Lipidomics Impact on Cell Biology, Structural Biochemistry and Immunopathology

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1. A lipidic constituent from Drosophila Lipophorin particles that binds to mammalian smoothened

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The Hedgehog (Hh) family of signaling proteins functions as morphogens important in embryonic patterning and adult tissue homeostasis. Stoichiometric binding of the Hh ligand to Patched (Ptc) releases the inhibitory effect of Ptc, leading to activation of Smoothened (Smo). The similarity of Ptc to resistance, nodulation, division (RND) proteins, which export substrates across the bacterial membrane by a proton antiport mechanism, and Smo regulation by small molecule-induced conformational change have led to the model of Ptc function as a transporter in regulating Smo. Lipophorin particles were recently shown to be important in transporting Hh protein in the *Drosophila* wing imaginal disc. We found that lipid extracts from lipophorin particles competed with the specific binding of a fluorescently-labeled Hh antagonist to membranes expressing mammalian Smo, and produced a concentration-dependent inhibition of a reporter for Hh pathway. We have partially purified the activity which may function as a conserved natural ligand for mammalian as well as fly Smo.

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2. Loss of 5-lipoxygenase results in increased inflammation during experimental Lyme arthritis

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The eicosanoid biosynthetic enzyme 5-lipoxygenase (5-LO) has been shown to regulate the immune response in animal models of autoimmune and infectious disease; however, contradictory results from different models have complicated the elucidation of precise mechanisms by which 5-LO participates in inflammation. To clarify the contribution of 5-LO in the immune response we utilized a murine model of Lyme arthritis, caused by infection with the bacterium *Borrelia burgdorferi* (*Bb*). Infection with *Bb* leads to severe joint inflammation in susceptible C3H/HeJ mice, which peaks approximately 21 days post-infection and then spontaneously resolves. Abrogation of 5-LO activity led to increased arthritis severity demonstrated by increased joint swelling, prolonged neutrophil infiltration, and multinucleate giant cell formation. The increase in severity was not due to increased *Borrelia* numbers in the joint, but did correlate with increased mast cell degranulation and altered pro- and anti-inflammatory eicosanoid and cytokine production in the joints of 5-LO$^{-/-}$ mice. These data suggest that 5-LO products may be crucial for controlling the inflammatory response to *Bb* infection, likely via the regulation of cell recruitment and activation, and may serve an underappreciated role in the resolution of inflammation following some infections. This work was supported by NIH grant AR052748.

3. Application of proteomic marker ensembles to mapping of subcellular lipidome macrophages

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Compartmentalization of biological processes and the associated cellular components within subcellular membrane structures, organelles, is crucial for cell function. Correspondingly, a *local* lipid environment controls (via phase state of the membrane, its fluidity and curvature, and/or specific binding) function of membrane proteins (e.g., receptors, channels, metabolite transporters, etc.). Therefore, a meaningful cellular lipidome has to be with necessity mapped at a subcellular level.

Typically, the location of a component (lipid, protein, etc.) is revealed through an association with an organelle marker. Therefore, the identification of reliable markers is critical for an understanding of cellular function and dysfunction. We fractionated RAW264.7 cells, both in the resting and in the endotoxin-activated state, into fractions representing the major organelles/ compartments: nuclei, mitochondria, cytoplasm, endoplasmic reticulum and plasma membrane. The identity of these fractions was confirmed via the distribution of conventional enzymatic markers. Through a quantitative liquid chromatography/mass spectrometry-based proteomic analysis of the fractions, we identified 50-member ensembles of marker proteins ("marker ensembles") specific for each of the corresponding organelles/compartments. Our analysis attributed 206 out of the 250 marker proteins (~ 82%) to organelles that are consistent with the location annotations in the public domain (EntrezGene, Swiss-Prot, and references therein). Moreover, we were able to correct locations for the majority of the remaining proteins. The marker ensembles were used to calculate the organelle composition of the above mentioned subcellular fractions. Knowledge of the precise composition of these fractions can be used to calculate the levels of metabolites in the pure organelles. As a proof of principle, we applied these calculations to known mitochondria-specific lipids (cardiolipins and ubiquinones) and demonstrated their exclusive mitochondrial location; this approach was further used to obtain a detailed subcellular lipidome. We speculate that the organelle-specific protein ensembles may be used to systematically redefine originally morphologically defined organelles as biochemical entities.

