Directing eicosanoid esterification into phospholipids.

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Eicosanoids are well known potent signaling mediators generated by cyclooxygenases (COX), lipoxygenases (LOX) and cytochrome P450 (CYP) enzymes in immune cells, platelets and inflammatory activated tissues. As free acids, they signal by binding to G protein-coupled receptors following secretion from their cell of origin. For many years, it has been known that when added to cells, exogenous eicosanoids can be incorporated into more complex lipids, including phospholipids (PL). However until recently this was considered little more than an epiphenomenon. This has changed in the last 10 years with the realization that phospholipid-esterified eicosanoids, otherwise known as enzymatically oxidized phospholipids (eoxPL) are formed acutely, on the same timescale as free acid analogs, and that these lipids are bioactive in their own right. In contrast to eicosanoids, eoxPL are not secreted, and remain cell bound where they exert their biological actions.

In the early 1990's, reports of incorporation of exogenously-added hydroxyeicosatetraenoic acids (HETEs) and epoxyeicosatetraenoic acids (EETs) into phospholipids, followed by their stimulated release led to the idea that these lipids could be a store for releasable eicosanoids (1-6). Brezinski and Serhan showed that 15-HETE was incorporated into neutrophil phosphatidylinositol (PI), then released following fMLP challenge. 15-HETE behaved differently to the 5-positional isomer, which was

incorporated into phosphatidylcholine (PC), suggesting isomer selectivity for different lysoPLs (1). Separately, Joulain characterized incorporation of 12-HETE into both PC and PI in mononuclear cells, also showing mitogen-stimulated release (2). In 1992, Bernstrom showed half maximal incorporation of EETs into mastocytoma PLs within 30 min, with primarily formation of PE species (4). In that study, fast atom bombardment/tandem MS was used for the first time to identify molecular species, including numerous plasmalogens of both PE and PC (4).

In 1998, Brinckmann observed that ionophore-activated eosinophils contain 15-HETE attached to membrane lipids (7). At that time, neither the molecular species involved nor their biology was characterized. Later, in 2005, while studying the cellular regulation of 15-LOX turnover, 15-HETE was found to be acutely generated attached to four individual phospholipids in IL-4–treated human monocytes (8). As explained below, this is a different process to incorporation of exogenous eicosanoids, as it is considerably faster and stimulated by inflammatory agonists. It also occurs on the same timescale as free eicosanoid generation, in contrast to incorporation of endogenous analogs, which is considerably slower. To characterize the molecular species of complex lipids, precursor scanning LC-MS/MS was used, thus "fishing" for molecular ions that incorporated a HETE functional group. Between 2007-2012, several families of eoxPL generated by LOXs were uncovered using this approach, not only in monocytes, but also in human platelets, neutrophils and airway epithelial cells (9-14). The most abundant were phosphatidylethanolamines (PE) but PC-derived forms were detected with many being plasmalogens. These are generated in a burst during the first 2-5 minutes of cell activation by pathophysiologic agonists, via the coordinated action of receptors and enzymes, and a slower rate of formation is maintained at least for several hours.

In tissues, the eoxPL profile reflects the oxidative enzymes expressed, for example, cells expressing 15-LOX generate PL that incorporate 15-HETE or 15-

ketoeicosatetraenoic acid (KETE), the latter via prostaglandin dehydrogenase activity downstream of 15-LOX (8,10). In platelets, 12-HETE or 14-HDOHE attached to PE or PC are found, while EET-PLs in liver predominate as positional isomers reflecting cytochrome P-450 activities (5,12,13). More recently, eoxPL generated by COX-1 have been found in human platelets. These include four forms of PGE_2-PE that are sensitive to aspirin inhibition *in vitro* and *in vivo* (15). The oxidized fatty acids that can be incorporated into PL not only include eicosanoids derived from arachidonate, but at least in platelets, a myriad of other oxidized fatty acids derived from 22:4, 22:5 and 22:6. Indeed, in platelets, recent estimates include over 100 individual molecular species acutely generated on thrombin activation (16).

