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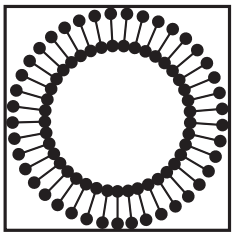

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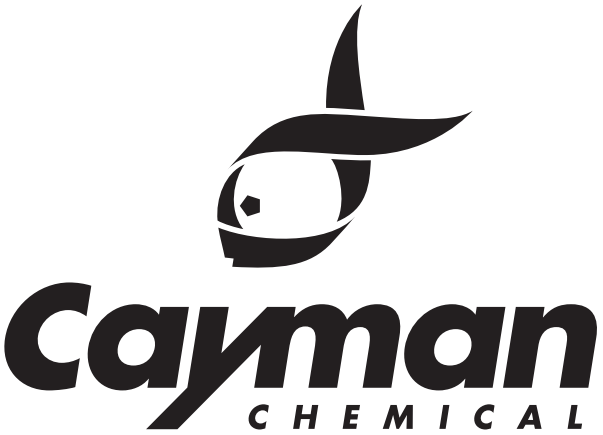

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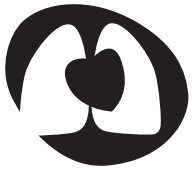

Department of Health and Human Services • National Institutes of Health
National **Heart Lung and Blood** Institute
People Science Health

(Grant# R13HL087685)

SERLC 2006 Awardees

Postdoctoral Trainee Travel Award

Dr. Archana Mukhopadhyay

Dr. Paramasivam Natarajan

Dr. Gabriel Schaaf

Dr. Motohiro Tani

Dr. Anelli Viviana

Graduate Student Travel Award

Stephanie E. Brock

Amanda Davis

Ashley Jones

Barbara Konik

Kannan Krishnamurthi

Poulami Mitra

Deanne Siow

SOUTHEASTERN REGIONAL LIPID CONFERENCE

November 1st-3rd, 2006

Schedule of Events

Wednesday, November 1st, 2006

4:00-6:00pm **Registration for all pre-paid SERLC participants**: Lobby of High Hampton Inn

5:00-6:00pm **Registration for all late registrants**: Lobby of High Hampton Inn

6:00-7:00pm **Reception**: Wade Hampton Room

7:00-8:00pm **Dinner**

8:10-8:30pm **Opening Remarks by SERLC Chair (Dr. Charles Chalfant) and Sponsor Recognition:** Pavilion

8:30-9:30pm **Keynote Speaker: Dr. Gordon Mills, University of Texas, MD Anderson Cancer Center, Houston, TX *"S1P and LPA: Validated targets for cancer therapy"***: Pavilion

9:30pm-Sometimes extremely late **Social Time:** Wade Hampton Room

Thursday, November 2nd, 2006

7:00-8:00am **Breakfast**

8:00-9:30am **Session I: Lipidomics, Enzyme Structure and Lipid Analysis**: Pavilion
Chair: Dr. Cameron Sullards, Georgia Institute of Technology, Atlanta, GA
Co-Chair: Dr. Brian "Binks" Wattenberg, University of Louisville-SOM, Louisville, KY

8:00- 8:15am "*Quantification of sulfatides by liquid chromatography, tandem mass spectrometry*"
Jeremy Allegood, Georgia Institute of Technology, Atlanta, GA

8:15-8:30am "*Membrane targeting of the pro-apoptotic protein Bax*"
Stephanie Brock, University of Louisville, KY

8:30-8:45am "*Elevation of sphingoid bases in HeK293 cells via overexpression of serine palmitoyltransferase or exogenous addition increases C18-(dihydro)ceramide biosynthesis via alteration of CerS1 expression, activity and subcellular localization*"
Ying Liu, Georgia Institute of Technology, Atlanta, GA

8:45-9:00am "*Crystallization and functional studies of sec14 family member sfh1 bound to phospholipids- Is PtdCho transfer critical for sec14 function*"
Gabriel Schaaf, University of North Carolina, Chapel Hill, NC

9:00-9:15am "*Fenretinide induces lethal autophagy via a novel ensemble of life and death regulators: dihydoceramide and sphinganine versus sphingosine-1-phosphate*"
Katie Fluke, Georgia Institute of Technology, Atlanta, GA

9:15-9:30am "*Mass isotopomer analysis of stable isotope-labeled palmitoyl-CoA*"
Christopher A. Haynes, Georgia Institute of Technology, Atlanta, GA

9:30-9:45am **Coffee Break**

9:45-12:00pm **Session II: The roles of bioactive lipids in cell signaling (focus on sphingolipids):** Pavilion
Chair: Dr. Besim Ogretmen, Medical University of South Carolina, Charleston, SC
Co-Chair: Dr. Carole Oskeritzian, Virginia Commonwealth University, Richmond, VA

9:45-10:00am "*Inhibitor 2 of Protein Phosphatase 2A (I2PP2A) Directly Binds Ceramide both in vitro and in situ: A Novel Mechanism for Ceramide-mediated Regulation of Nuclear Protein Phosphatase 2A Activity*"
Archana Mukhopadhyay, Medical University of South Carolina, Charleston, SC

10:00-10:15am "*Regulation of sphingosine kinase 1 during hypoxia in glioma cells: role of hypoxia inducible factor 2*"
Viviana V. Anelli, Medical University of South Carolina, Charleston, SC

10:15-10:30am "*Sphingosine-1-Phosphate Receptor Antagonists and Lymphocyte Trafficking*"
Ashley H. Snyder, University of Virginia, Charlottesville, VA

10:30-10:45am "*GSH depletion during aging upregulates nSMase2 activity and hepatic response to IL-1beta*"
Kristina Rutkute, University of Kentucky Lexington, KY

10:45-11:00am "*Acid Sphingomyelinase Mediates Cisplatin-Induced Cytoskeletal Remodeling*"
Youssef H. Zeidan, Medical University of South Carolina, Charleston, SC

11:00-11:15am "*$S1P_2$ receptor is essential for inner ear vascular development and hearing*"
Mari Kono, National Institute of Health, Bethesda, MD

11:15-11:30am "*The role of ABCC1 in export of sphingosine-1-phosphate from mast cells*"
Poulami Mitra, Virginia Commonwealth University, Richmond, VA

11:30-11:45am "*EGF Regulates Plasminogen Activator Inhibitor-1 by a Novel Pathway Involving c-Src, PKCδ, and Sphingosine Kinase 1 in Glioblastoma Cells*"
Barbara S. Konik, Virginia Commonwealth University, Richmond, VA