4. Eicosanoid dynamics during the development and resolution of Lyme disease-induced arthritis

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Eicosanoids are indispensable regulators of biological processes, such as cancer, hyperalgesia, atherosclerosis and arthritis. They are synthesized *de novo* from a limited set of poly-unsaturated fatty acids by cyclooxygenases, lipoxygenases and cytochrome P450s to generate a complex network of hundreds of interacting molecular signals that play distinct roles in regulating inflammatory and immune responses. We have developed LC-MS/MS methodology which comprehensively analyzes the known eicosanoid metabolome, and used it to characterize eicosanoid signaling during infection with *Borrelia burgdorferi*, the causative agent of Lyme disease. Using a murine model of Lyme arthritis, we determined the temporal production of eicosanoids *in vivo* from the induction of inflammation to its resolution. By comparing arthritis-susceptible mice to an arthritis-resistant strain, we have constructed a model for the regulation of Lyme arthritis pathology that integrates all the major eicosanoid biosynthetic pathways. Further studies utilizing COX-2 knockout animals identified significant off-target reduction in 5-lipoxygenase metabolites that can affect the pathology of disease. This demonstrates the utility of a comprehensive lipidomic approach for providing novel insight into the mechanisms of disease and the development of novel treatment strategies. This work was supported by the LIPID MAPS Large Scale Collaborative Grant from the NIH (GM069338), grant AR052748, and by a Gastroenterology training grant (T32 DK07202) from NIH.

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5. 17,18-Epoxyeicosatetraenoic acid, a potent anti-arrhythmic EPA metabolite: structure-activity relationships and development of stable analogs

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17,18-Epoxyeicosatetraenoic acid (17,18-EETeTr), a cytochrome P450 epoxygenase metabolite of eicosapentaenoic acid (EPA), exerts powerful negative chronotropic effects and protects against Ca^{2+}-overload arrhythmia in neonatal rat cardiomyocytes with an EC_{50} ~1 nM. Structure-activity-studies revealed the cis-$\Delta^{8,9}$-olefin and 17,18-epoxide are minimal structural elements for agonist activity. Several chemically and metabolically robust agonist analogs show promise as potential clinical candidates.

6. Genome-wide identification of phosphoinositide binding proteins

Michelle M. Ng[1], Holly C. Dippold[1], Suzette E. Farber-Katz[1], Sun-Kyung Lee[1], Monica L. Kerr[2], Marshall Peterman[1], Ronald Sim[1], Patricia Wiharto[1], Kenneth A. Galbraith[1], Swetha Madhavarapu[2], Greg J. Fuchs[3], Timo Meerloo[4], Marilyn G. Farquhar[4], Huilin Zhou[3], Seth J. Field[1]

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We have performed a large scale functional genomic screen to identify novel phosphoinositide binding proteins. The screen uses a highly validated *in vitro* binding assay to simultaneously assess binding to each of the phosphoinositides, plus several other lipids as well. The screen has identified previously known phosphoinositide binding proteins (including PH, PX, FYVE, Tubby, and Proppin domain proteins) and completely novel classes of phosphoinositide binding proteins (i.e., proteins that contain none of the previously known phosphoinositide binding domains). We will discuss in more detail one of the proteins identified in the screen as a proof of principle that the screen has identified new classes of phosphoinositide binding proteins. This is a novel protein that binds tightly and specifically to PtdIns(4)P, is highly conserved from yeast to humans, and provides new insight into Golgi function and the role of PtdIns(4)P.

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7. Quantification of glycerophospholipids in *Escherichia coli*

