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Review

Pathophysiology of the hepoxilins ☆

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ABSTRACT

There is increasing evidence from various scientific groups that hepoxilins represent novel inflammatory mediators. In vitro studies have shown that the hepoxilins cause mobilization of intracellular calcium in human neutrophils, cause plasma leakage, and potently stimulate chemotaxis of human neutrophils. In vivo, the hepoxilin pathway is activated in conditions of inflammation, e.g. after pathogen infection, in inflamed conditions (psoriasis, arthritis), and hepoxilins promote inflammatory hyperalgesia and allodynia. Although much work has demonstrated an effect of hepoxilins on neutrophils, the hepoxilin pathway has been demonstrated in a variety of tissues, including the lung, brain, pituitary, pancreatic islets, skin, etc. A genetic defect linked to a deficiency in hepoxilin formation has been described and believed to be responsible for the scaly skin observed in ichthyosis. Despite their biological and chemical instability, the involvement of the hepoxilin pathway in pathology has been demonstrated in vitro and in vivo through either isolation of the hepoxilins themselves (or their metabolites) or implied through the use of stable hepoxilin analogs. These analogs have additionally shown efficacy in animal models of lung fibrosis, cancer, thrombosis and diabetes. Research on these compounds has merely scratched the surface, but results published to date have suggested that the hepoxilin pathway is a distinct and novel pathway leading to inflammation and hepoxilin antagonists may provide the means of controlling early aspects of the acute inflammatory phase. This article is part of a Special Issue entitled "Oxygenated metabolism of PUFA: analysis and biological relevance".

1. Introduction

The hepoxilin pathway was discovered in the early 80s with the isolation of two compounds derived from the common intermediate, 12(S) hydroperoxy-eicosatetraenoic acid (12(S)-HPETE), a product of arachidonic acid (AA) formed through the 12S-lipoxygenase-induced reaction system present in the rat lung [1]. Scheme 1 provides an overview of the arachidonic acid cascade including formation of the hepoxilins and their metabolites.

Similar hepoxilin products derived from EPA [2] and DHA [3] have been reported. Analogous hepoxilin-like compounds derived from linoleic acid from skin have been reported [4]. The hepoxilin compounds possess both hydroxyl and epoxide groups formed from an intramolecular rearrangement of the hydroperoxy oxygen atoms of 12(S)-HPETE [5] (Scheme 2). Hence it was proposed that this was an enzymatic process, termed 'hepoxilin synthase' [6]. This was also shown to be a specific reaction as the 12(R)-HPETE was not a substrate for the isomerization reaction present in the rat pineal [7]. However

Abbreviations: AA, arachidonic acid; AMG9810, (2*E*)-*N*-(2,3-dihydro-1,4-benzodioxin-6-yl)-3-[4-(1,1-dimethylethyl)phenyl]-2-propenamide; Apaf-1, Apoptotic protease activating factor 1; BAPTA, 1,2-Bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid; CCCP, carbonyl cyanide *m*-chlorophenyl hydrazone; CDC, cinnamyl-3,4-dihydroxy-α-cyanocinnamate; DAG, diacylglycerol; DHA, docosahexaenoic acid; EH, epoxide hydrolase; eLOX3, epidermal lipoxygenase-3; EPA, eicosapentaenoic acid; fMLP, formyl-methionyl-leucyl-phenylalanine; GPCR, G-protein-coupled receptor; GppNHp, 5′-Guanylyl imidodiphosphate; GPx, glutathione peroxidase; GTP, Guanosine-5′-triphosphate; GTPγS, Guanosine-5′-O-[gamma-thio] triphosphate; HC030031, 1,2,3,6-Tetrahydro-1,3-dimethyl-N-[4-(1-methylethyl)phenyl]-2,6-dioxo-7H-purine-7-acetamide; HETE, hydroxyeicosatetraenoic acid; HODE, hydroxyoctadecadienoic acid; HP, hydroperoxy; HPETE, hydroperoxyeicosatetraenoic acid; HSP, heat shock protein; HX, hepoxilin; HXA_3-C, 11-Glutathionyl-hepoxilin A_3; IP3, inositol triphosphate; IP3R, inositol triphosphate receptor; K-562, chronic myelogenous leukemia human cell line; LOX, lipoxygenase; LTB_4, leukotriene B_4; NAT, n-Acetyl transferase; NECA, 5′-N-ethylcarboxamidoadenosine; PAF, platelet activating factor; PBT, proprietary bioactive therapeutics; PC, phosphatidyl choline; PE, phosphatidyl ethanolamine; PG, prostaglandin; PHGPx, phospholipid hydroperoxide gluthathione peroxidase; PPARγ, the Nuclear Peroxisome Proliferator Activator Receptor gamma isoform; ROS, reactive oxygen species; RVD, regulatory volume decrease; TCPO, 3,3,3-Trichloro-1,2-propene oxide; TRAP220, mediator of RNA polymerase II transcription subunit 1; TRPA1, transient receptor potential ankyrin 1 channel; TRPV1, transient receptor potential vanilloid 1 channel; TRX, trioxilin; U0126, 1,4-Diamino-2,3-dicyano-1,4-*bis*(2-aminophenylthio)butadiene, MEK Inhibitor VI; U937, cell line isolated from the histiocytic lymphoma of a 37-year old male patient; XOR, xanthine oxidoreductase

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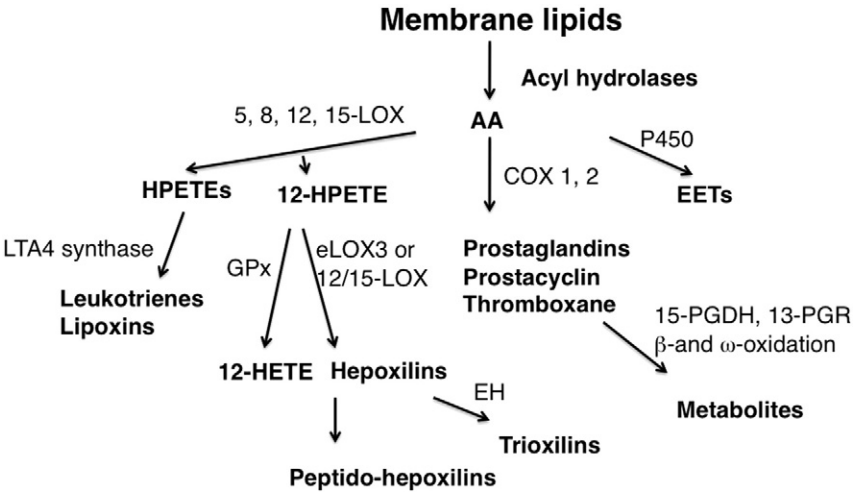


Scheme 1. Classical 'Arachidonic Acid Cascade' pathway showing diverse enzymatic mechanisms of oxidation of arachidonic acid. Several of these pathways have been described for EPA and DHA substrates.

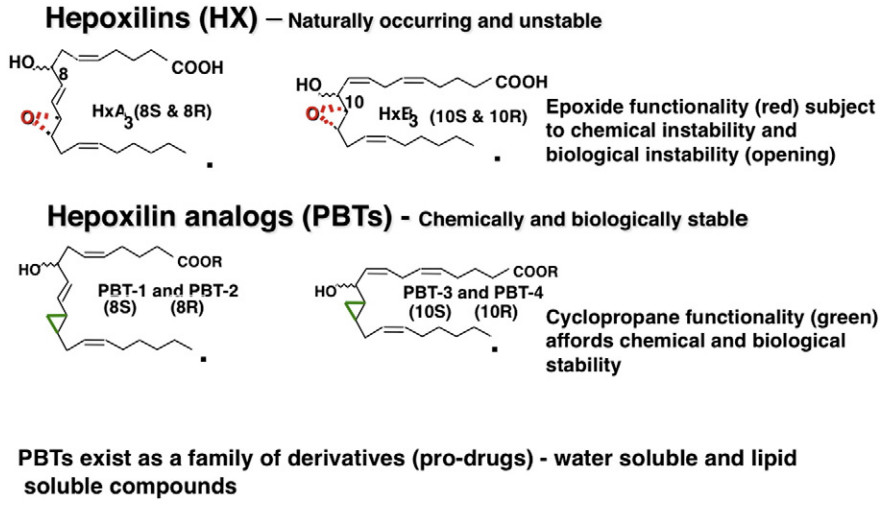
both 12(S) and 12(R)-HPETEs were converted into native (11S, 12S) and what we termed at the time, 'unnative' (11R, 12R) hepoxilins, when the reaction was catalyzed by heme-containing proteins in vitro [8]. Since then it has been shown that the 11R, 12R-enantiomer is uniquely formed in skin through a process initiated by 12(R)-lipoxygenase [9,11].

The enzymatic nature of the isomerization reaction to form the hepoxilins was later confirmed through two important findings 1) cloning experiments using rat insulinoma cells, RINm5F, demonstrated that the responsible isomerization activity was an intrinsic component of the 12(S)-lipoxygenase activity of the 12/15-lipoxygenase enzyme [10], and 2) demonstration that eLOX3, a protein which possesses no 12-LOX activity originally found in skin, acts in concert with 12(R)-LOX to convert the 12-LOX product, 12(R)-HPETE into 11R, 12R-hepoxilins [4,11,12] (see section on Control of hepoxilin/12-HETE formation).

Because the native hepoxilins are unstable chemically (degraded in acid environment) and biologically (degraded through cellular epoxide hydrolases) [13,14] to produce trihydroxy compounds (trioxilins) with much lesser (if any) biological activity, we developed stable analogs in the early 1990s to investigate the potential biological activity profile of the hepoxilins in vivo [15]. These analogs were designed to replace the unstable epoxide group with a stable cyclopropyl group (Scheme 2). In so doing, it was hoped that information could be obtained on the role of the endogenous hepoxilins. Indeed this review addresses the major findings from our group and those from several others showing the involvement of the hepoxilins in acute inflammation, the efficacy of the hepoxilin analogs as antagonists of the native hepoxilins, and to offer some insight as to the potential therapeutic interest generated by a recent finding that the hepoxilin analogs additionally cause some of their inhibitory effects in both the cancer animal model in vivo and on platelet aggregation in vitro through inhibition of the release of arachidonic acid, suggesting inhibition of an acyl hydrolase, likely a type of phospholipase A_2 [16].