11:45-12:00pm "*Opposing Functions of LASS1 and LASS6 Generated C18- and C16-Ceramides in the Regulation of Caspase Dependent Apoptosis in Head and Neck Squamous Cell Carcinomas*"
Can E. Senkal, Medical University of South Carolina, Charleston, SC

12:00-1:00pm **Lunch**

1:00-4:00pm **Free Time**

4:00-6:00pm **Poster Session:** Wade Hampton Room

6:00-6:15pm **Award Presentations**

6:15-7:15pm **Keynote Speaker: Dr. Charles Serhan, Harvard University, Boston, MA *"Novel Lipid Mediators in the Resolution of Inflammation: Resolvins and Protectins"***: Pavilion

7:15-8:00pm **Dinner**

8:30-11:00pm **Clogg Dancers and Country Band:** Lobby of High Hampton Inn

11:00pm-Sometimes extremely late **Social Time** Wade Hampton Room

Friday, November 3rd, 2006

7:00-8:00am **Breakfast**

8:00-9:45am **Session III: Lipid Biosynthesis/Metabolism, Enzymology, and Genomics**: Pavilion
Chair: Dr. Ashley Cowart, Medical University of South Carolina, Charleston, SC
Co-Chair: Dr. Huiping Zhou, Virginia Commonwealth University, Richmond, VA

8:00-8:15am "*Group VIA calcium-independent phospholipase A_2 as a novel regulator of sterol regulatory element-binding proteins*"
W. Palmer Wilkins, Viginia Commonwealth University, Richmond, VA

8:15-8:30am "*Arabidopsis PIPK1 is involved in a scaffold with F-actin and recruits PI4Kβ1 to the actin cytoskeleton*"
Amanda J. Davis, North Carolina State University, Raleigh, NC

8:30-8:45am "*A new pathway for NBD-phosphatidylserine flip in yeast*"
Haley Curtis Stevens, Emory University School of Medicine, Atlanta, GA

8:45-9:00am "*Suppression of drs28710; cold-sensitive growth and ergosterol localization defects by kes18710*"
Baby-periyanayaki Muthusamy, Vanderbilt University, Nashville, TN

9:00-9:15am "*Examining the puzzling synthetic interaction between the major yeast phosphoinositide transfer protein Sec14p and Tlg2p*"
Kimberly Tyeryar, University of North Carolina, Chapel Hill, NC

9:15-9:30am "*The rate of sphingolipid biosynthesis in liver affects utilization of palmitic acid for TAG synthesis*"
Gergana Deevska, University of Kentucky, Lexington, KY

9:30-9:45am "*Resistance to Dietary-Induced Obesity in Acid Sphingomyelinase Deficient Mice*"
Krasimira Rozenova, University of Kentucky, Lexington, KY

9:45:10.00-am **Coffee Break**

10:00-11:45am **Session IV: The roles of bioactive lipids in cell signaling (focus on glycerolipids)**:
Pavilion
Chair: Dr. Frank Fang, Virginia Commonwealth University, Richmond, VA
Co-Chair: Dr. Kimberly Paul, Clemson University, Clemson, SC

10:00-10:15am "*Age-dependent heart failure in mice with combined deficiency of LPA1 and 2*"
Hsin-Yuan Cheng, University of Kentucky, Louisville, KY

10:15-10:30am "*Regulation of a phospholipid flippase activity by phosphoinositides*"
Paramasivam Natarajan, Vanderbilt University, Nashville, TN

10:30-10:45am "*Regulation of PAFAH expression by PAF and oxidized lipids*"
Rachael Griffiths, Virginia Commonwealth University, Richmond, VA

10:45-11:00am "*The role of LPA production in the survival signaling of $SKOV_3$ ovarian cancer cells*"
Jerry A. Saunders, Wake Forest University, Winston-Salem, NC

11:00-11:15am "*Statins Induce Endosomal Sequestration of LPA_1 Lysophosphatidic Acid Receptors*"
Paul D. Salo, Georgia Institute of Technology, Atlanta, GA

11:15-11:30am "*Induction of Cox-2 expression by lysophosphatidic acid in ovarian carcinoma cells involves a permissive signaling input from a receptor Tyrosine Kinase and activation of Histone Deacetylases*"
Regina A. Oyesanya, Virginia Commonwealth University, Richmond, VA

11:30-11:45am "*Inhibition of calcium–independent phospholipase A_2 suppresses proliferation, survival and tumorigenicity of ovarian carcinoma cells*"
Yuanda Song, Virginia Commonwealth University, Richmond, VA

11:45-12:00 **Business Meeting:** Budget, Election of New Chair, Grant Renewal, Non-Profit Status

12:00-1:00pm **Lunch**

1:00pm **Depart**

Keynote Lecture I

Wednesday, November 1st, 2006, 8:30-9:30pm

S1P and LPA: Validated targets for cancer therapy

Gordon Mills

University of Texas, MD Anderson Cancer Center, Houston, TX

Lysophosphatidic acid (LPA) and sphingosine 1-phosphate (S1P), two small lysophospholipids, are potent inducers of many of the hallmarks of cancer including cell proliferation, survival, migration, invasion and neovascularization in in vitro and in vivo tumor models. Further the enzymes metabolizing LPA and S1P and their receptors are aberrant in multiple cancer lineages and exhibit transforming activity altering patterns and targets for metastasis. Several recent studies demonstrate remarkable activity of new chemical genomics and/or potential novel drugs in preclinical models. Combined with the physiologic and pathophysiologic activities of LPA and S1P, these studies mandate the implementation of preclinical and clinical evaluation of LPA and S1P as therapeutic targets.

Keynote Lecture II

Thursday, November 2nd, 2006, 6:15-7:15pm

Novel Lipid Mediators in the Resolution of Inflammation: Resolvins and Protectins

Charles N. Serhan

Center for Experimental Therapeutics and Reperfusion Injury, Department of Anesthesiology, Perioperative and Pain Medicine, Brigham and Women's Hospital and Department of Oral Medicine, Immunity and Infectious Disease, Harvard Medical School, 75 Francis Street, Boston, MA 02115

A well-integrated inflammatory response and it's ending, i.e. *resolution*, is essential in health and disease. This keynote lecture will focus on our understanding of the cellular and molecular events that govern resolution. This lecture will give an overview of recent advances from studies by the author and colleagues on the biosynthesis and actions of lipoxins and the novel anti-inflammatory and pro-resolving lipid mediators, namely resolvins and protectins that are generated from omega-3 fatty acids, EPA and DHA. These previously unappreciated families of lipid-derived mediators were originally isolated from murine models of acute inflammation captured during the natural spontaneous resolution phase. They possess potent anti-inflammatory, proresolving and protective properties that will be presented. Since the resolvins and protectins in animal models control the duration and magnitude of inflammation, mapping of these resolution circuits can provide new avenues for controlling the molecular basis of many inflammatory diseases. Moreover, defective resolution mechanism(s) may underlie the current appreciation of chronic inflammatory phenotype(s) that characterize some prevalent human diseases.