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Phosphatidic acid (PA), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and cardiolipin (CL) are the major classes of lipids produced in the Gram-negative bacterium Escherichia coli. Each class contains numerous possible molecular species due to differences in acyl chain length and saturation. A given molecular formula, i.e. a given m/z, can include multiple distinct molecular species. For example, a PA with m/z 645.45 contains 32 carbons and one un-saturation in the acyl chains. This m/z corresponds to PA with 14:0 and 18:1 acyl chains, and a PA with 16:0 and 16:1. The goal of this project is to quantify the total amount of each molecular species, as defined by the total number of carbons and un-saturations in the acyl chains, of PA, PE, PG, and CL in E. coli cells. Liquid chromatography electrospray ionization mass spectrometry (LC-ESI-MS) was used to quantify the lipids in this experiment. Lipids were extracted in the presence of synthetic internal standards non-isobaric with the endogenous species of E. coli. Following normal phase chromatography separation, peak areas for the extracted ion current were recorded for each endogenous species and compared to its corresponding synthetic standard. The amount of each species was summed to calculate the percent composition of PA, PE, PG, and CL in E. coli cells grown to late log phase at 37 °C in LB broth. Although issues were encountered while generating the standard curves used for calculating the absolute nmols of each analyte, the observed quantities, PE 79%, PG 19%, PA 1%, and CL 0.2 %, are consistent with previous literature. In future experiments, the phospholipid composition of E. coli will be measured under various conditions.

8. Genetic networks induced by LPS and oxPAPC in primary mouse macrophages

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The complexity of atherosclerosis, the major cause of coronary artery disease (CAD), is apparent from the long list of the genetic and environmental factors that affect disease susceptibility. It is clear that there are hundreds of genes, at least, that impact the common forms of the disease. Systems based approaches, such as developing gene expression networks, are useful for dealing with complex problems. Weighted co-expression gene network analysis, WCGNA, is one systems based approach we have previously used to both identify novel genes and to infer their mechanism. Specifically, this approach was successful at identifying endothelial responses to a specific moiety of oxidized lipid, oxPAPC. Expression networks identified the unfolded protein response as a novel pathway and follow up studies identified CHAC1 as a key mediator of this process.

Atherosclerosis is currently viewed as an inflammatory condition induced by oxidized lipids. The mouse strains C3H/HeJ and C57BL6/J vary tremendously in their susceptibility to atherosclerosis in the aortic root and their response to LPS. In order to better characterize the response of C3H/HeJ and C57BL6/J to inflammatory stimuli, we created macrophage-specific co-expression networks. We exposed thioglycolate-elicited macrophages to a series of doses and time courses of LPS and oxPAPC. Using WCGNA, we where able to identify several gene modules in response to both stimuli. We are currently characterizing these modules to identify novel key mediators, 'hub genes', of this process and to distinguish their role within the transcriptional network.

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9. Visualization of gene expression data for sphingolipid and glycosphingolipid metabolism via a pathway model and its application to cancer

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In this study, pathway maps for mammalian (glyco)sphingolipid biosynthesis were developed using the pathway visualization tool, Pathvisio v1.1 and an expansion of the Kyoto Encyclopedia of Genes and Genomes pathway diagrams to include newly identified gene isoforms, such as the subtypes of ceramide synthases, dihydroceramide desaturases, and others. This tool was applied to gene expression data that are publicly available for a wide range of cancer cell lines and tumors, and revealed interesting relationships. For example, comparison of the gene expression data for the breast cancer cell lines MCF7 and MDA-MB-231 predicted that the former has higher dihydroceramide desaturase form 2 (DES2), which is responsible for the biosynthesis of 4-hydroxyceramides (phytoceramides). Analysis of the sphingolipids of these cells by liquid chromatography, electrospray ionization-tandem mass spectrometry (LC ESI-MS/MS) found a 2 to 4-fold elevation in phytoceramides and other phytosphingolipids in MCF7 cells compared to MDA-MB-231, consistent with this prediction. The gene expression comparison also predicted differences in globoside synthase and SSEA3 synthase, which were consistent with the higher amounts of Gb4 and SSEA3 that have been previously observed in MCF7 cells. As another example, differences in gene expression for invasive ductal carcinoma (IDC) and normal ductal breast tissue predicted higher SSEA3 synthase and fucosyltransferase 2 (Fut2) which matched known elevations in SSEA3 and Globo-H antigen in breast tumors. A comparison of the subtypes of ceramide synthases expressed in different cancer cell lines and tumors versus normal cells also predicted interesting differences in ceramide subspecies that were verified by LC ESI-MS/MS, where the samples were available for analysis. Thus, these results illustrate how use of two types of "omic" technologies ("genomics" and "sphingolipidomics") can help identify new directions for research on (glyco)sphingolipid metabolism in cancer, with the possible identification of new intervention targets and/or biomarkers. These studies were supported by funds from NIH grants GM076217 and GM069338 (Lipid Maps).