2. Distribution of the hepoxilins

Studies in the early 1980s showed the presence of a unique transformation of 12(S)-HPETE in rat lung [1]. Two unstable products were isolated which were termed hepoxilin A_3 and hepoxilin B_3 (Scheme 2). Although the hepoxilins were further metabolized into their trihydroxy metabolites (trioxilins A_3 and B_3) through epoxide hydrolases present in the lung, hepoxilin synthetic/epoxide hydrolase activities were separated through ammonium sulfate fractionation of a low speed supernatant fraction of the lung homogenate [17]; thus, while 12(S)-HPETE and hepoxilins were formed by the 0–30% ammonium sulfate



Scheme 2. Structures of the native hepoxilins A_3 and B_3 and the corresponding synthetic stable analogs (PBTs). It should be noted that hepoxilin A3 is less stable than B3 due to the allylic nature of the epoxide.

fraction, the trihydroxy metabolites were formed from either hepoxilin A_3 or B_3 by an enzyme system (epoxide hydrolase) present in the pellet from the 30–50% saturation in ammonium sulfate. The hepoxilins were released intact from perifused rat pancreatic islets of Langerhans, on which they acted to release insulin [18–20]. Additional studies showed that the hepoxilins were formed by the rat pineal gland [7,21] and by the rat brain [3]. Hepoxilins were shown to be formed in blood after bolus intra-arterial administration of arachidonic acid in the rat [22,23]. Upon blockade of the epoxide hydrolase activity with TCPO (3,3,3-trichloropropene-1,2-oxide), thereby blocking metabolism of the hepoxilins into trioxilins A_3 and B_3, it was shown that the hepoxilins were substrates for the liver and brain glutathione S-transferase to form 11-glutathionyl hepoxilins termed HXA_3-C [24–27]. The glutathionyl compounds displayed biological actions in brain and on platelet cell volume regulation [27,28]. Intact skin (subcutis layer) was shown to metabolize 12(S)-HPETE into hepoxilins A_3 and B_3, and this transformation was heat labile [29].

3. Requirements for hepoxilin formation

3.1. Enzymatic reaction

Hepoxilins are formed through one of two divergent pathways from 12-HPETE, a product formed through the action of 12-lipoxygenase. The other pathway forms 12-hydroxy eicosatetraenoic acid (12-HETE) [8] (Scheme 1). The extent to which either the hepoxilins or 12-HETE is formed depends on the extent of the activity of peroxide reducing enzymes e.g. glutathione peroxidase and similar enzymes which reduce 12-HPETE to 12-HETE, and hepoxilin isomerase (synthase) which forms hepoxilins from 12-HPETE. Nigam's group has shown that the rat insulinoma cell line, RIN5mF, is low or devoid of glutathione peroxidase, so that the 12-HPETE could survive long enough to be transformed into hepoxilins [10,30]. In fact this formed the basis of further refinement of the view of the enzymatic nature of hepoxilin synthase by demonstrating that hepoxilin synthase was an intrinsic feature of 12(S)-lipoxygenase. Interestingly, by employing a site directed mutagenesis approach, it was shown that 15-LOX, which lacks intrinsic hepoxilin formation, could be transformed into 12-LOX with a recovery of hepoxilin synthase activity; the converse was also shown where 12-LOX with intrinsic hepoxilin synthase activity could lose its hepoxilin forming capacity by transforming it into 15-LOX [10]. Molecular models suggested that the extent of penetration of the arachidonic acid substrate into the enzymatic pocket of 12/15-LOX is essential to direct the substrate to be subjected to 12-LOX activity coupled with hepoxilin formation or 15-LOX activity (without hepoxilin formation).

3.2. Control of hepoxilin/12-HETE formation

Since metabolism of 12-HPETE follows two pathways, i.e. 12-HETE or hepoxilins, it was shown that both pathways were inversely controlled by the reducing potential within the cell. Nigam proposed this to be carried out by the level of both the abundant cytosolic glutathione peroxidase (GPx-1) and the phospholipid hydroperoxide gluthathione peroxidase (PHGPx), a hydroperoxide scavenger, such that cells containing a high amount of these peroxidases shifted metabolism of the 12(S)-HPETE into 12(S)-HETE with little or no hepoxilins formed [31]. Blockade of these peroxidases with iodoacetate or in the absence of the peroxidases (as in RIN5mF cells) resulted in hepoxilins being formed [31]. The importance of the lipid reduction/peroxidation balance (tone) in cells has been pointed out in independent reviews on the enzymatic formation of hepoxilins by Nigam [32] and by Pattabhiraman [33].

The recent evidence has shown at least in human skin, that hepoxilin synthase is identified in a protein, eLOX3 that is devoid of 12-LOX activity [34]; eLOX3 appears to be associated with this 12(R)-lipoxygenase in skin and few other tissues; it has been found defective in ichthyosis, a genetic defect involved in scaly skin, dry skin, infections and other conditions related to water loss, suggesting that products of eLOX3 may be important (possibly essential?) in normal water barrier function [9,11,34–37]. eLOX3 has been implied in inflamed skin and tonsils [38,43], although other organ systems appear to implicate its existence, e.g. lung and spinal cord. eLOX3 may indeed be ubiquitous, but the product, hepoxilin A_3, may only be found if the reduction pathway (i.e. 12-HETE formation) is suppressed. Is eLOX3 selective in vivo for 12(R)-HPETE, converting it into the 11R, 12R-hepoxilin (as in skin) [9,39] as this is a better substrate in vitro than 12(S)-HPETE which is converted into the corresponding 11S, 12S hepoxilin [39]? In other words is the hepoxilin derived from 12(S)-HPETE exclusively formed through 12/15-LOX? A new form of ichthyosis has been reported in a patient with lamellar ichthyosis (LI) whose scales expressed high amounts of 12(R)-LOX without conversion of the 12(R)-HPETE into the corresponding 11R, 12R-hepoxilin A_3. This new form of ichthyosis has been termed hepoxilin A(3) synthase-linked ichthyosis (HXALI) [40].

Through Aloxe3 knockout mice, Krieg's group demonstrated an important role of eLOX3 and therefore of hepoxilins playing an essential role in water barrier function [37]. Two excellent recent reviews on the properties of skin lipoxygenases and their potential role in the activity of the water permeability barrier have recently appeared [4,12]. In these reviews, the potential formation of hepoxilins derived from arachidonic acid or of '*hepoxilin-like*' compounds derived from linoleic acid, the main PUFA esterified to omega-hydroxylated acyl ceramides, is discussed. It is of interest that normal murine skin expresses both leukocyte-type and platelet-type 12-LOX although 12R-LOX is in low amount [11,41]; once activated through inflammation (psoriasis [42,43]) or in tumors [41], or in ichthyosis [4,34], skin expresses 8(R)- and 12(R)-lipoxygenase activity [9]. Interestingly, it was recently shown through the use of 12R-LOX$^{-/-}$, or eLOX-3$^{-/-}$ mice that formation of dermal hepoxilins derived from 12S-HPETE was unchanged in the former model, while they were totally abolished in the eLOX-3$^{-/-}$ mice, suggesting that hepoxilins are NOT formed from 12R-LOX (the precursor HPETE is) but that eLOX-3 is essential for their formation. While animals deficient in eLOX-3 reproduced normally, the pups died within 12 h of birth due to defective skin barrier function [37]. While eLOX3 is important as a 'hepoxilin synthase', it has been proposed that its function in the skin is to form 'hepoxilin-like' compounds derived after the action of 12R-LOX on the abundant C18 fatty acids esterified to omega-hydroxylated ceramides [4,11,12]. AA is both deficient in these ceramides and is also a poor substrate for 12R-LOX [44]. Skin is the only tissue in which 12(R)-lipoxygenase has been observed with its subsequent formation of 12(R)-HETE and hepoxilins of the R,R epoxide configuration [9].

Indeed 12(S)-lipoxygenase activity has been linked to conditions of oxidative stress. Under these conditions hepoxilin formation is elevated since the reduction pathway (PHGPx) which converts 12(S)-HPETE to 12(S)-HETE is diminished. This was initially shown in platelets depleted of GSH through selenium deficiency where 12(S)-HPETE formation became elevated many fold and this was accompanied by increases in the trihydroxy compounds later recognized to be formed from the hepoxilins [45–47]. Hence the metabolism of 12(S)-HPETE can be diverted to 12(S)-HETE or the hepoxilins depending on the state of peroxidaase activity in the cell with biological consequences as both pathways produce compounds with differing biological actions. For example 12(S)-HETE causes proliferation of cancer cells [48], it is implicated in diabetes [49], hypertension [50], and certain other diseases, whereas hepoxilin A_3 is a pro-inflammatory mediator and potentiates vascular reactivity of constrictor hormones [51,52]. The stable hepoxilin analogs are pro-apoptotic, anti-inflammatory, anti-thrombotic and anti-diabetic (see review in [53]). Through their effects on inhibiting phospholipase A_2 [16] the hepoxilin analogs may very well prevent the formation of 12-HETE and actions of hepoxilins and other eicosanoids

(such as in inflammation, cancer and other diseases, see sections below).

3.3. Involvement of phospholipase A_2

Phospholipase A_2 is critically involved in the biosynthesis of the oxygenated family of compounds formed, by releasing the substrate arachidonic acid or other polyunsaturated fatty acids (EPA and DHA) from membrane phospholipids. These oxygenated compounds are formed from the cyclooxygenases-1 and -2, lipoxygenases (5-, 8-, 12- and 15-) and cytochrome P450 as well as through combinations of these enzymatic reactions (5- and 12-; 12- and 15-; 5- and 15-; 12- and hepoxilin synthase). Thus multiple end products with diverse biological function are derived within the so called 'Arachidonic Acid Cascade' (Scheme 1). These functions relate to inflammatory disease, cancer, diabetes, thrombosis, and pain.