Most eicosanoid generating enzymes require free fatty acid as substrate, and are unable to oxidize intact PL, thus phospholipase A_2, normally the cytosolic isoform, is essential for eoxPL formation. An exception is 15-LOX in human monocytes or airway epithelia (the murine 12/15-LOX) which can also oxidize membrane PLs directly. Thus, in most tissues eoxPL formation will require not only formation of the oxidized free acid, but importantly, its reacylation into lysoPL pools. Up to now, little was known about the process that reacylates eicosanoids acutely in immune cells other than it is sensitive to thimerosal or triascin C, both rather crude pan inhibitors of Co-A-dependent fatty acid acylation pathways. Given that eoxPL form on a similar timescale to free acid analogs, the process of fatty acid hydrolysis, oxidation and reacylation must be fast and tightly controlled by enzymes.

Fatty acyl attachment to PLs is a two step process requiring first the formation of fatty acyl-CoAs (FA-CoA) via the action of one of five long-chain acyl-CoA synthetase isoforms (ACSL-1,-3,-4,-5,-6) (EC 6.2.1.3) (17-20). Following this, headgroup specific lysophospholipid acyl transferases such as lysophosphatidylethanolamine acyltransferase (LPEAT), otherwise known as MBOAT2 (membrane bound O-acyl

transferase) or lysophospholipid acyltransferase (LPAT) that catalyze the coupling of the FA-CoA into lysoPLs to form PL (21). Human cells express at least 5 LPATs also termed MBOATs that have a varying degrees of specificity for both the FA-CoA and the lysophospholipid acceptor (22).

Up to now, nothing was known regarding how these enzymes regulate eoxPL formation. However, there were that hints that cellular acylation of shows selectivity beyond simply utilizing the most abundant oxidized fatty acid and lysoPLs. For example, despite multiple attempts, our own groups have never detected PL-esterified thromboxane in platelets, even though this is one of the more abundant eicosanoids made (unpublished observations). Also, 12-HETE-d8 is not incorporated into platelet PE during the timescale of agonist-stimulated 12-HETE-PE generation, indicating that exogenous and endogenously generated eicosanoids are somehow sensed differently and suggesting that cell compartmentalization is an important factor (13). Last, two recent studies by Kagan, Conrad and co-workers showed that ACLS4 shapes lipid composition including formation of oxidized arachidonate and adrenic acid-PEs by lipoxygenase, during a cell death process called ferroptosis (23,24)

In this issue of the Journal of Lipid Research, Klett *et al.* take the first steps towards defining individual eicosanoid esterification pathways. Building on a previous observation that ACSL4 can metabolize EETs, they elegantly show that all five ACSL enzymes can utilize either HETEs or EETs, forming analogous FA-CoAs using LC/MS/MS. With recombinant enzymes, they found differences in Michaelis-Menten kinetics for substrates and isoforms. Intriguingly, substrate preferences were somewhat altered when ACSLs were expressed in COS7 cells, indicating that cellular environment exerts a significant influence on ACSL activities that could relate to differences in membrane composition, presence of co-activators or inhibitors, presence of other enzymes, their cellular location, expression level and post-translational modification. Indeed at least

one ACSL is known to be subject to differential cellular phosphorylation and acylation (25). Mammalian cellular ACSL and MBOAT expression patterns are complex, and this means that tissue specific incorporation in terms of rates and eoxPL species formed endogenously will likely vary widely; however, there is no information on this topic as of yet. These intriguing new studies by Klett and coworkers provide the first evidence about how cellular reacylation of eicosanoids is controlled, paving the way for further investigations in primary mammalian cells in order to define how specific individual eoxPL are formed during physiological and pathophysiological situations. The studies are important as they place another piece in the jigsaw concerning the formation and action of new bioactive phospholipids of likely importance to innate immunity, and acute and chronic inflammatory disease.

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