10. Quantification of ceramide-1-phosphate by LC-ESI MS/MS

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Sphingolipids are a structurally diverse category of compounds found in all eukaryotes as well as some prokaryotes and viruses. They have been shown to be components of biological membranes and also regulators of cell metabolism1,2. Ceramide-1- phosphate, formed by the phosphorylation of ceramide via ceramide kinase, has been shown to play a role in phagocytosis, eicosanoid synthesis, mast cell degranulation, cell proliferation and survival3. Because of its importance in cell function, the quantification of Cer-1-P is of interest. Past methods for used to quantify amounts of Ceramide-1-Phosphate using HPLC-ESI MS/MS demonstrated a large amount of carryover on the analytical column. An alternative method for quantifying Ceramide-1-Phosphate was developed. Positive mode ionization tandem MS allows for the identification of ceramide-1-phosphate by the production of a structurally specific product ion, *m/z* 264.4. Separation by chain length of ceramide-1-phosphate molecular species is achieved by using reverse phase liquid chromatography without carryover of analyte in the analytical column. In RAW cells it is shown that there is a strong bias towards C16. An increase of ceramide-1-phosphate was also observed with addition of Kdo_2-Lipid A.

11. Participation of de novo sphingolipid biosynthesis in the regulation of autophagy in Kdo2-Lipid A stimulated RAW264.7 macrophages

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Kdo_2-Lipid A, a lipopolysaccharide (LPS) sub-structure with endotoxin activity equal to LPS [1], is a potent inducer of autophagy in RAW264.7 macrophage cells, as monitored by following the redistribution of GFP-LC3 into discrete punctate vesicles, Western blotting, and mRNA analysis of the autophagy genes, *LC3* and *Atg12.* Ceramide [2] and, more recently, dihydroceramide [3] have been shown to be inducers of autophagy; therefore, this study determined whether the induction of autophagy by Kdo_2-Lipid A is mediated via changes in sphingolipid metabolism in RAW264.7 cells using liquid chromatography, electrospray ionization tandem mass spectrometry (LC ESI-MS/MS). When RAW264.7 cells were treated with Kdo_2-Lipid A there were significant increases in dihydroceramide and ceramide due to upregulation of the *de novo* biosynthetic pathway, as evidenced by LC ESI-MS/MS analysis of cellular sphingolipids [4, 5], stable isotope labeling of sphingolipids, and gene array analysis (www.lipidmaps.org). Furthermore, the increased *de novo* sphingolipid biosynthesis is necessary for the Kdo_2-Lipid A induced autophagy, as the autophagic response was inhibited by myriocin/ISP-1, an inhibitor of serine palmitoyltransferase. Since the *de novo* biosynthesis of dihydroceramide and ceramide originates in the endoplasmic reticulum (ER), which has been the proposed source of membranes for the formation of autophagosomes, we explored whether ceramides can be detected in autophagosomes using confocal microscopy and anti-ceramide antibodies with visualization by a fluorescently conjugated secondary antibody. Consistent with previous literature, the majority of ceramide fluorescence was localized within the Golgi region in untreated RAW264.7 cells; however, following the addition of Kdo_2-Lipid A, ceramide fluorescence shifts to punctate vesicles, which co-localize with autophagosomes. Therefore, these findings strongly suggest that sphingolipids may be essential membrane components of the autophagic process. This work is supported by the LIPID MAPS grant GM069338.

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12. Vα24-invariant natural killer T Cells mediate anti-tumor activity via killing of tumor-associated macrophages

Liping Song[1,2], Shahab Asgharzadeh[1], Jill Salo[1], Kelly Engell[1], Hong-wei Wu[1], Richard Sposto[1], Tasnim Ara[1], Ayaka M. Silverman[1], Yves A. DeClerck[1], Robert C. Seeger[1], Leonid S. Metelitsa[1, 2]