4. Second messenger systems

4.1. Adenylyl cyclase

Hepoxilin A_3 inhibited cyclic AMP formation in the rat pineal gland that was stimulated with NECA, an A1/A2 adenosine receptor agonist [21]. Interestingly, an increase in intracellular calcium release is accompanied by receptor stimulation as well as an increase in phospholipase A_2 activity thereby releasing the hepoxilin precursor, arachidonic acid. Also of interest is the finding that 12- and 15-HPETE were found to stimulate NAT activity (involved in melatonin synthesis) and melatonin release [54]. Other HPETEs and HETEs were ineffective. It has been shown that 12-HPETE is formed in the pineal and that 12-HPETE is transformed to the hepoxilins [21] (see above discussion). This action of hepoxilins inhibiting cyclic AMP formation was stereospecific as the 8(S) (11S, 12S) hepoxilin was approximately 7 fold more active than the corresponding 8(R) hepoxilin (11S, 12S) enantiomer [7].

Hepoxilins could negatively affect adenosine-induced cyclic AMP formation, or the receptors negatively-coupled with adenylate cyclase, or the catalytic unit of this enzyme [7]. In this respect, the hepoxilin analogs inhibit platelet aggregation although while this effect may be coupled to inhibition of cyclic AMP formation, it could as well result from the demonstrated antagonistic effects on the thromboxane [55,56] receptor as well as phospholipase A_2 inhibition [16] thereby negatively affecting thromboxane A_2 formation. Hence it is probable that hepoxilins may play a significant role in melatonin synthesis through their stimulatory effects on calcium and cyclic AMP and NAT although direct measurement of melatonin production as influenced by hepoxilins is lacking.

4.2. G-protein interaction

Hepoxilin A_3 evokes a biphasic release of intracellular calcium in human neutrophils [57]. An initial fast response is observed that is independent of extracellular calcium, accompanied by a secondary slower phase that is dependent on the presence of extracellular calcium. The slow phase of intracellular calcium release was blocked by the incorporation of the non-hydrolyzable form of GTP, i.e. GTP-γ-S into 'shocked' cells or through the use of GppNHp suggesting involvement of a G-protein [58].

4.3. The nuclear peroxisome proliferator activator receptor — PPARγ

PPARγ is at the center stage of adipogenesis, the differentiation of pre-adipocytes to mature adipocytes. Adipocyte differentiation is an important process required to control obesity and insulin sensitivity. Many factors are involved in the control of adipogenesis, and thiazolidinedione drugs are most widely used for PPARγ activation, control and management. Dysfunction of adipogenesis leads to metabolic disease. Oxidative stress affects adipogenesis. For example, Reactive Oxygen Species (ROS) appear to facilitate the early mitotic clonal expansion phase of adipogenesis. The antioxidant protein thioredoxin increases adipocyte differentiation in vivo, leading to improved insulin sensitivity through increased expression and activity of PPARγ [59].

Fatty acids and some derivatives are known to bind to and activate PPARγ. Interestingly, although lipoxygenases are relatively ubiquitous, eLOX3 which transforms 12(R)-HPETE into R,R-hepoxilins is uniquely found in pre-adipocytes (3T3L1) and skin [60]. Hence although the natural ligand for PPARγ activation has been proposed to be 15-deoxy-delta 12-PGJ2 (15d-delta12-PGJ2) [61,62], the occurrence of eLOX3 in pre-adipocytes thereby implicating the formation of hepoxilins, together with the known inhibition of PPARγ activation by baicalein, a lipoxygenase inhibitor, might suggest that a lipoxygenase-derived product could prove to be another endogenous ligand for PPARγ activation [63]. Indeed, 3T3L1 pre-adipocytes are equipped with eLOX3, yet surprisingly devoid of conventional lipoxygenases suggesting that they would produce epidermal-type hepoxilins [63]. In fact, hepoxilins A_3 and B_3 stimulate maturation of pre-adipocytes into mature adipocytes through a selective stimulation of PPARγ [60]. Further, hepoxilin B_3 enhanced binding of TRAP220 (Mediator Subunit 1, Med1) to the PPARγ LBD to a level comparable to that of rosiglitazone. PPARγ agonists increase transactivation by facilitating binding of coactivators. TRAP220 is part of the mediator complex required for adipogenesis [64,65]. It was found that hepoxilins are transiently formed during the initial stages of adipogenesis, a stage where they would be involved in the activation of the differentiation process [60]. Additionally, ROS generation by xanthine oxidoreductase (XOR) synergistically activated eLOX3 in producing the lipoxygenase-derived ligand that activated PPARγ during the early stages of pre-adipocyte to adipocyte differentiation [60]. Such conditions of ROS are known to stimulate hepoxilin formation presumably through enhanced eLOX3 expression/activity. Hence the data appear to strongly support an essential role for the hepoxilins in adipogenesis.

It is interesting to note that 15d-delta12-PGJ2 has been shown through its electrophylic properties to covalently bind to nucleophiles in proteins such as glutathione and cysteine in proteins [66]. Additionally, 15d-delta12-PGJ2 has been shown to bind covalently to a unique cysteine residue in PPARγ suggesting that its irreversible covalent association with PPARγ may alter the binding affinities/actions of classical PPAR agonists [67]. This may apply to the hepoxilins as the epoxide group of hepoxilin A_3 is a reactive functionality able to react with sulfhydryl groups (e.g. in proteins) or water. Indeed hepoxilin A_3 is metabolized into a glutathione conjugate, HxA3-C, suggesting that hepoxilin A_3 (and probably also B_3) may be found covalently linked to protein. Could hepoxilin's actions as described in this Review be related to covalent binding to specific elements of certain proteins?

4.4. Heat shock protein (HSP70)

Heat shock proteins constitute a family of chaperone proteins. They are involved in cell's machinery for folding and refolding of proteins to prevent their retention in the endoplasmic reticulum. Their role is to protect proteins from aggregating and becoming non-functional. HSP expression has been associated with cell injury and with the repair process. They are induced in human leukocytes during infection and inflammation suggesting a role in inflammatory processes. 12-HETE is formed by human leukocytes and is known to induce the expression of HSP70 [68,69]. 12-HETE is chemotactic to human neutrophils and evokes intracellular calcium effects and second messenger release [70]. Hepoxilin A_3 was shown to stimulate HSP70 synthesis in human neutrophils to a greater extent than exposure of the cells to 42 °C heat shock, but its effect was slightly less than that of 12(S)-HETE, while an unrelated compound, PGA_1, was ineffective [71].

HSP70 is believed to directly inhibit apoptosis [72]. A hallmark of the intrinsic pathway of apoptosis is the activation of cytochrome c release from the mitochondria leading to a recruitment of Apaf-1 and dATP/ATP into an apoptosome complex. Caspase-3 is activated through the activation of caspase-9 by this apoptosome complex thereby causing apoptosis. Although it has not been shown that the natural hepoxilins A_3 and B_3 affect apoptosis, it is interesting to note that the hepoxilin analogs evoke apoptosis in the leukemic K562 cell line in vitro and in K562-derived tumors in vivo in immunocompromised mice [73]. In vitro studies have shown that the hepoxilin analogs cause apoptosis through a stimulation of the release of cytochrome c and activation of caspase-3 [74]. These studies suggest that the hepoxilins may be involved in the stress/HSP/anti-apoptosis pathways and that the hepoxilin analogs oppose this, probably directly as hepoxilin A_3 antagonists [75] or indirectly as phospholipase A_2 inhibitors [16], potentially leading to a novel therapeutic approach for cancer control (see later section on hepoxilin analogs in cancer).

5. Inflammation

5.1. General actions

The recent studies from several laboratories have demonstrated that the hepoxilin pathway represents a novel approach to acute inflammation [76–82]. The hepoxilins had previously been shown to be formed by human neutrophils, to raise intracellular calcium in neutrophils, promote chemotaxis of human neutrophils, and promote skin vascular permeability, hallmarks of acute inflammation (reviewed in [53]). The hepoxilins act on intracellular calcium in a GPCR-coupled response, and neutrophils have specific hepoxilin binding, essential for hepoxilin's calcium mobilizing actions [75,83,84] and chemotaxis [85]. They are formed during pathogen-evoked inflammation [78] in which they act as chemoattractants to recruit neutrophils to the apical surface of the intestinal epithelium; in a Lyme disease model infected with the Lyme bacterium *Borrelia burgdorferi* [80] where they are temporally the first eicosanoids formed after infection; in inflammatory hyperalgesia and tactile allodynia [81,82] where they appear to activate TRPV1 and TRPA1 receptors. The hepoxilin PBT analogs antagonize the onset of lung fibrosis in vivo in a mouse model of human idiopathic pulmonary fibrosis evoked by intratracheal administration of bleomycin, an antibiotic with potent cancer chemotherapeutic effects [86]. These analogs appear to inhibit the rate of collagen synthesis and deposition, and the marked accumulation of macrophages, events characterized in this acute inflammatory model of pulmonary fibrosis.

Acute inflammation is associated with vascular events and white cell events. The former involves initial vasoconstriction of the blood vessels causing a decrease in blood flow to the injured site, followed by vasodilatation leading to an increase in blood flow and an increase in the release (vascular permeability) of plasma and fluids (leakiness) into the surrounding tissue. This essentially allows access of important serum proteins (complement and antibodies) to enter the tissue site to promote antimicrobial action and phagocytosis by leukocytes. Influx of fibrinogen with resultant fibrin formation limits the spread of infection. A second important event in acute inflammation results from the invasion of white blood cells to the site of infection in characteristic events. The hepoxilins have been shown to be intrinsically involved in the early part of the acute phase, i.e. vascular permeability and migration of leukocytes in a pathogen-evoked inflammatory model [77].

