride and leptin levels [57]. The finding that plasma levels of 12,13-DiHOME were negatively associated with obesity was consistent with a previous report [77] and has since been validated in a larger sample of volunteers [92]. Similarly, cold-exposed mice (4°C) had increased serum levels of 12,13-DiHOME and increased expression of sEH in the BAT but not in other tissues that express sEH [57]. Interestingly, cold-exposed mice that have a defect in normal BAT development (*Myf5*^{Cre}*Bmpr1a*$^{f/f}$ mice) produced 12,13-DiHOME from the compensated sWAT instead of the BAT; suggesting

that 12,13-DiHOME secretion may be a component of all thermogenic adipocytes rather than just brown adipocytes. Thus, it was suggested that 12,13-DiHOME could be a thermogenic BATokine activated by cold exposure.

Pharmacologic experiments testing the effects of 12,13-DiHOME in mice further confirmed that it played a role in thermogenesis. Injection of 1 μg/kg of 12,13-DiHOME increased oxygen consumption, carbon dioxide production and cold resistance. Moreover, injection of 1 μg/kg of 12,13-DiHOME resulted in an increase in BAT-specific lipids, FA and glucose uptake in mice. Furthermore, in diet-induced obese mice injected daily with 10 μg/kg of 12,13-DiHOME for 2 weeks, a decrease in circulating triglycerides and an increase in expression of lipoprotein lipase in BAT were observed with no significant change in weight, glucose tolerance or circulating nonesterified fatty acids [57]. Together, these results suggested that 12,13-DiHOME may activate BAT by increasing the availability and oxidation of free fatty acids.

The increased fatty acid uptake in BAT observed with 12,13-DiHOME treatment has been attributed to the increases of membrane translocation of the low glycosylated form of fatty acid transporters CD36 and oligomeric fatty acid transport protein 1 [57]. 12,13-DiHOME did not have a significant effect on maximal respiratory capacity or uncoupling in the BAT, but basal respiration was increased, indicating increased basal fuel uptake and metabolism [57]. Taken together, 12,13-DiHOME serves as a link between cold exposure and thermogenesis by promoting fatty acid uptake in brown adipocytes in an autocrine or paracrine manner. Increased fatty acids provide more substrates for CYP and sEH, consequently increasing more 12,13-DiHOME production as a feedforward mechanism [57].

3.6. Exercise and skeletal muscle regulation

Several studies have reported that 12,13-DiHOME is increased in response to exercise. In a series of studies investigating exercise-regulated metabolites in athletes, blood levels of 12,13-DiHOME were increased immediately postexercise in both long-distance runners [69] and cyclists [70]. It was found that both 9,10-DiHOME and 12,13-DiHOME were increased after the cycling exercise and were subsequently reduced back to baseline once the exercise was over. Only postexercise 12,13-DiHOME levels were positively correlated with postexercise oxidized LA derivative 13- and 9-hydroxy-octadecadienoic acid (13-HODE+9-HODE), a new oxidative stress biomarker for acute exercise [70]. Similar changes in 12,13-DiHOME were observed over the course of a 45-min bout of exercise in young or old healthy male subjects of varying levels of physical fitness [86]. Additionally, it was found that routinely active subjects had significantly higher pre-exercise 12,13-DiHOME levels compared to subjects who did not regularly exercise [86]. Resting 12,13-DiHOME concentration was positively correlated with cardio-respiratory fitness, as measured by peak oxygen uptake, and negatively correlated with total fat mass, BMI, body weight and triglycerides. However, covariate analysis showed that when percentage fat mass is accounted for, the only significant correlation was with triglycerides [86].