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Tumor infiltration with Vα24-invariant NKT cells (NKTs) associates with favorable outcome in neuroblastoma and other types of cancer. Although NKTs can be directly cytotoxic against CD1d-positive cells and activate NK cells in mouse models, the majority of human tumors are CD1d-negative and not infiltrated with NK cells. Therefore, the role of NKTs in cancer has remained largely unknown. We demonstrate that CD68+ tumor-associated monocytes/macrophages (TAMs) represent the majority of CD1d-expressing cells in primary neuroblastomas. TAMs in vitro and in vivo stimulated neuroblastoma growth, and gene expression analysis of 129 primary neuroblastomas revealed that high level expression of TAM genes predicted poor outcome. While NKTs were not cytotoxic against neuroblastoma cells, they effectively killed monocytes pulsed with tumor cell lysate. The killing was CD1d-restricted since it was inhibited by anti-CD1d mAb. The pre-treatment of tumor cells with butyl-deoxygalactonojirimycin, an inhibitor of glucosylceramide synthase abrogated NKT cell IFNγ production in response to lysate-pulsed monocytes while no effect was observed when monocytes or monocyte-NKT mixture were treated with the same inhibitor. These data indicate that monocytes cross-present to NKTs yet unknown glycosphingolipid(s), derived from neuroblastoma cells instead of generating new ligands themselves. Co-transfer of human monocytes and NKTs to tumor-bearing NOD/SCID mice decreased monocyte number at the tumor site and suppressed tumor growth compared to mice transferred with monocytes alone. Thus, killing of TAMs reveals a novel mechanism of NKT cell anti-tumor activity that relates to the disease outcome.

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13. Validation of phospholipid concentrations in rat brain by imaging mass spectrometry

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Although the two-dimensional representations commonly employed in MALDI-Mass Spectrometric Imaging (MSI) apparently indicate the distribution of an ion across sub-regions of a tissue, several complex biological and mass spectrometric factors suggest that the interpretation of these images is not straightforward. Relative molecular abundance, ease of ion formation and ejection, water content, cell type, density of tissue structure, and the like could all affect the signal of ions viewed in an image.

In validation studies for cellular phospholipids, we have determined how the intensity of signal in a sagittal section of rat brain, as determined by MALDI-MSI, relates to the abundance of molecules in a tissue sample. Tissue was sectioned normally (10 μm-thick) for MALDI-MSI, both before and after a thicker section was cut. Microdissected regions of thick sections were weighed and phospholipids extracted and quantitatively determined by HPLC-ESI/MS/MS with respect to internal standards for PA, PC, PE, PG, PI, and PS.

The intensities of ions arising from two abundant phosphocholine species within the same region of cerebral cortex, as measured by ESI/MS/MS, are inconsistent with the corresponding relative intensities of molecular ions observed for the same species in MALDI-MSI of an adjacent slice of tissue. For instance, the ion arising from16:0/16:0 PC at m/z 734.5 for [M+H]+ is more abundant by ESI/MS/MS than for the protonated ion arising from 16:0/18:1 PC, while the opposite relative intensities are observed by MALDI-MSI in the same region of tissue. In other cases, relative ion intensities are different depending on which region of brain is studied for a particular phospholipid. Although comparison of like molecules within the same anatomical region of tissue is expected to yield a linear response of signal intensity to amount of each species, the differential signals obtained by MALDI and ESI are difficult to interpret because of salt adduct formation, isomers, overlapping signals from other compounds, and ion suppression effects.

14. Artifactual formation of N-acylphosphatidylethanolamine and related molecules during chloroform-methanol based lipid extractions

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Recently, Gillum *et al* (2008) reported the presence of the lipid N-acylphosphatidylethanolamine (NAPE) in plasma, and suggested a physiological role in the control of appetite[1]. Based on this publication, a number of pilot experiments using human plasma and fetal bovine serum were undertaken to isolate and further characterize this lipid via high performance liquid chromatography coupled with negative-ion electrospray ionization mass spectrometry. Initially, we noticed a correlation between NAPE abundance and extraction pH, with striking reductions in NAPE levels under acidic extraction conditions. To determine whether this phenomenon was due to the instability of NAPEs in low pH, we performed lipid extractions of human plasma under neutral conditions, verified the presence of NAPEs, and then re-extracted the lipids under acidic conditions. The NAPEs were shown to be stable under these acidic re-extraction conditions, suggesting that plasma NAPEs may be artifactual in nature. Using synthetic diacyl or plasmalogen forms of phosphatidylethanolamine (PE) and free decanoic acid, we show that abundant NAPEs can be formed chemically under neutral Folch extraction conditions, along with a number of other PE derived artifacts. Consistent with the absence of NAPEs under acidic extraction conditions initially shown in our human plasma and fetal bovine serum pilot extractions, chemical formation of NAPEs and other adducts from synthetic PE and decanoic acid did not occur under acidic extraction conditions. Based on these experiments, we propose a mechanism of chemical NAPE formation that is phosgene dependent. To verify that the chemical NAPE formation was phosgene dependent, a chloroform purification technique described by Cone *et al* (1982) was employed in an attempt to remove phosgene. Using chloroform filtered through an activated alumina column in neutral lipid extractions of fetal bovine serum, we produced an approximate 50 percent reduction in NAPE levels strongly suggesting that chemical NAPE formation is phosgene dependent.