5.2. Intracellular calcium release

Calcium is an essential intracellular regulator of many cellular processes, including gene transcription, muscle contraction and cell proliferation. Intracellular Ca^{2+} concentration fluctuates upon excitation of the cell leading to cell activation, or modulation or termination of response [87]. This involves a variety of plasma membrane molecular channels and ion pumps to mobilize calcium into or to exit the cell. Of particular interest to this review are store-operated Ca^{2+} channels regulating entry in non-excitable cells present in blood, neutrophils. These channels open as a response to the stimulated depletion of Ca^{2+} through interaction with IP3 and its receptor IP3R. IP3 and DAG are generated from PIP2 upon activation of PLC [88,89]. Indeed it has been shown that hepoxilin A_3 activated the release of DAG from human neutrophils [90]. Intracellular stores release their calcium from organelles through the activation of the ryanodine receptor channels and IP3R [91].

The native hepoxilins were shown to affect intracellular calcium responses in human neutrophils [57,92]. Both the methyl ester and the free acid were active in causing a rise in intracellular calcium in human neutrophils [93]. Hepoxilin A_3 caused a biphasic rise in intracellular calcium, with an initial rapid phase followed by a slow secondary phase. Extracellular depletion of calcium blunted the secondary phase but not the initial phase, as the mitochondrial uncoupler CCCP also did [92]. The effect of hepoxilin A_3 was coupled to GTP-binding proteins as it was abolished by pertussis toxin [57]. Subsequent studies showed that neutrophils that had incorporated an analog of GTP, i.e. GTP-γS, through the cellular shock or through the use of GppNHp were unable to respond to hepoxilin A_3 treatment indicating the involvement of GPCR in their action [58]. Of interest was the finding that hepoxilin A_3 blunted the calcium mobilizing actions of a variety of unrelated inflammatory agents such as FMLP, LTB_4, PAF and thapsigargin, suggesting that hepoxilin A_3 acted on some common pathway affected by these agents [94], i.e. initial effect/suppression of calcium stores by hepoxilin A_3 rendering action by the diverse inflammatory mediators largely ineffective. Could this be explained by an effect of hepoxilins on IP3 release? Hepoxilin A_3 produced acidification that was decreased in cells loaded with BAPTA, a calcium chelator. Importantly, hepoxilin A_3 increased acidification evoked by N-ethyl-N-(methyl ethyl)amino ameloride, an inhibitor of the Na+/H+ antiport and failed to elicit a recovery phase suggesting that the recovery phase was due to activation of the antiport [57].

It is of interest that the hepoxilin analogs behave in a similar way to the natural hepoxilin A_3 on the blockade of intracellular release of neutrophil calcium by the inflammatory mediators except that unlike hepoxilin A_3, the hepoxilin analogs weakly affect calcium on their own [95].

Hepoxilin A_3 dose dependently blocks its own effect in releasing intracellular calcium in human neutrophils supporting the notion that it may inhibit further release of intracellular calcium stores through which it then inhibits the action of several unrelated inflammatory mediators. There are no reports as yet relating to how quickly the recovery of normal calcium release takes place after either the hepoxilin A_3 or the hepoxilin antagonists.

Other studies showed hepoxilin A_3 to attract calcium ions across membranes [96]. The guinea pig visceral yolk sac was used whereby [45] Ca and [14] C-hepoxilin A_3 were followed in Ussing chambers at the maternal and the fetal side of the membranes. While radiolabeled hepoxilin was not transported across the membrane, radiolabeled calcium was transported to either direction of the membrane (fetal or maternal) that hepoxilin was placed.

Important to subsequent studies showing the involvement of hepoxilins in the onset of bacterial infection (see 'specific conditions' below), was the demonstration by Nigam's group [85] that hepoxilin A_3 caused strong chemotaxis of human neutrophils. Hepoxilin A_3 proved to be similar in potency to LTB_4 although it showed an apparent biphasic effect. Hepoxilin A_3 was more potent than FMLP. Its effect was abolished by pretreatment of the cells with pertussis toxin, confirming previous reports that hepoxilin action was mediated through G-protein interaction [57,58].

While native hepoxilin A_3 (11S, 12S) potently releases intracellular calcium in human neutrophils and causes neutrophil chemotaxis, we observed that native hepoxilin B_3 showed marginal effect in releasing

calcium but it did inhibit the calcium mobilizing actions of native hepoxilin A_3 suggesting that hepoxilins A_3 and B_3 may share binding to a putative receptor. On the other hand hepoxilin A_1, an analog devoid of delta 5 and 13 double bonds was ineffective showing the importance of the chain orientation in the molecule for receptor binding/action.

The role of hepoxilins in causing potentiation of effects that may be calcium related was also demonstrated on blood vessels. Indeed, hepoxilin A_3 was inactive on its own but it potentiated norepinephrine-evoked constriction of rat thoracic strips with a threshold dose at 10^{-8} M, the effect lasting beyond washing out of the compound. In this model, the 8(S) epimer was the active species of hepoxilin A_3, while the 8(R) epimer was inactive [51]. The hepoxilin effect was blocked by nifedipine, a calcium channel blocker. In another study the vascular effects of the hepoxilins and related compounds on the vascular tone of the guinea pig isolated trachea was evaluated [52]. Again, neither of the compounds tested had any effects on the resting tension by themselves. However the 8(S) hepoxilin A_3 potentiated the vasoconstriction of neurokinin A with a threshold dose at 10^{-8} M while the 8(R) epimer was inactive. The corresponding trihydroxy compound was inactive indicating significant specificity. These studies suggested that hepoxilins act in a stereospecific fashion to modulate vascular tone and contractility. In contrast, trihydroxy derivatives derived from 15-HPETE (11-hydroxy-14,15-epoxyeicosatrienoic acid and 15-hydroxy-11,12-epoxyeicosatrienoic acid) appear to be endothelium-derived 15-lipoxygenase metabolites that relax the rabbit aorta with specificity for the 11(R),12(S),15(S)-trihydroxyeicosa-5(Z),8(Z),13(E)-trienoic acid stereoisomer [97,98]. 12-HPETE has been shown to be converted by the rabbit aorta into 8,9,12-trihydroxyeicosatrienoic acid termed trioxilin C3, with potent concentration-dependent relaxant properties on phenylephrine-precontracted aortas [99].

5.3. Vascular permeability

Vascular permeability plays an important role in acute inflammation by causing the leakage of plasma proteins from blood in preparation for the migration of leukocytes to the site of damage, this being bacterial in origin or other physical trauma evoked to the tissue (skin or other organ site). Prostaglandins have been recognized as signals that evoke plasma exudation in the skin in vivo [100]. We have demonstrated a similar effect of the native hepoxilins (11S, 12S) at similar potency to that evoked by prostaglandin E_2 [101]. Although the hepoxilins are not powerful in causing an increase in vascular permeability on their own, they greatly potentiate the action of sub-threshold doses of bradykinin. Interestingly, while the 8(S) epimer of hepoxilin A_3 was essentially inactive, the 8(R)-epimer was quite active in a dose-dependent fashion from 10 ng to 1000 ng/spot tested. In the presence of bradykinin (50 ng) which did not cause changes in vascular leakage on its own, 8(R) hepoxilin A_3 greatly potentiated the leakage 100-fold within the range 0.1–10 ng/spot tested [102].

5.4. Migration of neutrophils

Models of intestinal infection (Salmonella and Pseudomonas) [76–79,103] have been shown to produce hepoxilin A_3 as an essential mediator of the process whereby white cells migrate to the infection site at the apical epithelial layer. It has been proposed that hepoxilin A_3 affects this process by establishing a gradient for the chemoattraction of the leukocytes. Hence the bacterial infection must activate a phospholipase A_2 which releases the hepoxilin precursor, arachidonic acid, at an early stage and this is then transformed into hepoxilin A_3.

How does hepoxilin A_3 function to attract leukocytes? It had been demonstrated by us and others that hepoxilin A_3 has potent effects on human neutrophils in vitro. It causes release of intracellular calcium, it causes vascular permeability, and it is a potent chemoattractant to human neutrophils. A putative selective hepoxilin binding protein has been observed [75]. Hence the hypothesis that hepoxilin A_3 may cause the attraction of neutrophils across epithelial barriers is well-founded. Additional support of this hypothesis is derived from the abolition by 12-LOX inhibitors of neutrophil migration to bacterial infection, indicating that not only is arachidonic acid release required after bacterial infection, but also its transformation into the hepoxilins. Taking Nigam's findings of the regulation of hepoxilin formation by peroxidase activity (GPx/PHGPx), thereby shifting metabolism of 12-HPETE away from 12-HETE (reductase pathway) to hepoxilins (isomerase pathway), this must imply that the bacterial infection somehow must increase 12-HPETE survival for it to be transformed selectively into hepoxilins. Does bacterial infection downregulate GPx/PHGPx activity? An additional point remains to be explained. How does hepoxilin A_3 exert its specificity on neutrophils? The next section shows the presence of a specific protein on human neutrophils that binds hepoxilin A_3.

5.5. Specific hepoxilin binding

In 1995 we observed binding of tritium labeled 8(S) hepoxilin A_3 to human neutrophils [83,84]. This binding was specific to hepoxilin A_3 as other eicosanoids including the prostaglandins, LTB_4 and 12-HETE did not antagonize hepoxilin binding. The trihydroxy metabolite (trioxilin A_3) was ineffective. The 8(R) epimer of hepoxilin A_3 was equally active in displacing the radioligand. A single population of binding sites was calculated with binding parameters $K_D = 79.3 \pm 9.1$ nM and $B_{max} = 8.86 \pm 1.4$ pmol per 2×10^6 cells approximating 2.67×10^6 sites/cell. Specific binding was inhibited by the pretreatment of broken cells with proteinase K, while intact cells were unaffected indicating that the binding protein was intracellular [75]. In additional studies it was shown that the action of hepoxilins in releasing intracellular calcium was coupled to its ability to bind to the putative binding protein as the methyl ester form of hepoxilin A_3 gave a strong fast intracellular calcium release as well as good competition binding to the radioactive hepoxilin ligand in intact neutrophils [58]. Further studies in which the non-hydrolyzable GTP analog, GTP-γS, was incorporated into neutrophils through shock treatment of the cells, showed that the binding and calcium action of the hepoxilins were inhibited indicating the involvement of a G-protein in the response [58]. This was also inhibited by pertussis toxin [57]. Further studies with a radioiodinated photo affinity analog of hepoxilin A_3 [104] showed competition binding of the radioligand by unlabeled hepoxilin A_3 for a single protein in intact human neutrophils (Pace-Asciak, Demin, Reynaud, unpublished). The photo affinity analog antagonized the effects of hepoxilin A_3 to the mobilization of intracellular calcium in human neutrophils demonstrating binding of this ligand to the hepoxilin binding site.