Session I: Lipidomics, Enzyme Structure and Lipid Analysis

Quantitation of Sulfatides by Liquid Chromatography, Tandem Mass Spectrometry

Jeremy Allegood, Elaine Wang, M. Cameron Sullards and Alfred H. Merrill Jr.

Schools of Biology, Chemistry & Biochemistry and the Parker H. Petit Institute for Bioengineering and Bioscience, Georgia Institute of Technology, Atlanta, GA 30332

Sulfatides are sulfate esters of galactosylceramide (GalCer), a small number of higher order galactosyl-series sphingolipids, and—more rarely—glucosylceramides. They are found primarily in myelin membranes, kidney and a number of other vertebrate cell types, such as macrophages, and have important structural roles as well as to influence cell growth, signal transduction, adhesion and morphogenesis. This study established the conditions for extraction, chromatography, and quantitative analysis of the major sulfatide of most tissues, GalCer 3-sulfate, and used the method to measure the induction of sulfatide biosynthesis in RAW264.7 cells stimulated with Kdo_2 Lipid A following the Lipid MAPS protocols (www.LipidMaps.org). The sulfatide molecular subspecies are first identified in crude extracts by precursor ion scanning in negative ion mode with the product fragment being the sulfate moiety, which gives a strong signal at *m/z* 96.9. Ionization and fragmentation conditions were then optimized by syringe to develop a multiple reaction monitoring method for the high abundance subspecies (sulfatides with ceramide backbones with fatty acid chain lengths of C16:0 through C26:0 as well as their alpha-hydroxy counterparts) and an unusual chain length homolog (C12-sulfatide, available from Avanti Polar Lipids) as an internal standard. For liquid chromatography, the sulfatides were separated using a 2.1 x 20 mm Ace c18 column and binary solvent system. Mobile Phase A consisted of MeOH:H_2O 50:50 (v/v) with 5 mM ammonium acetate and 0.01% NH_4OH. Mobile Phase B consisted of MeOH with 5 mM ammonium acetate and 0.01% NH_4OH. LC profile involved a 2 min hold at 80% B, followed by a 5 min linear gradient to 100% B, which is held for 2 min, and then the column is re-equilibrated at 80% B for 2 min. Application of the method to RAW264.7 cells found that sulfatides were undetectable in unstimulated cells but increase after Kdo2 Lipid A stimulation to approximately 100 pmol/μg of DNA after 24 h, with the major subspecies being the C18:0, C24:1 and hC24:1 fatty acid containing ceramides, in the ratios 23:18:29, respectively. (This work was supported by funds from GM069338.)

Membrane targeting of the pro-apoptotic protein Bax

*S.E. Brock and B. Wattenberg.

Brown Cancer Center and Department of Biochemistry and Molecular Biology, University of Louisville.

Action of the pro-apoptotic protein Bax requires a signal-dependent targeting to the mitochondrial outer membrane (MOM). Recently, bax has been shown to induce a separate apoptotic pathway by localizing to the endoplasmic reticulum (ER). The mechanism that distinguishes between targeting to these two lipid membranes is unknown. A hydrophobic stretch of amino acids within the C-terminal tail of Bax serves as the targeting signal as well as the lipid membrane anchor. This places Bax in a class of proteins referred to as tail-anchored proteins. We have utilized artificial tail-anchor sequences to define features that specifically direct targeting to the MOM or the ER. An artificial sequence consisting of 16 leucines fused to a reporter protein (Green Fluorescent Protein) was used as a template for mutagenesis. It was demonstrated that increasing the polarity of this sequence, as well as introducing positive charge at the very C-terminus, directs targeting from the ER to the mitochondria. Circular dichroism studies were also performed on these sequences. These demonstrate that mitochondrial-targeted tail-anchor has a greater propensity to form α-helices in solution compared to ER-targeted tail-anchor. Therefore, structural differences of the tail-anchor could facilitate specific membrane targeting. It is our belief that the ER membrane is a more promiscuous membrane that serves as default localization for tail-anchored proteins. Increasing polarity of the hydrophobic tail-anchor primary sequence allows for greater recognition by the MOM, thereby driving targeting from the ER default membrane to the MOM. Currently, the data described above is being used as a basis to examine the structural requirements of the Bax mitochondrial targeting signal. Site-directed mutagenesis is being employed to determine specific polarity characteristics within the tail that are required for MOM recognition. The importance of these elements is being assessed by measuring targeting efficiency (fluorescence microscopy and subcellular fractionation). Nuclear Magnetic Resonance (NMR) is also being used to assess the 3-dimensional structure of the Bax tail-anchor as well as select mutants.
(Research Support: American Heart Association)

Elevation of sphingoid bases in Hek293 cells via overexpression of serine palmitoyltransferase or exogenous addition increases C18-(dihydro)ceramide biosynthesis via alteration of CerS1 expression, activity and subcellular localization

Ying Liu, Christopher Haynes, Jeremy Allegood, Samuel Kelly, Elaine Wang, and Alfred H. Merrill, Jr.

Schools of Biology, Chemistry and Biochemistry & the Petit Institute for Bioengineering and Bioscience, Georgia Institute of Technology, Atlanta, GA 30332

Biosynthesis of the ceramides (Cer), backbones of complex sphingolipids, involves the acylation of sphinganine (primarily from de novo biosynthesis) or sphingosine (from sphingolipid recycling) by ceramide synthases (CerS) that are selective for different categories of fatty acyl-CoA's; for example, CerS1 (LASS1) is highly selective for stearoyl-CoA and produces C18-(dihydro)Cer. We have observed that increasing the rate of production of sphinganine by stable overexpression of serine palmitoyltransferase increases C18-(dihydro)Cer relative to other Cer subspecies as determined by liquid chromatography electrospray tandem mass spectrometry. This increase was found to be due to an increase in CerS1 mRNA expression (by QRT-PCR) and enzymatic activity (by in vitro enzymatic assay). Addition of exogenous sphingosine or sphinganine to HEK293 cells (and similar findings were made with HeLa and HepG2 cells) also increased the proportion of C18-(dihydro)Cer; however, the increases in C18-(dihydro)Cer biosynthesis appear to be faster than expected for up-regulation of CerS1 expression. Therefore, we have examined additional effects of sphingosine on CerS1 using various means, including transfection of Hek293 cells with a CerS1-GFP construct. These studies have found that CerS1-GFP is located in both the endoplasmic reticulum and Golgi, and that exogenous addition of sphingosine decreases the apparent rate of reappearance of CerS1-GFP in the Golgi after photobleaching. Therefore, the increase in C18-(dihydro)Cer biosynthesis may be due to retention of CerS1 in the ER where it could have greater access to its substrates, or the activity may be modulated by other topologic factors. These studies have uncovered multiple mechanisms for CerS1 to respond to sphingoid base amounts, which suggests that this enzyme plays an important role in controlling the amounts of free sphingoid bases versus this subclass of ceramide. (Acknowledgements: These studies were conducted in collaboration with David Uhlinger, Johnson & Johnson Company, and by funding from NIH grant GM069338.)