1. M. Gillum, D. Zhang, X. Zhang, D. Erion, R. Jamison, C. Choi, J. Dong, M. Shanabrough, H. Duenas, D. Frederick DW, Hsiao JJ, Horvath TL, Lo CM, Tso P, Cline GW, Shulman GI (2008) N-acylphosphatidylethanolamine, a gut-derived circulating factor induced by fat ingestion, inhibits food Intake, Cell, **135**(5), 813-24.

15. Lipid dynamics in clathrin mediated endocytosis

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Clathrin-mediated endocytosis (CME) is the major mechanism of cargo internalization from the plasma membrane. CME initiates as a result of recruitment of adaptors, clathrin, and other proteins into a small invaginating region of the plasma membrane termed a clathrin-coated pit (CCP), which eventually pinches off and forms a clathrin-coated vesicle. Numerous proteins function in CME; however, much less is known about the role of lipids. A detailed spatial and temporal understanding of CME has thus far remained elusive, in part due to a paucity of information available about the dynamics of this process. While it is known that plasma membrane phosphatidylinositol-(4,5)-bisphosphate (PIP_2) is required for CCP initiation, it is unclear whether PIP_2 is synthesized or enriched within a CCP, and how this might participate in CCP maturation. In addition, PIP_2 levels can regulate synthesis of phosphatidic acid (PA); however, the consequence of possible PA dynamics within CCPs is also not well understood. Hence, we sought to determine how the possible localized, coordinated dynamics of these two lipids species may control CCP maturation.

We have previously described the use of time-lapse TIRF microscopy imaging of cells expressing fluorescently-labelled clathrin, followed by computational analysis of CCP dynamics. Monitoring the lifetimes of CCPs allows isolation of discrete populations of CCPs, delineating several temporal stages of CCP maturation. Using this method, we have here observed that perturbation of PIP_2 dynamics *via* siRNA gene silencing of phosphatidylinositol-4-phosphate-5-kinase and synaptojanin isoforms result in perturbation of early and late stages of CCP maturation, respectively. Perturbation of PA synthesis by siRNA gene silencing or chemical inhibition of PLD1/2 reveals that PA synthesis may function to negatively regulate CCP maturation. Thus, our results are consistent with localized PIP_2 and PA dynamics within CCPs, and suggest that each lipid may have distinct functions during CCP maturation.

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16. Comparing global and targeted lipid and fatty acid shotgun profiling of brain tissue extracts by NanoESI-infusion

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The growth in lipidomics research is uncovering a need for complete and comprehensive workflows for identifying and quantifying lipid species from biological extracts. In choosing analytical methods for lipidomics, different yet complementary mass spectrometry approaches can provide a more complete and comprehensive data set leading to a detailed characterization of lipid molecular species from complex extracts. A preliminary strategy carried out as global “shotgun” tandem mass spectrometry by direct infusion electrospray ionization or LC-MS/MS analysis, uses information dependant MS/MS scanning in both polarities for unbiased lipid profiling. The second approach involves multiple lipid-class-specific precursor ion and neutral loss scanning whose resulting spectra can be used directly to identify and characterize lipids and fatty acids in tissue-derived lipid extracts. The multiple precursor ion scanning (MPIS) methods have been published and recently reviewed by Ekroos et al, describing the advantages of targeted MPIS techniques for generating comprehensive lipid arrays from small sample volumes. Fully characterizing these lipid components by high quality MS/MS for fatty acid chain length and double bond positioning is a critical step for understanding their biological implications in cell signaling and lipid-initiated disease progression. Taking advantage of the speed, selectivity, and sensitivity of hybrid triple quadrupole technology, whole lipid extracts from rat brain tissue can be analyzed by direct nanoESI infusion for in depth glycerophospholipid profiling - achieving both qualitative and quantitative data (with the use of synthetic lipid internal standards) in very fast analysis times. Lipid species identification and quantitation is carried out using LipidView™ Software enabling post acquisition processing of precursor ion, neutral loss, MRM, and MS/MS data via lipid database searching and accurate peak integration. We present robust targeted and global workflows for the identification and quantitation of glycerphospholipids in total lipid extracts from rat brain tissue.