6. Specific conditions

6.1. Insulin secretion

Elevated blood glucose leads to a sequence of events including a rise in potassium and calcium ions in the cells within the islets of Langerhans in the pancreas ending by the release of insulin from these cells. This rise in calcium ions activates phospholipase C causing cleavage of phosphatidyl inositol 4,5-bisphosphate into 1,4,5-trisphosphate (IP3) and diacylglycerol. IP3 activates receptor release of calcium ions from the ER into the cytosol and provokes the release of insulin from pre-formed stores in secretory vesicles [105].

The first biological action observed of exogenously applied hepoxilin A_3 to perifused intact rat islets of Langerhans was the release of insulin [18]. In this early study it was shown that the islets of Langerhans actually converted 12S-HPETE into hepoxilins A_3 and B_3 besides 12-HETE, hence demonstrating the presence of hepoxilin synthase enzymatic activity. The hepoxilin A_3 so formed potentiated glucose (10 mM) stimulated release of insulin almost 3-fold at 2 μM concentration over

control. At this concentration, PGE_2 stimulated insulin secretion by only 2-fold over control. Subsequent studies showed that the effect of hepoxilin A_3 on insulin secretion from incubated islet cells was strongly dependent on the glucose concentration used; there was an optimal stimulation by hepoxilin A_3 versus control, of insulin secretion at 5–6 mM glucose throughout the tested range of glucose concentrations between 3 and 15 mM (A. Beney and C. Pace-Asciak, unpublished). Subsequently, it was shown that hepoxilins were able to release insulin in vivo [106] after intraarterial administration to rodents.

The effect of hepoxilin A_3 on the release of insulin from the β cells may be a direct effect on the ER initiated by a specific mobilization of calcium [57]. It is not known whether the hepoxilin binding protein is located on the ER, or whether the effect of the hepoxilin is mediated through the phospholipase C pathway thereby releasing calcium through an IP3 pathway. It has been reported that hepoxilin A_3 stimulates the release from human neutrophils of arachidonic acid and diacylglycerol [90] suggesting an involvement of phospholipase A_2 and C and its inhibition by pertussis toxin through receptor mediation.

The deleterious effects of 12-HETE in diabetes onset (type 1 and 2) and obesity shown through 12-LOX knockout mice have been discussed in a recent review from Nadler's group [107]. 12-LOX knockout mice are resistant to diabetes induction with streptozotocin, or with a high-fat diet suggesting a causative role of 12-HETE in diabetes development and to β-cell dysfunction in the obese state. Unfortunately the presence of hepoxilins was not studied to determine whether they were additionally responsible for the anti-diabetic effects. Although it would be expected that hepoxilins would not be formed in 12-LOX knockout mice, it is possible that these compounds may be formed through pathways other than the classical AA cascade (see section on Skin).

6.2. Brain function

There is limited information regarding the potential role of hepoxilins in the brain except that the hepoxilins are formed in the brain and that they modulate electrical and neurotransmitter effects [108]. Hepoxilin A_3 was shown to potentiate neurite regeneration [109]. Hepoxilin A_3 also caused the release of calcium from intracellular stores in neurons [109]. Whereas normal neurons showed a similar biphasic calcium pattern as previously observed in human neutrophils with the second slow phase returning to a baseline significantly above the initial baseline, injured neurons apparently lack this second phase of calcium release and return to baseline immediately after the initial robust rise in intracellular calcium concentration. This suggests the possibility that the injured neurons lack the mechanisms to release calcium from the mitochondria as observed in the normal second phase pattern being refractory to CCCP, the mitochondrial uncoupler, or in the absence of extracellular calcium [109].

In separate studies hepoxilin A_3 displayed neuromodulatory effects on hippocampal CA1 neuronal slices in vitro [26,110]. Hepoxilins were shown to be formed by these intact and electrophysiologically functional hippocampal slices treated with arachidonic acid. Electrophysiological recordings demonstrated that hepoxilin A_3 at concentrations as low as 0.5–10 μM prolonged AHP and the early and late stages of the IPSP suggestive of a post-synaptic effect of the hepoxilins in the former through increased potassium ion conductance due to increased calcium ion entry or to increased sensitivity of potassium channels to ambient intracellular calcium concentrations. The IPSP changes are presynaptic events likely related to intracellular changes in calcium concentrations evoked by hepoxilin A_3. Interestingly, hepoxilin A_3 was shown to blunt the release of norepinephrine from rat hippocampal slices indicating a presynaptic action of the hepoxilin [111].

Hepoxilin A_3 is subject to metabolism through the glutathione S-transferase route as is known for the leukotrienes [112–116]. This transformation into glutathionyl hepoxilin A_3 (named hepoxilin A_3-C in keeping with the leukotriene nomenclature) is carried out by enzymes in the liver [26] and enzyme activity has been ascribed to isozymes containing the Yb_2 subunit and not the Ya or Yc subunit [24]. Hepoxilin A_3-C was formed in rat hippocampal slices in the presence of the epoxide inhibitor, TCPO thereby blocking the transformation of the hepoxilin A_3 precursor into the inactive trihydroxy metabolite. Hepoxilin A_3-C displayed robust activity at concentrations as low as 16 nM causing membrane hyperpolarization, enhanced amplitude and duration of the post spike train after hyperpolarization, a marked increase in inhibitory post synaptic potential and a decrease in spike threshold [27]. Hence it is postulated that hepoxilins and their glutathione metabolites might modulate synaptic neuromodulation in the brain.

6.3. Potentiation of vasoconstriction

It appears that the hepoxilins do not have actions on their own in most cases but appear to modulate (enhance or reduce) the actions of endogenous compounds which affect vascular tone. For example it was indicated above that hepoxilin A_3 blunted the release of norepinephrine from hippocampal slices evoked by 4-aminopyridine [111]. Exception to this was their effect on the release of intracellular calcium in human neutrophils where hepoxilin A_3 produced a robust biphasic action on its own, but blocked the action of unrelated inflammatory mediators [94], such as FMLP, LTB_4, PAF and thapsigargin. Also mentioned above was the robust effect of hepoxilin A_3 on the chemoattraction of human neutrophils, similar in potency to that evoked by LTB4 [85]. In contrast, hepoxilin A_3 showed an epimer specific sensitization of blood vessels to norepinephrine [51]. In this study, the 8(S) epimer of hepoxilin A_3 was active, while the 8(R) epimer was not. However, the 8(R) epimer of the glutathione adduct, hepoxilin A_3-C, was active in sensitizing the blood vessels to norepinephrine, while the 8(S)-epimer of this metabolite was inactive. The threshold concentration for the compounds was 10^{-8} M. Neither compound influenced the tone of the blood vessel when administered on its own. The noted sensitization to norepinephrine did not occur either in calcium-free medium or in the presence of nifedipine, a calcium channel blocker. In another study, the effect of hepoxilin A_3 on the guinea pig trachea was investigated [52]. Contraction was induced by neurokinin A and hepoxilin A_3 caused potentiation of the contraction within the concentration range 10^{-9}–10^{-6} M. Again as in the previous study with aorta and portal vein, the 8(S)-epimer of hepoxilin A_3 was active, the 8(R)-epimer was inactive, while the 8(R) epimer of the hepoxilin A_3-C was active, the corresponding 8(S)-hepoxilin A_3-C was inactive in modulating the tone of the trachea. The corresponding trihydroxy metabolite of hepoxilin A_3 was inactive.

6.4. Cell volume regulation

Regulation of cell volume is an important physiological function that cells have conserved throughout evolution in response to changes of osmolarity of the extracellular medium [117].

Cells swell as a result of a decrease in extracellular osmolarity and are able to counteract this increased volume by producing a substance(s) which restore(s) cell volume, a process known as regulatory volume decrease (RVD). This involves an outward movement through appropriate channels of K^+ and Cl^- followed by a passive redistribution of water [118]. This is an important process as cell volume affects a myriad of intracellular pathways that depend on co-factor concentrations, ion concentrations, receptor interactions, cell proliferation and cell death. Pathologically, maintenance of cell volume is affected by trauma to the brain and apoptosis [117].

Several years ago the involvement of a short-lived lipoxygenase product in RVD was observed in human platelets that were subjected to a hypotonic cell swelling [119]. The substance was termed LP. LP was formed and released during the swelling of platelets and LP-enhanced retraction of expanded cell volume. In these studies it should be indicated that cells that are initially volume expanded quickly return to a normal volume through the release of an endogenous substance

(LP) that opposes the swelling. Hence experimentally, in order that compounds can be tested for their ability to oppose swelling (i.e. with RVD properties), cells must be maintained in the swollen state; It was found that the addition of a lipoxygenase inhibitor (NDGA, nordihydroguiaretic acid) to the platelets before hypotonic volume expansion retained the cells in a swollen state until LP was added to retract the expanded cell volume. LP was later isolated and identified as hepoxilin A_3 [28]. Furthermore authentic hepoxilin A_3 showed the same biological properties in decreasing platelet cell volume as LP. Consistent with the identity as hepoxilin A_3, having an unstable allylic epoxide, the study showed that the addition of an epoxide hydrolase inhibitor (TCPO) rendered both LP and hepoxilin A_3 a 4-fold increased activity on RVD. This was an important early study identifying LP with hepoxilin A_3. RVD is driven by a K^+ gradient associated with the outward movement of KCl; hence, hepoxilin A_3 may represent an endogenous mediator required for normal homeostasis [28]. It is interesting to note that hepoxilin A_3 was earlier shown to activate distinct K^+ channels in the marine mollusk, *Aplysia californica* [120–122] suggesting that the effects of hepoxilin A_3 on RVD may indeed result from hepoxilin causing K^+ efflux.