Crystallization and functional studies of Sec14 family member Sfh1 bound to phospholipid – Is PtdCho transfer critical for Sec14 function?

Gabriel Schaaf[a], Eric A. Ortlund[c], Matthew R. Redinbo[b,c], Vytas A. Bankaitis[ab]

[a]Department of Cell & Developmental Biology [b]Lineberger Comprehensive Cancer Center, [c]Department of Biochemistry & Biophysics, University of North Carolina School of Medicine at Chapel Hill, NC 27599-7090, USA

Sec14p is the major phosphatidylinositol (PtdIns)/phosphatidylcholine (PtdCho) transfer protein in yeast and the founding member of a large eukaryotic protein superfamily. The protein catalyzes the exchange of either PtdIns or PtdCho between membrane bilayers *in vitro* in an energy independent fashion. Despite the recent resolution of a detergent-bound apo structure of Sec14p, it remains unclear how Sec14 and its homologs bind and exchange their various phospholipid substrates. Here we report high resolution crystal structures of yeast Sfh1p, the closest homolog of Sec14p, in complex with phosphatidylcholine and phosphatidylinositol. The structures led us to rationally design Sec14 mutants that either abolish overall phospholipid binding or exhibit specific defects in either PtdIns or PtdCho binding and *in vitro* transfer activity. Functional assays were performed to assess the ability of these mutants to complement defects associated with Sec14 dysfunction. This study provides insights on how Sec14 regulates membrane trafficking at the interface of lipid metabolism and Golgi secretory function and provides a new understanding of life without Sec14.

Fenretinide induces lethal autophagy via a novel ensemble of life and death regulators: dihydroceramide and sphinganine versus sphinganine 1-phosphate.

Katie Fluke, Kacee Sims, Holly Symolon, Wenjing Zheng, Elizabeth Munter, Amin Momin, Carrie Pack, Chris Haynes, Samuel Kelly, Jeremy Allegood, Elaine Wang & Alfred H. Merrill, Jr.

Schools of Biology, Chemistry and Biochemistry, and the Petit Institute for Bioengineering and Bioscience, Georgia Institute of Technology, Atlanta, GA 30322

Fenretinide (4-hydroxyphenylretinamide, 4HPR) is a promising anticancer agent that is being evaluated in human clinical trials. One of the proposed mechanisms of action for fenretinamide is to increase the biosynthesis of ceramide, an inducer of apoptosis. Using LC ESI MS/MS for “sphingolipidomic” analysis of MCF7 cells, we find that fenretinide elevates dihydroceramides instead of ceramides (due to inhibition of dihydroceramide desaturase) and increases the amounts of sphinganine and sphinganine 1-phosphate. In exploring how these metabolites could mediate fenretinide cytotoxicity, we have discovered that dihydroceramides induce autophagy, and whether this is cytotoxic depends on the relative amounts of sphinganine 1-phosphate versus sphinganine--a highly toxic compound due to its effects on multiple signaling pathways as well as being lysosomotrophic. These findings reveal new facets of sphingolipid biology and autophagy, and suggest that the efficacy of fenretinide might be increased by concomitant inhibition of sphingosine kinase. (Acknowledgement: These studies were conducted in collaboration with Dr. Myles Cabot at the John Wayne Cancer Institute. The studies were conducted using funding from the Lipid MAPS Consortium grant GM069338 and NCI U19-CA87525).

Mass Isotopomer Analysis of Stable Isotope-Labeled Palmitoyl-CoA

Christopher A. Haynes, Elaine W. Wang, M. Cameron Sullards, and Alfred H. Merrill, Jr. Schools of Biology, Chemistry & Biochemistry, and the Petit Institute for Bioengineering and Bioscience, Georgia Institute of Technology, Atlanta, GA 30332

Mammalian cells cultured with stable isotope-labeled palmitate (such as [U-^{13}C]-palmitate) take up this fatty acid from the medium and activate it to the CoA thioester (Pal-CoA) that can be utilized for the biosynthesis of various categories of complex lipids directly, or after chain elongation and/or desaturation. By determining the isotope enrichment of the Pal-CoA pool by liquid chromatography-tandem mass spectrometry (LC-MS/MS), the rate of appearance of these stable isotope labeled precursors in downstream metabolites, such as sphingolipids, can be used to estimate metabolic flux. Alternatively, cells can be incubated with stable isotope-labeled acetate (eg., [1-^{13}C]-acetate), which is taken up from the medium, and polymerized by fatty acid synthase into fatty acyl-CoAs that contain a spectrum of mass isotopomers (molecules with identical chemical compositions and structures, but different isotopes of an element at a structural position). Using acetate labeling combined with LC MS/MS analysis of the Pal-CoA isotopomers followed by MIDA (mass isotopomer distribution analysis) or ISA (isotopomer spectral analysis) allows the estimation of two metabolic flux parameters: the dilution of labeled acetate utilized by fatty acid synthase, and the dilution of newly synthesized Pal-CoA. We have used both stable isotope-labeling protocols to measure the biosynthesis of Pal-CoA by RAW264.7 cells for subsequent analysis of Pal-CoA-derived sphingolipids because [U-^{13}C]-palmitate treatment results in either completely unlabeled Pal-CoA (M + 0) or completely labeled Pal-CoA (M + 16), whereas, [1-^{13}C]-acetate treatment results in a distribution of Pal-CoA isotopomers (M + 0 through M + 8) that allows the statistical estimation of: 1) the fraction of labeled acetyl-CoA precursors utilized by fatty acid synthase, and 2) the fraction of newly synthesized (labeled) Pal-CoA. Using 0.1 mM [U-^{13}C]-palmitate (as the BSA complex), 62 mol % of the Pal-CoA pool was stable isotope-labeled after 6 h, as determined by the quantitation of M + 0 and M + 16 Pal-CoA. Using 0.1 mM [1-^{13}C]-acetate, 64 mol % of Pal-CoA was stable isotope-labeled after 6 h, as determined by mass isotopomer distribution analysis (MIDA) of M + 0 through M + 5 (mass isotopomers greater than M + 5 were below the limit of quantitation). The similar labeling of the Pal-CoA pool of RAW264.7 cells despite the source of the stable isotope is somewhat surprising, but the information allows follow-up analysis of the utilization of this precursor for complex lipid biosynthesis. (Supported by NIH Grant GM69338 (Lipid MAPS).)