17. A comprehensive classification system for lipids

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In 2005, the International Lipid Classification and Nomenclature Committee (ILCNC) under the sponsorship of the LIPID MAPS Consortium developed and established a “Comprehensive Classification System for Lipids” based on well-defined chemical and biochemical principles, and using an ontology that is extensible, flexible and scalable. This classification system which is compatible with contemporary databasing and informatics needs has now been accepted internationally and widely adopted. In response to considerable attention and requests from lipid researchers from around the globe and in a variety of fields, the comprehensive classification system has undergone significant revisions over the last few years to more fully represent lipid structures from a wider variety of sources and to provide additional levels of detail as necessary. We present the various categories, classes and subclasses which comprise this updated classification system for lipids.

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18. LIPID Metabolites And Pathways Strategy (LIPID MAPS)

Manish Sud[1], Dawn Cotter[1], Eoin Fahy[1], Robert Byrnes[1], Shakti Gupta[1], Sean Li[1], Mano Maurya[1], Yihua Zhao[1], Shankar Subramaniam[1,2], H. Alex Brown[3], Christopher K. Glass[4], Alfred H. Merrill, Jr.[5], Robert C. Murphy[6], Christian R.H. Raetz[7], David W. Russell[8], Edward A. Dennis[9]

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Lipids and their metabolites play a key role in the regulation and control of cellular function and disease. In order to develop compounds of therapeutic interest, it is vital to not only identify and characterize existing and novel lipids but also quantify changes in their metabolites, and develop biochemical pathways and interaction network maps. LIPID MAPS consortium is involved in this endeavor. To integrate, analyze, track, and disseminate large volumes of heterogeneous chemical, biological, and analytical data being generated by multidisciplinary research groups, we have developed a computational infrastructure which includes a variety of tools with both web-based and batch access. We present our current and on-going work on development of these tools to support LIPID MAPS research activities.

19. Integrated lipids, genes, and pathways data across timecourse experiments for RAW 264.7 cells treated with Kdo2-lipid A

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The macrophage cell type plays an important role in innate immune responses and the development of various chronic inflammatory diseases. In addition to investigating macrophage transcriptome response to stimulation with Kdo2-lipid A, a chemically defined lipopolysacchride (LPS) specific for the TLR-4 receptor, LIPID MAPS consortium has also systematically evaluated the responses to macrophage lipidome by quantitative mass spectrometric measurements and correlated these changes with transcriptomic responses. We present here integrated lipidome and transcriptome data, mapped on to pathways, collected across timecourse experiments for RAW 264.7 macrophage cells in response to Kdo2-lipid A activation.

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20. Cholesteryl ester hydroperoxides induce lipoprotein accumulation in macrophages via TLR4-dependent fluid phase uptake

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Toll-like receptor-4 (TLR4) recognizes microbial pathogens, such as lipopolysaccharide (LPS), and mediates LPS-induced proinflammatory cytokine secretion, as well as microbial uptake by macrophages. In addition to exogenous pathogens, TLR4 recognizes modified self, such as minimally oxidized low-density lipoprotein (mmLDL). Here we report that mmLDL and its active components, cholesteryl ester (CE) hydroperoxides, induce TLR4-dependent fluid phase uptake typical of macropinocytosis. We show that mmLDL induced recruitment of spleen tyrosine kinase (Syk) to a TLR4 signaling complex, TLR4 phosphorylation, activation of a Vav1-Ras-Raf-MEK-ERK1/2 signaling cascade, phosphorylation of paxillin, and activation of Rac, Cdc42 and Rho. These mmLDL-induced and TLR4- and Syk-dependent signaling events and cytoskeletal rearrangements lead to enhanced uptake of small molecules, dextran and, most importantly, of both native and oxidized LDL, resulting in intracellular lipid accumulation. An intravenous injection of fluorescently labeled mmLDL in wild type mice resulted in its rapid accumulation in circulating monocytes, which was significantly attenuated in TLR4-deficient mice. These data describe a novel mechanism leading to enhanced lipoprotein uptake in macrophages that would contribute to foam cell formation and atherosclerosis. These data also suggest that CE hydroperoxides are an endogenous ligand for TLR4. As TLR4 is highly expressed on the surface of circulating monocytes in patients with chronic inflammatory conditions, and CE hydroperoxides are present in plasma, lipid uptake by monocytes in circulation may contribute to monocytes' pathological roles in chronic inflammatory diseases.