Plasma levels of 12,13-DiHOME are similarly increased in either trained or untrained male mice subjected to acute exercise [86]. Chronic exercise training also significantly increased 12,13-DiHOME and decreased body and fat mass, consistent with the negative correlation between resting 12,13-DiHOME levels and body and fat mass observed in humans. The surgical removal of interscapular BAT from the mice blunted the effects of exercise on 12,13-DiHOME levels despite no difference in basal 12,13-DiHOME compared to mice that underwent sham surgery. A single bout of exercise and 3 weeks of exercise training increased mRNA expression of *Ephx*1 in BAT but not in other tissues. No changes in *Ephx2* gene expression were found in any of the tissues. Taken together, it was concluded that even though various tissues produce 12,13-DiHOME, BAT is responsible for the exercise-induced increase in 12,13-DiHOME [86].

Similar to brown adipose tissue, mice injected with 12,13-DiHOME had increased fatty acid uptake in skeletal muscle and a decrease in the respiratory exchange ratios. This increase in fatty acid uptake was also observed *in vitro* in differentiated C2C12 myotubes but not in differentiated 3T3-L1 white adipocytes [86]. Further, 12,13-DiHOME increased basal and maximal respiration in C2C12 cells. Thus, 12,13-DiHOME may support active skeletal muscle function by increasing lipid uptake and mitochondrial activities.

4. Conclusion

Recent studies have brought new interest in the roles of CYP-derived LA metabolites EpOMEs and DiHOMEs in health and disease. Despite the toxic effects observed at high concentrations, pharmacologic strategies that target 12,13-DiHOME may hold promise as a therapeutic approach for treating pain, obesity and related metabolic dysfunction. Both cold and exercise induce secretion of 12, 13-DiHOME from the BAT into circulation. At low concentrations that appear to be safe, 12,13-DiHOME has been shown to effectively increase fatty acid uptake in the BAT and skeletal muscle and decrease circulating triglycerides in mice. Moreover, this low dose of 12,13-DiHOME had no effect on blood pressure or pulse (except for a brief increase in diastolic pressure) [57]. Therefore, 12,13-DiHOME can mimic the beneficial effects of cold exposure and exercise on improving lipid metabolism.

On the other hand, the therapeutic use of sEH inhibitors, which can block the production of DiHOMEs, is currently being explored for many different disease states, including obesity [54], insulin resistance [56] and metabolic syndrome [45]. Therefore, it is important to have a better understanding of the biological activities of CYP-derived LA metabolites, including the EpOMEs and DiHOMEs, in health and disease.

Many questions remain with respect to the roles of EpOMEs and DiHOMEs in physiology. One of these is whether a change in LA content in the diet would affect the processes in which EpOMEs or DiHOMEs are involved, such as immune response, pain perception, and BAT activation by cold and exercise. LA is the most abundant PUFA in the human diet, and consumption has increased over the past century [6]. Since increased dietary intakes of LA lead to increased plasma and tissue levels of EpOMEs and DiHOMEs, it seems plausible that there may be physiological consequences of increased DiHOMEs on health.

An additional remaining question is whether common molecular mechanisms exist that mediate the mitochondrial toxicity of 12,13-DiHOME at high doses and the beneficial effects on metabolism that occurs at lower doses. Common to both effects is an increase in uncoupling of the electron transport chain (ETC). In the case of mitochondrial toxicity, uncoupling of the ETC can reduce efficiency of oxidative phosphorylation, leading to reduced cellular function, increased oxidative stress and eventually cell death, as observed with high doses of 12,13-DiHOME. These effects likely underlie many of the toxic effects that have been observed in a variety of organs, including cardiovascular, pulmonary and renal systems. By comparison, the "inefficient" use of chemical fuel is a feature of BAT, and uncoupling of the ETC drives nonshivering thermogenesis. It is unclear whether 12,13-DiHOME-mediated uncoupling observed in other cell types occurs in brown adipocytes and contributes to the thermogenic phenotype observed in BAT. If this is true, understanding how dose regulates uncoupling responses in BAT relative to other organ systems may be the key to the pharmacologic application of 12,13-DiHOME for treating metabolic diseases, such as hyperlipidemia and type 2 diabetes.

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