Session II: The roles of bioactive lipids in cell signaling (focus on sphingolipids)

Inhibitor 2 of Protein Phosphatase 2A (I2PP2A) Directly Binds Ceramide both in vitro and in situ: A Novel Mechanism for Ceramide-mediated Regulation of Nuclear Protein Phosphatase 2A Activity

Archana Mukhopadhyay and Besim Ogretmen

Department of Biochemistry and Molecular Biology, Medical University of South Carolina, 173 Ashley Avenue, SC 29425

Inhibitor 2 of protein phosphatase 2A (I2PP2A) was identified as a ceramide-binding protein both *in vitro* and *in situ* in A549 human lung adenocarcinoma cells. *In vitro* assays indicate that ceramide is able to prevent the inhibitory effect of I2PP2A to both the PP2A trimer and the catalytic subunit of PP2A. However, it was determined that ceramide did not prevent the inhibition of PP2A by I1PP2A, showing a high degree of specificity for I2PP2A. This prevention of the inhibitory effect of I2PP2A to PP2A was not observed toward sphingosine, dihydroceramide, ceramide-1-phosphate or sphingosine-1-phosphate. More importantly, results suggest that I2PP2A also binds endogenous ceramide in A549 cells. It was examined that the association of I2PP2A is specific to C_{18}-ceramide and it does not bind to C_{16}-ceramide generated by sphingosine recycling pathway in A549 cells, after pulsing the cells with biotinylated sphingosine together with either stearoyl CoA or palmitoyl CoA, respectively. Recombinant I2PP2A, expressed in *E. coli*, revealed a strong association with D-erythro-C_6-ceramide *in vitro.* Results displayed that I2PP2A appears to have a higher affinity to D-e- stereoisomers of ceramide. In addition, it was observed that I2PP2A-ceramide interaction is involved in regulation of the promoter of hTERT, the catalytic subunit of telomerase, via the regulation of c-Myc dephosphorylation. The dephosphorylation of c-Myc is involved in the control of its ubiquitination by PP2A in A549 cells. Results suggest that this lipid-protein, ceramide-I2PP2A, interaction may play major biological roles in the regulation of cancer pathogenesis and/or therapy.

Regulation of sphingosine kinase 1 during hypoxia in glioma cells: role of hypoxia inducible factor 2

Viviana V. Anelli, Amy B. Cheng, Christopher R Gault and Lina M. Obeid

Department of Medicine, Division of General Internal Medicine, Medical University of South Carolina, Charleston, SC 29425, USA

Gliomas are the most common primary tumors arising in the central nervous system. The degree of hypoxia within these solid tumors has been reported to correlate with tumor progression and patient survival. One of the primary cellular events evoked upon exposure to hypoxia is the induction of the hypoxia-inducible factor (HIF) family members, HIF-1 and HIF-2. Those proteins play important roles as transcription factors which modulate a battery of more than 40 target genes whose protein products play crucial roles in tumor progression. Both HIF-1 and HIF-2 become stabilized under hypoxic conditions, however HIF-2 is the less well studied of the pair. Therefore, the identification of HIF-2 specific target genes and their downstream functions remain unclear. Importantly, several studies have demonstrated that HIF-2 expression is important for angiogenesis. Recent microarray evidence suggests that HIF-2 may specifically upregulate the enzyme sphingosine kinase 1. Sphingosine kinase 1 is the major enzyme responsible for the formation of sphingosine-1-phosphate, a sphingolipid metabolite that plays an important role in the regulation of cell survival, cell growth, cell migration, and angiogenesis. Here we investigated the role of HIF-2 in the regulation of sphingosine kinase 1 during hypoxic conditions. $CoCl_{2,}$ a well-studied and commonly used hypoxia-mimetic was employed. Incubation with $CoCl_2$ increased sphingosine kinase 1 mRNA levels, protein expression, and SK1 enzyme activity in U87 glioma cells. Coincident with the above changes, increases in intracellular sphingosine-1-phosphate were also observed. Importantly, knock-down of HIF-2 by small interfering RNA abolished the induction of sphingosine kinase 1 and the production of sphingosine-1-phosphate under hypoxic conditions. Therefore, we demonstrate that HIF-2 not only up-regulates expression of sphingosine kinase 1 during hypoxia, but also that increases in intracellular sphingosine-1-phospate also occur. Taken together, we demonstrate that sphingosine-1-phosphate levels are increased in glioma cells under hypoxia which may contribute to tumor progression.

Sphingosine-1-Phosphate Receptor Antagonists and Lymphocyte Trafficking

Ashley H. Snyder‡, Frank W. Foss§, Michael D. Davis‡, Michael Rouse◊, Mark D. Okusa◊, Timothy L. Macdonald§, Kevin R. Lynch‡¶

From the Departments of ‡Biochemistry and Molecular Genetics, ¶Pharmacology, ◊Medicine, and §Chemistry, University of Virginia, Charlottesville, Virginia, 22908.

Sphingosine-1-phosphate (S1P) is a lysophospholipid signaling molecule that regulates numerous cellular processes including proliferation, migration and survival. S1P signals via a set of five G protein-coupled receptors ($S1P_{1\text{-}5}$). S1P signaling was validated as a target of immunomodulatory drugs when the sphingosine analog, FTY720, was found to be metabolized in vivo to a pan-S1P receptor agonist. FTY720 alters lymphocyte trafficking such that lymphocytes accumulate in secondary lymphoid tissues; the index of its action is lymphopenia. We synthesized a series of S1P analogs to use as tools to explore S1P biology. One compound, VPC44116, is a competitive antagonist at $S1P_1$ and $S1P_3$ receptors. Although FTY720-P is thought to be a functional antagonist, administration of the receptor antagonist VPC44116 caused neither lymphopenia nor lymphocytosis. Further, VPC44116 antagonized the lymphopenia evoked by its positional isomer, VPC44152, an $S1P_{1,4,5}$ agonist, and the selective $S1P_1$ agonist SEW2871. Recently, injections of VPC44116 and similar compounds have been shown to cause extravasation of Evans blue dye from circulation into lung and kidney. VPC44116 and follow on compounds will enable further understanding of S1P signaling.