21. X-ray structure of Sonepcizumab, a novel humanized monoclonal antibody against the bioactive lipid sphingosine-1-phosphate

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Biologically active lipids are now recognized as important mediators of critical physiologic and pathological processes, including cancer, inflammatory, neurodegenerative diseases and dysfunctional fibrosis. Using the proprietary ImmuneY2™ technology, Lpath Inc. has developed a potential first-in-class therapeutic agent, a humanized monoclonal antibody, sonepcizumab, which functions as a molecular sponge to target and neutralize the bioactive lipid, sphingosine-1-phosphate (S1P). Sonepcizumab blocks the tumorigenic and angiogenic effects of dysregulated S1P produced by cancer cells and during pathological angiogenesis. Here we present the x-ray crystal structure of the Fab fragment from a humanized monoclonal antibody raised to specifically recognize and bind S1P. Fab fragments were prepared by papain treatment of whole humanized IgG and then combined with S1P. The complex structure was solved by molecular replacement and refined against a complete set of indexed diffraction intensities to 1.9 Å resolution. This structure elucidates the molecular basis for the high selectivity of S1P by the antibody. This specificity is achieved through electrostatic interactions between the complementary determining regions (CDRs) of the light chain and the S1P headgroup, while the complex is stabilized by packing of the hydrocarbon tail within a hydrophobic pocket formed by the CDRs of the heavy chain. Unexpectedly, the structure reveals two metal atoms that bridge the intermolecular interaction between aspartic acid residues in the light chain and the phosphate group of S1P. This structure accounts for the high affinity and specificity of the antibody-S1P interaction seen in vitro and confirms that the ligand binds to the hypervariable CDR regions of the antibody as predicted.

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22. Regulation of the brain isoprenoids farnesyl- and geranylgeranyl pyrophosphate is altered in Alzheimer disease

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Post-translational modification of proteins by farnesyl- (FPP) and geranylgeranylpyrophosphate (GGPP) is receiving increasing attention in diseases diverse as cancer, cardiovascular disease and neurodegenerative diseases. FPP and GGPP covalently attach to proteins with a C-terminal CaaX motif such as small GTPases enabling those proteins to be inserted into membranes resulting in activation of pathways involved in inflammation, oxidative stress, and cell proliferation and growth. Elevated levels of FPP and GGPP have been previously proposed to occur in Alzheimer disease (AD) but have never been quantified. Impeding progress on understanding brain FPP and GGPP regulation and consequences on protein targets have been the analytical difficulties of isolation and detection sensitivity. We recently reported FPP and GGPP levels in normal human brain tissue using fluorescence HPLC. In the present study, we determined if FPP and GGPP are increased in brain tissue of human AD patients as compared with controls. This study demonstrates for the first time that FPP (36%) and GGPP (56%) levels are significantly elevated in human AD brain but not cholesterol, indicating a specific targeting of isoprenoid regulation independent of HMG-CoA-reductase. Gene expression of FPP synthase and GGPP synthase were also significantly elevated in brain tissue of the AD samples whereas the expression levels of HMG-CoA reductase did not differ between the AD and control samples. Further underscoring the selective disruption of FPP and GGPP homeostasis in AD, we showed that simvastatin-induced inhibition of HMG-CoA reductase *in vivo* significantly reduced FPP, GGPP and cholesterol abundance in mice. The specific targeting of FPP and GGPP in AD brain may stimulate protein prenylation and contribute to AD neuropathophysiology and suggests that FPP and GGPP regulation is independent of HMG-CoA reductase in the AD brain. Supported by the Hanna Bragard-Apfel Foundation, NIH grants AG23524, AG18357, Medical Research Council, and Department of Veterans Affairs.