6.5. Bacterial infection

The studies by McCormick's group are mostly responsible for the concept that hepoxilin A_3 may represent an intrinsic mediator of bacterial infection which creates a chemical gradient through which neutrophils are attracted to the site of infection (the apical surface of intestinal epithelial cells) [76,78]. Previously, it was known that the hepoxilins (A_3) showed pro-inflammatory properties, e.g. caused release of intracellular calcium in human neutrophils, acted through a specific binding protein in neutrophils, showed increased dermal vascular permeability, and was a potent chemoattractant to human neutrophils. The McCormick group importantly applied and extended these findings by demonstrating that hepoxilin A_3 was indeed formed by intestinal apical epithelial cells after infection with *Pseudomonas aeruginosa* [78], and the compound when added to *P. aeruginosa*-infected epithelial cells caused neutrophils to migrate across an epithelial barrier.

An important feature described in mice infected with *P. aeruginosa* is rectal prolapse. Rectal prolapse is a condition in which the rectum turns itself inside out. As the condition worsens, the rectum may protrude with weakness of the anal sphincter often resulting in leakage of stool or mucus. This was successfully treated in vivo with baicalein, a 12-lipoxygenase inhibitor [123]. This showed that the resolution of this pathology was mediated through the blockade of hepoxilin A_3 formation.

Of great interest to the hepoxilins and 12-HETE pathways is the recent observation by Hurley et al. [103] that infection of lung epithelial cells with *P. aeruginosa* (PA01 strain) resulted in the expected enhanced release of arachidonic acid as well as PGE_2; PGE_2 was blocked by a specific phospholipase $A_{2\alpha}$ inhibitor (U0126), but unexpectedly, it was observed that hepoxilin A_3 was not affected by this 'specific' inhibitor and 12-HETE was in fact slightly increased. These experiments also showed that neutrophil transmigration in vitro was unaffected by this specific inhibitor further supporting the notion that neither arachidonic acid nor PGE_2 is involved in the transmigration of neutrophils but the hepoxilin pathway is. This observation suggested that phospholipase $A_{2\alpha}$, thought to be responsible for the release of arachidonic acid for eicosanoid synthesis (prostaglandins and lipoxygenase products etc.), actually does not affect the hepoxilin pathway. This is a difficult result to interpret as 12-HETE and hepoxilin A_3 are produced by the same 12-lipoxygenase pathway which depends on released arachidonic acid substrate and usually the pathway is directed to either 12-HETE formation or to hepoxilin by the amount of GPx/PHGPx present. Interestingly, these authors also showed that hepoxilin-evoked transepithelial migration is inhibited by a phospholipase A_2 inhibitor (ONO-RS-082) which also prevents PGE2 formation. Hence it is suggested that hepoxilin and 12-HETE are produced through the action of an *alternate* phospholipase, other than the generally accepted eicosanoid generating cytosolic isoform of phospholipase $A_{2\alpha}$ [103]. Is it possible that this 'alternate' phospholipase may act on hepoxilin A_3 and 12-HETE that may have been previously stored/esterified rather than one that involves de novo synthesis as we are accustomed to think?

The report by Anton et al. [42] showing that hepoxilin B_3 and trioxilins can be acylated to PC and PE suggest that a phospholipase may cause the release of these compounds. Could they be formed directly on the phospholipid backbone possibly through the action of 12/15-LOX or 12LOX/eLOX3 in response to stress or are they formed through the conventional pathway and then acylated (stored) on phospholipids? It is difficult to imagine that these compounds would be acylated intact as the hepoxilins (especially hepoxilin A_3) are very unstable. The concept of lipoxygenation of arachidonic acid esterified to phospholipids in the membrane followed by a concerted isomerization into hepoxilins is more plausible especially if the isomerization reaction is an intrinsic component of the 12/15-lipoxygenase enzyme as Nigam suggested [10] (see earlier). However to date there have been no reports of the isolation of intact hepoxilin A_3 esterified to phospholipids (only the stable hepoxilin B_3 and the trioxilins [42]) or to any other lipid that can serve to release free intact hepoxilins and 12-HETE, whose release can be activated by bacterial infection or any other trauma or pathology, except for skin [4,12] (see below section on Skin). This alternate pathway, if present, would revolutionize our thinking about this particular area in the hepoxilin field but this may also be applicable to other eicosanoids (lipoxins, protectins, resolvins, nitrolipids). We ought to search for esterified versions of these lipid mediators and investigate whether their release can be demonstrated from these 'stores'.

6.6. Pain

Pain is part of the body's defense to external harmful stimuli. It protects the injured site through a reflexive retraction from the painful stimulus, while the injury heals.

The involvement of prostaglandins in pain and inflammation dates back to the 1970s (reviewed in [124,125]). The prostaglandins were initially reported to be detected in inflammatory exudates after a scalding injury [126]. Essentially, prostaglandins have been shown not to produce pain on their own but to mediate painful responses induced by other mediators (bradykinin, histamine, serotonin) and to cause increased sensitization to a painful stimulus acting through pain receptors to produce hyperalgesia [127]. It was also shown early on that various hydroperoxides produced during the lipoxygenase reactions on fatty acids when infused intradermally produced pain that was more intense than that observed with the prostaglandins (for example PGE_1). It was of immediate onset [128].

Relevant to this review on the hepoxilins, it was shown a few years ago that hydroperoxy metabolites of arachidonic acid formed through the lipoxygenase pathways caused hyperalgesia [129,130]. More recently a cloned capsaisin receptor (VR1) in HEK cells was directly activated by 12- and 15-HPETEs and LTB_4, while prostaglandins and unsaturated fatty acids had no effect [131]. 12-HPETE was more active than 15-HPETE. These authors note the structural similarity of the capsaicin molecule with that of 12-HPETE and suggest that this may afford 12-HPETE with the properties to bind to the capsaicin receptor and to activate the appropriate channel. Of course the active substance is unlikely to be 12-HPETE itself as ample evidence exists (see above section on the 'Enzyme Reaction') that this unstable compound is actively transformed into 12-HETE (weakly active on nociception [131]) and hepoxilin A_3 by hepoxilin synthase. In fact subsequent studies from Yaksh's group identified hepoxilin A_3 as the lipoxygenase metabolite which caused inflammatory hyperalgesia and tactile allodynia (pain, generally on the skin, caused by something that would not normally cause pain) in spinal neurons through the activation of the TRPV1 and TRPA1 receptors [82,132] Intraplantar administration of

carrageenin causes hyperalgesia. Pre-administration of intrathecal nordihydroguiaretic acid (NDGA), a lipoxygenase inhibitor, attenuated tactile allodynia in a dose-dependent fashion, confirming the involvement of a product of lipoxygenase, i.e. hepoxilins A_3 and B_3.

12-LOX metabolites (12(S)-HPETE, 12(S)-HETE, HXA_3, or HXB_3) evoke profound, persistent tactile allodynia, with modest, transient heat hyperalgesia. The involvement of the hepoxilins as mediators of nociception was further confirmed through the use of lipoxygenase inhibitors (baicalein and CDC) which blocked the effect while 5-lipoxygenase blocker, Zileuton, had no effect on tactile allodynia. Cells stably overexpressing TRPV1 and TRPA1 receptors displayed increased intracellular mobilization of calcium by hepoxilin A_3, as reported by us previously in human neutrophils [58,92] and in hippocampal neurons [109]. Additional support for the hepoxilin effects was derived from studies from constitutive deletion or antagonists of TRPV1 (AMG9810) or TRPA1 (HC030031) which attenuated the hepoxilin-evoked actions. Spinal hepoxilin A_3-evoked allodynia was also suppressed by anti-hyperalgesic doses of AMG9810 or HC030031 [132]. These findings strongly support a role of hepoxilins A_3 and B_3 in mechanisms related to the onset of inflammatory hyperalgesia and tactile but not heat-evoked allodynia. Will the hepoxilin stable analogs (PBTs) be effective in controlling pain?

6.7. Skin

Ceramides form a structural component of membranes but are known to have in addition, functional activities; these include regulating apoptosis, cell growth arrest, differentiation, cell senescence, cell migration and adhesion [133]. Omega-hydroxylated ceramides are the predominant lipid species in the corneocyte lipid envelope in the epidermis. They are formed through a cytochrome P450 dependent reaction. The resulting omega-hydroxy ceramides are acylated with fatty acids, the predominant fatty acid being linoleic acid, thereby forming linoleyl esters of the structural omega-hydroxy acyl ceramides prevalent in the stratum corneum extracellular lamellae, which regulate cutaneous permeability [134]. The involvement of the omega-hydroxy acyl ceramides in water permeability homeostasis was demonstrated by the slower barrier recovery of hairless mice subjected to acute barrier disruption through tape-stripping in the presence of a P-450 inhibitor aminobenzotriazole (ABT) [134]. Importantly, and probably of great functional significance is that the linoleate esterified to omega-hydroxy ceramide can be converted into epoxy alcohols (hepoxilin-like) through the corresponding 12(R)-lipoxygenase coupled with eLOX3. It is proposed that the C18 hepoxilin-like product so formed may act as a signal for a lipase to release it from the ceramide in a complex enzymatic chain reaction involved in water membrane homeostasis [4]. It was earlier shown that the R-type hepoxilins (8R hepoxilin A_3) had greater specificity than the 8S enantiomers for potentiating bradykinin-evoked vascular permeability in the rodent skin [135].