GSH depletion during aging upregulates nSMase2 activity and hepatic response to IL-1beta

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Oxidative stress and upegulated inflammatory response are fundamental for the onset of aging and appear to be causatively linked. In our previous study we showed that hepatocytes from aged (20 mo) rats, in contrast to hepatocytes from young (3 mo) rats, are hyperresponsive to IL-1beta stimulation and exhibit more potent JNK activation and attenuated interleukin receptor-associated kinase–1 (IRAK-1) degradation. We demonstrated that this phenomenon was caused by age-dependent increase in the activity of neutral sphingomyelinase-2 (nSMase2). The results reported here show that the elevation of nSMase activity during aging is caused by 60 to 70 % depletion of GSH levels in aged hepatocytes. We demonstrated that GSH inhibits both the endogenous and the overexpressed nSMase2 activity in a biphasic dose-dependent manner at concentrations that are typical for hepatocytes in young animals, e.g., 5 to 20 microM. Therefore, as GSH levels decline with age, nSMase2 activity may increase. Also, pharmacological inhibition of GSH synthesis in hepatocytes from young animals activated nSMase, and was sufficient to potentiate JNK activation and to stabilize IRAK-1 in response to IL-1beta, thus leading to hyperresponsiveness. Vice versa, elevation of GSH content in hepatocytes from aged animals by treatment with N-acetyl cysteine (NAC) inhibited nSMase activity and restored normal IL-1beta responsiveness. Furthermore, overexpression of nSMase2 in the NAC-supplemented aged hepatocytes rescued the aging phenotype, suggesting that nSMase2 acts downstream of GSH depletion. Importantly, the aging-associated GSH decline, nSMase activation and IL-1beta hyperresponsiveness were found to be significantly attenuated in aged calorie restricted rats, which are known to exhibit delayed aging and extended lifespan. In summary, this study shows that depletion of cellular GSH during aging augments hepatic response to pro-inflammatory stimuli, e.g. IL-1beta, by upregulating nSMase2 activity. (Supported by NIA award RO1 AG019223 (to MNK) and AHA pre-doctoral fellowship (to KR).)

Acid Sphingomyelinase Mediates Cisplatin-Induced Cytoskeletal Remodeling

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Sphingomyelin (SM) hydrolysis is a key pathway during several cellular stress responses including apoptosis, cell cycle arrest and senescence. Sphingomyelinases (SMases) are key enzymes in this pathway regulating conversion of membrane sphingomyelin to the lipid second messenger, ceramide (Cer). Besides its well established cytotoxic effects, other tumor suppressor properties of ceramide are still widely unknown.

In this study, we investigated the role of the SM/Cer pathway in the cytoskeletal changes induced by the DNA-damaging agent cisplatin. Treatment of the breast cancer cell line, MCF-7, with cisplatin induced a transient activation of acidic sphingomyelinase (ASMase) with a concomitant elevation in total Cer levels. In addition, translocation of ASMase from endolysosomes to the plasma membrane (PM) was observed. This translocation process was blocked upon infection with a dominant negative PKCδ adenovirus or transfection of ASMase^{S508A} mutant. Interestingly, cisplatin treatment induced clear morphologic changes including loss of cellular lamellipodia / filopodia and appearance of membrane ruffles. These changes were reproduced upon exogenous delivery of D-e-C16-Cer but not dihydro-C16-Cer. Phalloidin staining revealed that cisplatin and Cer treatment caused loss of filamentous actin, its dissociation from the PM and the appearance of cortical stress fibers. Further investigations showed that Cer generated by cisplatin treatment induced dephosphorylation (inactivation) of the actin binding protein, ezrin. Indeed, immunofluorescence studies showed relocation of ezrin from membrane protrusions such as lamelli/filopodia to the cytosol after cisplatin treatment. Importantly, knockdown of ASMase using specific RNAi sequence protected MCF-7 cells from cisplatin-induced cytoskeletal changes including ezrin dephosphorylation. Taken together, these results highlight a novel tumor suppressor property for Cer and an important role for ASMase in mediating cisplatin-induced cytoskeletal remodeling. On the translational level, our study indicates that modulation of the SM/Cer cycle offers a potential novel pathway for modulating tumor invasion and metastasis.

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$S1P_2$ receptor is essential for inner ear vascular development and hearing

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Sphingosine 1-phosphate (S1P) is a sphingolipid metabolite that triggers diverse signaling responses through five G protein-coupled receptors ($S1P_1$-$S1P_5$). A major physiological role of the S1P signaling system has been shown to be for vascular development and integrity. Here we report that $S1P_2$ receptor is essential for inner ear development and hearing. Auditory brainstem response analysis demonstrated that *$S1P_2$* null mice had profound hearing loss at one month, the age that mice normally establish hearing ability. Histological analysis demonstrated degeneration of sensory hair cells and spiral ganglion neurons in *$S1P_2$* null cochlea. However, the earliest lesion was found within the stria vascularis, a structure that contains the primary vasculature of the cochlea. We conclude that $S1P_2$ signaling is crucial for the proper establishment of the inner ear vasculature and maintenance of the auditory system.

The role of ABCC1 in export of sphingosine-1-phosphate from mast cells

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Mast cells play a pivotal role in inflammatory and immediate-type allergic reactions by secreting a variety of potent inflammatory mediators, including sphingosine-1-phosphate (S1P). However, it is not known how S1P is released from these cells. Here we report that S1P is exported from mast cells independently of degranulation. We demonstrate that S1P secretion is mediated by ATP binding cassette (ABC) transporters. Constitutive and antigen-stimulated S1P release was inhibited by MK571, an inhibitor of ABCC1 (MRP1), but not by inhibitors of ABCB1 (MDR-1, P-glycoprotein). Moreover, downregulation of ABCC1 with small interfering RNA, which decreased its cell surface expression, markedly reduced S1P export from both rat RBL-2H3 and human LAD2 mast cells. Transport of S1P by ABCC1 influenced migration of mast cells toward antigen but not degranulation or calcium mobilization. These findings have important implications for S1P functions in mast cell-mediated immune responses.

EGF Regulates Plasminogen Activator Inhibitor-1 by a Novel Pathway Involving c-Src, PKCδ, and Sphingosine Kinase 1 in Glioblastoma Cells

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Patients with gliomas expressing high levels of Epidermal Growth Factor Receptor (EGFR) and Plasminogen Activator Inhibitor-1 (PAI-1) have a shorter overall survival prognosis. Moreover, EGF enhances PAI-1 expression in glioma cells. Although multiple known signaling cascades are activated by EGF in glioma cells, we show for the first time that EGF enhances expression of PAI-1 via sequential activation of c-Src, protein kinase C delta (PKCδ), and sphingosine kinase 1 (SphK1), the enzyme that produces sphingosine-1-phosphate. EGF induced rapid phosphorylation of c-Src and PKCδ and concomitant translocation of PKCδ as well as SphK1 to the plasma membrane. Downregulation of PKCδ abolished EGF-induced SphK1 translocation and upregulation of PAI-1 by EGF; whereas, downregulation of PKCα had no effect on the EGF-induced PAI-1 activation, but enhanced its basal expression. Similarly, inhibition of c-Src activity by PP2 blocked both EGF-induced translocation of SphK1 to the plasma membrane and upregulation of PAI-1 expression. Furthermore, SphK1 was indispensable for both EGF-induced c-Jun phosphorylation and PAI-1 expression. Collectively, our results provide a functional link between three critical downstream targets of EGF, c-Src, PKCδ, and SphK1 that have all been implicated in regulating motility and invasion of glioma cells.