This is a fascinating novel concept from the eicosanoid point of view in which lipoxygenation would occur on a fatty acid (linoleic acid) esterified to a ceramide backbone (omega-hydroxy). The isolation of C18-hepoxilin-like structures suggests that these compounds may be present in lipid 'stores' and released through the activation of specific acyl hydrolases. The ceramide pathway is a unique pathway restricted until now to skin, but if similar lipids acting as stores of these mediators are discovered in other tissues, this may provide a new understanding about the formation/occurrence of these oxidized products separate from the well-established classical 'arachidonic acid cascade' of reactions emanating from free arachidonic acid (and other PUFAs). It is known that lipoxygenases could directly oxygenate PUFAs esterified to phospholipid (see review by O'Donnell and Murphy [136]) or involve the acylation of oxidized fatty acids (HETEs) [136] to membranes but this has not yet been shown with lower chain fatty acids (hydroxylated or hydroperoxy linoleic acid) esterified to an omega-hydroxylated ceramide with further metabolism into hepoxilin-like structures while still in the membrane. Hence lipoxygenases (e.g. 12R-LOX) do not necessarily require the fatty acid to be in the free form. Trioxilin B_3 (metabolite of the hepoxilin B_3) and hepoxilin B_3 can be incorporated into phospholipids (PE and PC) in psoriatic lesions [42]. But hepoxilin A_3 is so unstable, that it cannot survive acylation into membrane structural lipids; it would have to be formed directly on the membrane — indeed Murphy has indicated that 12-HPETE is found esterified in phospholipids; could this be transformed into hepoxilin A_3 or B_3 through the action of eLOX3? If the omega-ceramide oxidized lipids as well as those esterified to phospholipids do indeed act as stores of these oxidation products, they could be readily released as needed upon activation by cell mediators, probably through some pathological signal. This generates the concept of membrane lipid modification (through controlled enzymatic oxidation) in preparation for the release of certain bioactive lipid mediators to support a specific pathological function (pro-inflammation, pro-cancer, pro-thrombosis, potentiated vascular constriction, hyperalgesia and other conditions). So it is possible that the ceramide-bound as well as phospholipid-esterified HETEs (and shorter chain HODEs) and hepoxilins (and C18-hepoxilins) formed on the cell membrane would be easily and rapidly released. Another point relates to omega-hydroxylation. Brash et al. have suggested that an omega-hydroxylated hepoxilin or trioxilin may be the active principle of the 12-LOX pathway involved in the water permeability function of the epidermis [11]. This would probably have to be a C18 product derived from linoleate, the major fatty acid bound to the omega-hydroxy ceramide of the stratum corneum of the skin.

It is unfortunate that omega-hydroxylated hepoxilins (C20 or C18) or ceramides containing these lipids have not yet been tested for their actions, if any, on transepidermal water loss (TEWL) to support the hypothesis that these compounds (in the free form or in their acylated form to ceramides) may indeed be the responsible mediators for this important activity in the skin. Phospholipid-esterified hepoxilins may have different biological activities in other tissues. Indeed we had identified omega-hydroxylated hepoxilin A_3 in incubates of human neutrophils with hepoxilin A_3 as substrate [137] and found it capable of releasing intracellular calcium in these cells as does the native hepoxilin A_3. Unfortunately preliminary tests with the hepoxilins and the hepoxilin antagonists on TEWL have not afforded conclusive results.

7. Hepoxilin stable metabolites and analogs

7.1. Structures

The natural hepoxilins are unstable compounds subject to metabolism both chemically as well as enzymatically due to the presence of an allylic epoxide [1,13] (Schemes 1, 2). The biological activity of hepoxilin A_3 in vitro is surprising in view of its instability and in fact suggests that it may be much more powerful than the biological assays indicate. One pathway of metabolism of hepoxilin A_3 relates to an action by epoxide hydrolase [13,14] which gives rise to trihydroxy compounds (trioxilins A_3 and B_3) having little or no biological activity with respect to intracellular calcium release, vascular permeability, platelet aggregation, and hepoxilin binding; a second pathway of hepoxilin transformation is to glutathionyl derivatives through glutathione S-transferase reaction to generate highly potent compounds (hepoxilin A_3-C) on mammalian neurotransmission [27]. A third pathway identified is the formation by intact neutrophils of an omega-hydroxylated hepoxilin A_3 [137]. To allow investigation of the basic hepoxilin structure on in vivo models of disease, we designed compounds in which the unstable epoxide grouping was replaced with a stable cyclopropyl grouping to produce the least structural modification and achieve compound stability. A family of compounds (PBTs) was chemically synthesized [15] which we subjected to biological testing (see below). The basic structures are shown in Scheme 2 in comparison to the natural hepoxilin structures.

Recently another group of compounds has been described in which the epoxide functionality was deleted and replaced with an ether bridge [79] between carbon atoms 11 and 12.

7.2. General actions of the hepoxilin analogs

7.2.1. Antagonists to hepoxilins

The PBTs display hepoxilin A_3 antagonist activity. They selectively antagonize the binding of tritiated hepoxilin A_3 to intact human neutrophils and neutrophil membranes [75] and they inhibit the hepoxilin A_3 evoked release of intracellular calcium in these cells [138]. Additionally the PBTs block aggregation of human platelets in vitro [56]. The PBTs displayed interesting actions in vivo as anti inflammatory and anti cancer drugs which will be described below. Other actions reviewed earlier relate to anti-thrombotic and anti-diabetic actions [53,138,139].

7.3. Disease implication

7.3.1. Anti-inflammatory

Our first in vivo study showed that the PBTs could prevent the occurrence of lung fibrosis in an accepted mouse model employing a single intratracheal administration of bleomycin [86]. Bleomycin is an antibiotic anti-cancer agent that causes interstitial lung fibrosis in humans; in mice a single dose administered intratracheally evoked changes that resembled human idiopathic pulmonary fibrosis histopathologically [140] with marked accumulation of inflammatory cells (macrophages) and increased rate of collagen synthesis and deposition [141–143]. Upon administration of PBTs intraperitoneally, the bleomycin-evoked acute pulmonary inflammatory phase was totally abolished at the three doses used (400, 1000, 2000 μg/kg); hence PBTs abolished macrophage influx and increased collagen synthesis. Four PBTs were tested as the methyl ester pro-drug form, results showing that PBT-1 and -2 whose structure is based on hepoxilin A_3 were the most potent while PBT-3 and -4 (based on the hepoxilin B_3 structure) were less active. Results also indicated that the potency of the active PBTs was below 400 μg/kg when administered intraperitoneally daily for the 8 days of the study [86]. The PBTs inhibited the vascular permeability response in the rat skin produced by bleomycin in this study.

A recent report indicated that a novel type of hepoxilin-like analogs lacking the epoxide or cyclopropane functionalities, but instead having an ether linkage was synthesized. Used in vitro in transwell filters, these analogs were added to the apical side at the same time as the pathogen was added, while the neutrophils were added to the basolateral side; these products dose-dependently antagonized *P. aeruginosa* (PAO1)-induced transepithelial migration of neutrophils across H292 epithelial barriers [79]. Importantly, the analogs antagonized migration of neutrophils more effectively that were stimulated by *P. aeruginosa* than by the peptide, FMLP, indicating specificity to the activated pathway. These authors provided additional support for hepoxilin A_3 formation showing that arachidonic acid was released by *P. aeruginosa* (PAO1 strain) or by K12 *Escherichia coli* (MC1000 strain). When infection with *P. aeruginosa* was carried out in vivo in mice through intranasal administration, and BAL fluid was collected 18 h thereafter, hepoxilin A_3 and 12-HETE were observed albeit in a 1:100 ratio, together with the appearance of abundant PMNs with significant monoamine oxidase activity reflecting the presence of an acute inflammatory response. Unfortunately in this report, the effect of the hepoxilin analogs on the in vivo characterization of inflammatory cells, arachidonic acid, 12-HETE and hepoxilin A_3 formation and release into the BAL fluid was not reported to determine whether the analogs studied are effective in vivo as already shown to be the case for the PBTs in a variety of animal models (recently reviewed in [53]).

A recent report from Hurley's group showed that hepoxilin A_3-induced chemoattraction across transepithelial barriers was selective toward neutrophils but not eosinophils indicating specificity toward infective rather than allergic conditions. Additional confirmation was derived from the observation of a selective effect of pathogen (which produces hepoxilin A_3) on neutrophil but not of eosinophil transepithelial migration [144].

7.3.2. Anti-cancer

The PBTs effectively inhibit the growth of neoplastic cells in vitro and tumors derived from the subcutaneous implantation of neoplastic cells in vivo in nude mice. Efficacy was shown in leukemic, breast, and prostate cancer cell lines [73,74]. The effect was selective to neoplastic cells as normal cell lines were unaffected. Both in vitro and in vivo, the cells/tumors underwent apoptosis after PBT treatment showing the classical features (DNA fragmentation, DNA laddering, cell cycle analysis, Annexin V binding). PBTs affected the intrinsic pathway, causing the release of cytochrome c from the mitochondria and activating the degradation of caspase-3 [74]. Nude mice were used that had been transplanted subcutaneously with various neoplastic cell lines; solid tumors grew in 2–3 weeks (80–100 mm^3 volume). An 8-day administration of PBT-3 or -4 intraperitoneally caused inhibition of growth of these tumors, while in vehicle treated animals these tumors grew rapidly so that the animals had to be sacrificed by the end of this 8-day period [73]. Considering the size (volume) of the tumors between the two groups, the PBT-treated groups produced a remarkable cessation of growth, an effect that would have high clinical relevance. Importantly, after the 8-day treatment with PBT was stopped, tumor growth did not start immediately but was delayed until about 40 days into the study, and then the tumors grew rapidly as in the vehicle treated group. If, however, PBT treatment is started again for another 8-day period just before tumor growth began, a further cessation of growth is observed such that a delay in tumor growth is observed until approx 100 days [53,145]. Thus, 2 periods of PBT treatment (8-days each) could prevent tumor growth by 100 days as opposed to the 8-day uncontrolled rapid growth in the absence of PBT. In other studies we noticed that PBT was effective in controlling tumor growth when the tumors were relatively small in size (around 80–100 mm^3 volume, but possibly even smaller) but not when the tumor growth was well established (300 mm^3 in volume). This suggests that the PBTs may have positive effects on metastatic growth, either in inhibiting metastasis or in inhibiting the growth of metastatic cells since these cells would be expected to be within the size range where PBTs would be effective in controlling their growth.