Opposing Functions of LASS1 and LASS6 Generated C18- and C16-Ceramides in the Regulation of Caspase Dependent Apoptosis in Head and Neck Squamous Cell Carcinomas

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Previously we have shown that human head and neck squamous cell carcinoma (HNSCC) tumor tissues have decreased $C_{18:0}$-ceramide and increased $C_{16:0}$-ceramide contents compared to their adjacent normal tissues (J. Biol Chem. 279:44311-44319, 2004). These data suggested a novel hypothesis that C18-ceramide and C16-ceramide might play distinct and opposing roles in the regulation of apoptosis in HNSCC cells. In fact, reconstitution of $C_{18:0}$-ceramide levels by overexpression of longevity assurance gene homolog 1 (LASS1), which is the ceramide synthase for the specific generation of $C_{18:0}$-ceramide, induced apoptosis in HNSCC cell lines, and enhanced chemotherapy-mediated activation of caspases. On the other hand, down-regulation of LASS6, which mainly generates $C_{16:0}$-ceramide, by siRNA in UM-SCC-22A (SCC of hypopharynx) cells inhibited cell growth (about 50%), which mechanistically involves mitochondrial membrane potential collapse, juxta-nuclear re-positioning of mitochondria, and activation of upstream caspases -8, and -9, and effector caspase-3. As expected, siRNA mediated knock down of LASS1, caused hyperpolarization of the mitochondria, and inhibited basal and chemotherapy induced caspase activation. These results suggest that two different ceramide species have opposing roles in the regulation of apoptosis in HNSCC, such that, LASS1/$C_{18:0}$-ceramide is pro-apoptotic, whereas LASS6/$C_{16:0}$-ceramide might have a pro-survival role in HNSCC.

Session III: Lipid Biosynthesis/ Metabolism, Enzymology, and Genomics

Group VIA calcium-independent phospholipase A_2 as a novel regulator of sterol regulatory element-binding proteins

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Disruption of lipid homeostasis is central to the progression of atherosclerosis, diabetes, fatty liver disease and other disorders. Sterol regulatory element-binding proteins (SREBP) constitute a crucial family of transcription factors that globally regulate lipid metabolism by activating the transcription of genes involved in lipid synthesis. SREBP undergo posttranslational processing from an inactive precursor to an active mature form. Exogenous unsaturated fatty acids (UFA) suppress SREBP processing and expression and hence SREBP-mediated gene expression. To date, an endogenous source of UFA capable of modulating SREBP remains elusive. Group VIA calcium-independent phospholipase A_2 ($iPLA_2$) releases UFA from the *sn*-2 position of glycerophospholipids and is localized to the same compartment as precursor SREBP. Based on these observations, it is hypothesized that $iPLA_2$ provides endogenous UFA that suppressSREBP-mediated transcription. To test this hypothesis, siRNA and chemical inhibitors were used to suppress $iPLA_2$ (reduce endogenous UFA) and plasmid and adenovirus strategies were used to overexpress $iPLA_2$ (increase endogenous UFA) in CHO and HepG2 hepatoma cells. $iPLA_2$ inhibition increased both SREBP expression and SREBP-mediated transcription. In contrast, $iPLA_2$ overexpression attenuated SREBP expression and SREBP-mediated transcription. Importantly, the decline in SREBP correlated with reduced expression of SREBP target genes (fatty acid synthase and acetyl CoA carboxylase) and attenuated overall fatty acid synthesis in HepG2 hepatoma cells, a model relevant to lipid metabolism. These data support the hypothesis that $iPLA_2$ generates endogenous UFA that limit the functioning of SREBP and further suggest that this enzyme may hold therapeutic promise to control lipid disorders.

Arabidopsis PIPK1 Is Involved In a Scaffold with F-actin And Recruits PI4Kβ1 to the Actin Cytoskeleton

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Phosphatidylinositol phosphate 5-kinases (PIPK) are a family of enzymes that phosphorylate phosphatidylinositol-4-phosphate (PtdIns4P) to form phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P_2). Nine of the 11 Arabidopsis isoforms contain Membrane Occupation and Recognition Nexus (MORN) repeats, including *At*PIPK1. The MORN repeats are unique to this family of plant lipid kinases as they are not found in lipid kinases from any other system.

*At*PIPK1 was found to interact directly with both G- and F-actin in vitro, and plant PtdInsP kinase activity was found associated with an F-actin fraction polymerized from plasma membranes. In contrast, when the human PIPK1α was expressed in tobacco cells it associated with the plasma membrane but was not recovered with the F-actin fraction polymerized from the plasma membrane.

In further experiments, *At*PIPK1 was found to interact with bundled F-actin, suggesting that the lipid kinase binds on the outside of the filaments and is not non-specifically trapped as the filaments form. *At*PI4Kβ1, which synthesizes PtdIns4P, was found with the F-actin fraction only when *At*PIPK1 was present indicating that *At*PIPK1 can recruit *At*PI4Kβ1 to the cytoskeleton. The MORN domain was neither necessary nor sufficient for interaction with actin or *At*PI4Kβ1 but interacted directly with *At*CDC48, a protein involved in proteosome targeting and vesicle fusion. These results describe a dynamic process where *At*PI4Kβ1 could be recruited to F-actin by *At*PIPK1 thereby providing a mechanism for phosphoinoside production on F-actin by supplying PtdIns4P for the production of PtdIns(4,5)P_2. The interaction of the MORN domain with *At*CDC48 demonstrates a potential link between the inositol lipids and actin-mediated vesicle trafficking where the PtdIns(4,5)P_2 produced on F-actin is targeted to sites of vesicle fusion.

A new pathway for NBD-phosphatidylserine flip in yeast

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The regulation of phosphatidylserine (PS) distribution across the plasma membrane of eukaryotic cells has been implicated in numerous cell functions (e.g., apoptosis and coagulation). Given the biological significance of PS movement across the plasma membrane, it is important to understand the transport mechanisms involved.