7.3.3. PBTs and phospholipase A_2

Since it is known that 12-HETE and some prostaglandins especially PGE_2 are tumor promoters [48,146], we decided to analyze the eicosanoid profile in tumors at different stages of growth and treatment with PBTs in vivo to get an idea of their potential contribution to tumor growth. In this study we used the leukemic U937 bcl-xL cell line and transplanted the cells subcutaneously as we did before with the leukemic K562 CML cell line. It was interesting to observe that 12-HETE was the major eicosanoid present in this tumor as well as its precursor, arachidonic acid [16]. All other eicosanoids including other HETEs, prostaglandins, EETs and hepoxilins were found in very small amounts. Importantly, tumors analyzed after 8-days of treatment with PBT-4 showed drastic reduction in arachidonic acid and 12-HETE and all other eicosanoids, suggesting that the observed tumor growth inhibition may be linked with the much decreased presence of arachidonic acid and 12-HETE. In fact, as the PBT dose started to wear off (tumors at 19 days were studied in this model), arachidonic acid and 12-HETE became greatly overexpressed; they decreased back to very low levels by day 29 as a spurt in tumor growth took place and the tumor growth was unresponsive to PBT treatment. These experiments suggest that a specific acyl hydrolase may be present in small tumors that are inhibited by PBT, causing inhibition of arachidonic acid release and of 12-HETE formation to result in blockade of tumor growth. It is expected that tumor size and tumor status may be essential to the positive anti-

tumor effect of the PBTs, and therefore it is possible that the PBTs may be very effective in the control of metastatic growth.

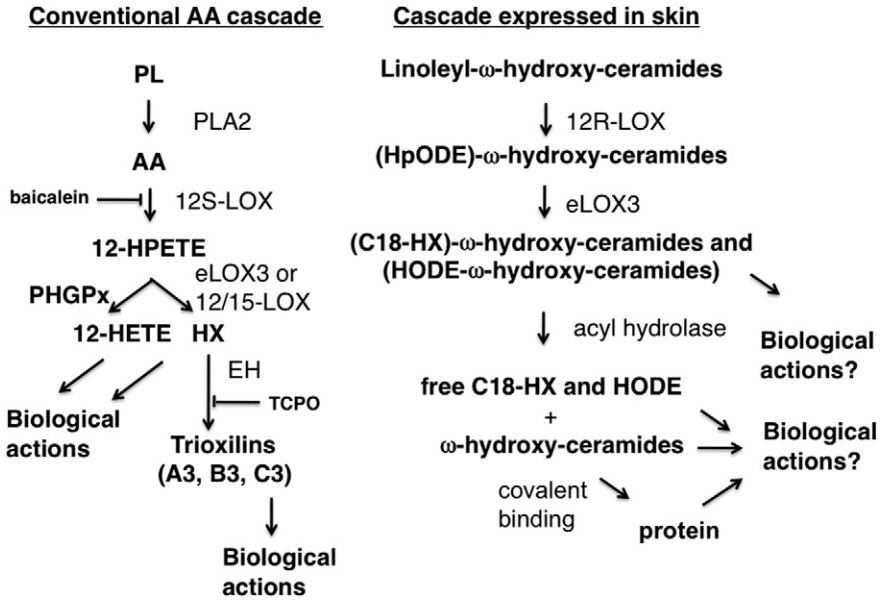
It is possible that a specific phospholipase (acyl hydrolase) is activated in the early stages to cause tumor growth and this phospholipase may be responsive to blockade by the hepoxilin-related analogs, PBTs. Increased expression of phospholipase has been associated with breast and prostate cancer [147–150]. From the previous sections, it appears that we should now also consider other specialized forms of acyl hydrolases, those acting on ceramide omega esters and phospholipids, that could release pre-formed eicosanoid mediators derived from lipoxygenase and related enzymes for certain well-defined pathological roles related to infection, inflammation, cancer and other conditions.

8. General comments — therapeutic potential

It is well known that hepoxilins are formed through the bidirectional metabolism of 12-HPETE, the initial unstable intermediate derived from the action of 12-lipoxygenase on *FREE* unesterified arachidonic acid (Schemes 1, 3, left panel). While blockade of this pathway would take place through the use of 12-lipoxygenase inhibitors, to date there is little known about co-factors that stimulate the hepoxilin route, except by preventing the enzymatic degradation of hepoxilins through the use of epoxide hydrolase inhibitors such as TCPO as we have shown. Nigam's group has shown that GPx/PHGPx can alter the metabolism of 12-HPETE in favor of 12-HETE, while the absence of GPx/PHGPx in cells favors the formation of hepoxilins as shown in his studies with the insulinoma cell line, RIN5mF. It has also been demonstrated by McCormick's group that the formation of hepoxilin A_3, a potent neutrophil chemoattractant, is induced as a result of bacterial infection, e.g. *Salmonella typhimurium*, *P. aeruginosa*, *Staphylococcus aureus and E. coli*. The studies from Brown's group further indicated an important temporal relationship between infection with the Lyme bacterium, *B. burgdorferi* and hepoxilin formation in vivo. The latter studies demonstrated that the hepoxilin pathway is the initial pathway activated after infection, presumably because of its desired role as a neutrophil chemoattractant to initiate the inflammatory process. This is followed by the formation of other eicosanoids (PGs, LTs and 12-HETE, resolvins) involved in supporting inflammation and promoting resolution.

Hurley's studies clearly suggested an alternate isoform of phospholipase $A_{2\alpha}$ may be involved in the formation of hepoxilin A_3 since hepoxilin A_3 and 12-HETE formations were not blocked when a specific inhibitor of phospholipase $A_{2\alpha}$ was used, yet PGE_2 formation was inhibited indicating that arachidonic acid release had been inhibited. More general phospholipase A_2 inhibitors however, blocked the formation of the 12-HPETE products. These studies suggest that hepoxilins are formed through the classical pathways resulting from the release of arachidonic acid from membrane phospholipids. Such a pathway is followed by selective 12-lipoxygenase activation and hydroperoxidation followed by isomerization (eLOX3 in skin or an intrinsic activity of a 12/15-lipoxygenase) into hepoxilins for the required biological activity. This biological activity is initiated through hepoxilin's known effects on calcium mobilization in cells, its chemoattractant properties and its selective binding to neutrophils as we and others have shown. Which specific acyl hydrolase is responsible for the release of arachidonic acid and subsequent 12-lipoxygenase/hepoxilin synthase activity remains to be determined.

Measurement of levels of arachidonic acid and other eicosanoids (including HETEs) are important to evaluate the mechanism of formation of these eicosanoids to determine if the classical pathways from unesterified fatty acids are involved or other new pathways. A novel and potentially important concept arises from the recent reviews of Brash et al. [4] and Krieg et al. [12] that hepoxilin-like compounds (and presumably HODE compounds as well) derived from linoleic acid were detected in skin and these compounds were derived from linoleate esters of omega-hydroxylated ceramides. This novel finding (although not showing yet biological action of these complex lipids on dermal function i.e. water permeability barrier) suggests that lipoxygenases, such as 12-lipoxygenase and presumably other lipoxygenases, can oxygenate polyunsaturated fatty acids (linoleate) esterified to a ceramide backbone and presumably other structural lipids in membranes. The skin ceramides appear to be deficient in AA but are abundant in LA. Are the C18-hepoxilin-like compounds biologically active as the C20 compounds? For many years it was known that polyunsaturated fatty acids esterified to phospholipids could be



Scheme 3. Comparison of two cascades for the generation of hepoxilins and 12-HETE in the conventional AA cascade mechanism [151] (left) and one proposed for the generation of 'hepoxilin-like' compounds derived from linoleic acid present in the omega-hydroxy-acyl ceramides in skin [4,12]. The important novelty of the ceramide concept is that hepoxilin-like and HETE-like (HODE) compounds can be formed directly on the ceramide backbone as a structural component of membranes, thereby providing the possibility of 'storage' of these bioactive compounds as omega esterified ceramides, available to be released under specified pathological conditions for rapid and localized biological action. Could this apply to other lipid mediators 'stored' in other membrane lipids and released rapidly through some pathological stimulus?

oxygenated by lipoxygenases (or even peroxidized non enzymatically) and that these oxidized fatty acids esterified to phospholipids were biologically active (Murphy's group) [136] — could these oxidized fatty acids be better substrates for acyl hydrolases than the intact fatty acids esterified to structural lipids in the membrane?

An emerging concept may be put forward in this review suggesting that conditions leading to pathology (e.g. stress, infection, vasoconstriction, pain, cancer, various ichthyoses, skin psoriasis and other conditions) may cause the release of hepoxilins and other similar mediators (maybe lipoxins, resolvins, protectins and nitrolipids) from preformed stores (esterified to structural membrane lipids) for *immediate release and biological action* (including intracellular calcium release, vascular permeability, potentiation of certain events as vasoconstriction, neurotransmission, chemotaxis for neutrophils, insulin release, possibly supporting epidermal water permeability barrier) at the local site needed immediately as these mediators are short-lived (Scheme 3 right side showing the ceramide pathway but can also be extended to other lipid mediators in other membrane lipids (not shown)); a more physiological role for these products would involve the multi step *classical cascade* of reactions (Scheme 3 left side) initiated by the release of arachidonic acid and similar fatty acids through various phospholipases and metabolism through a series of enzymatic steps which may not necessarily all be in the same location in the membrane resulting in the slower continuous formation/release/action of these mediators. It rests to be seen how general a concept it is for ceramides and other membrane lipids to contain such mediators esterified intact to them as discussed herein and how easily they are released upon specific stimulation. Acyl hydrolases which release such esterified mediators from structural membranes may not be the classical phospholipases identified to date. Interesting surprises may yet be in store.

If hepoxilin A_3 is intrinsically involved in the early stages of infection (or other stress) as a neutrophil chemoattractant as the abovementioned studies indicate and if this and other hepoxilins (e.g. glutathione metabolites of the hepoxilins) also play an important role in potentiating actions of various mediators (norepinephrine, serotonin, nerve growth factor, bradykinin, neurokinin-A etc.), an important role for specific antagonists of hepoxilin action, such as the PBTs, may have significant therapeutic value. We have already shown such compounds to have good efficacy without detectable side effects in animal models of disease (cancer, inflammation, thrombosis, diabetes and pain). We need to take our proof-of-concept findings into clinical development. There is great potential for the use of hepoxilin antagonists in a variety of conditions and diseases.

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