A recent study identified two members of a subfamily of P-type ATPases, Dnf1p and Dnf2p, that are necessary for normal, inward-directed transport (flip) of fluorescent (NBD) tagged phospholipids in yeast. In this report, double deletion of *DNF1* and *DNF2* was shown to inhibit both NBD-PC and NBD-PS flip. Although results from our laboratory confirm the inhibition of NBD-PC in the *Δdnf1Δdnf2* strain, we found contradictory results for the flip of NBD-PS. Rather than being inhibited, NBD-PS flip was increased ~2 fold in the *Δdnf1Δdnf2* strain relative to its isogenic parent. This observation suggests that the mechanism for NBD-PS flip differs from that of NBD-PC. To further support this hypothesis, we found, in wild type cells, that NBD-PC flip was down-regulated as a function of increasing culture density, whereas NBD-PS flip was unaffected. In addition, the up-regulated flip of NBD-PS observed in the *Δdnf1Δdnf2* strain was inhibited with increasing culture density, suggesting a second mechanism of NBD-PS flip that is sensitive to the growth phases of the cell. The combined data argue that NBD-PS flips by a different mechanism than NBD-PC in wild type cells and, that a second NBD-PS flip mechanism exists, which is upregulated in the absence of Dnf1p and Dnf2p. (*This work was supported by National Institutes of Health Grant GM064770 and a grant from the University Research Fund.)

Suppression of drs28710; Cold-Sensitive Growth and Ergosterol Localization Defects by kes18710;

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Drs2p is an integral membrane type 4 P-type (P4) ATPase required for a phospholipid translocase activity in the trans-Golgi network (TGN) of Saccharomyces cerevisiae. The ATPase activity of Drs2p, and presumably its flippase activity, is required for clathrin-dependent protein transport from the TGN to the cell surface and between the TGN and early endosomes. Disruption of DRS2 yields a viable strain that grows well at 24C or above but fails to grow at 20C or below. Growth of drs2; at 30C requires the presence of three closely related P4 ATPases (Dnf1p, Dnf2p and Dnf3p); however, Drs2p provides an essential function at low temperatures that cannot be replaced by the Dnf ATPases. To better understand this essential function of Drs2p at low temperatures, we have screened for spontaneous bypass suppressors of the drs2; cold-sensitive (cs) growth defect. These suppressors fell into two complementation groups called sdk1 and sdk2 for suppressor of drs2 knockout. SDK1 was cloned and found to be allelic to KES1/OSH4, encoding an oxysterol binding protein previously identified as a suppressor of sec14. kes1; suppresses drs2; cs growth defects but cannot by-pass the essential function of the DRS2/DNF gene family, nor does it suppress dnf mutant phenotypes. Surprisingly, kes1Δ does not suppress drs2; protein trafficking defects tested thus far. However, drs2; cells show altered ergosterol localization indicated by increased filipin staining of intracellular membranes with a decrease in plasma membrane staining, and this phenotype is partially suppressed by kes1;. kes1; also suppresses the nystatin hypersensitivity of drs2, suggesting restoration of an ergosterol organization in the plasma membrane that resists nystatin binding. These results suggest that the extreme cs growth defect of drs2; is caused by a defect in ergosterol trafficking that is induced by excessive Kes1p activity.

Examining the puzzling synthetic interaction between the major yeast phosphoinositide transfer protein Sec14p and Tlg2p

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Phosphatidylinositol transfer proteins (PITPs) selectively bind and transfer phospholipids between membrane bilayers. Cumulative evidence favors a critical, yet undefined role for PITPs in regulating the coupling of phospholipid metabolic reactions with lipid-signaling and vesicle trafficking events. Indeed, the major PITP in *Saccharomyces cerevisiae*, Sec14p, is required for the biogenesis of *trans*-Golgi network (TGN)-derived secretory vesicles. To better understand how Sec14p stimulates vesicular biogenesis, we took advantage of a synthetic gene array (SGA) analysis to identify non-essential genes that have a synthetic interaction with *sec14-1^{ts}* on a genomic scale. The most unanticipated, yet validated interaction involving *sec14-1^{ts}* suggests a functional linkage between Sec14p and the TGN/endosomal t-SNARE Tlg2p. Currently, we are interpreting the implications of the Sec14p/ Tlg2p interaction on Sec14p cellular function. We initially hypothesized that Sec14p and Tlg2p function in the recycling of trafficking machinery components from endosomes to the TGN. Surprisingly, pulse-chase data show a striking block in the modification of secretory proteins in *tlg2Δ sec14^{ts}* and prominent p1CPY levels, which suggests an early block in the secretory pathway. The early secretory block in the double mutant was further justified by the discovery of exaggerated endoplasmic reticulum in electron microscopy images. Collectively, these results support the possibility that Sec14p and Tlg2p may have previously unidentified functions in membrane transport into the Golgi as well as transport from the TGN.

The rate of sphingolipid biosynthesis in liver affects utilization of palmitic acid for TAG synthesis

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Triacylglycerols (TAG) are the main store of excess energy within the cell. TAG synthesis is metabolically linked to that of phospholipids at the level of DAG, which is a precursor for both. Previous studies have shown that increased demand for phosphatidylcholine (PC) in the cells can inhibit the utilization of DAG in TAG synthesis efficiently decreasing the levels of TAG. This study provides evidence that the rate of sphingolipid biosynthesis also influences the synthesis and accumulation of TAG in liver.

We have found (see abstract by Rozenova et al.) that Acid Sphingomyelinase (ASMase) deficient mice placed on a high fat (Western) diet for 10 weeks are resistant to obesity and, in contrast to ASMase (+/+) mice, do not accumulate fat in adipose and non-adipose tissues. However, ASMase(-/-) mice seem to accumulate SM, dihydroceramide, ceramide and sphinganine, as well as some phospholipids like PC, phosphatidylserine (PS), and phosphatidylethanolamine in the liver, and show an increased liver weight.

The high fat diet also significantly increased the activity of serine palmitoyltransferase in liver microsomes, suggesting that elevated sphingolipid content in the liver could be due to stimulation of de novo synthesis in ASMase-deficient mice, but not in control mice. To test this, we have used an *in vitro* model, HepG2 cells treated with desipramine, an inhibitor of ASMase, and supplemented with palmitic acid, the major saturated fatty acid in the high fat diet. Studies with radiolabeled L-serine showed that the addition of palmitic acid stimulated *de novo* sphingo- and phospholipid synthesis at significantly higher extent in cells with inhibited ASMase activity as compared to control cells. These differences were Fumonisin B1 inhibitable, supporting that the increases in ceramide were due to increases in acylation of free sphingoid bases rather than to changes in SM turnover. Importantly, the increased incorporation of palmitic acid into TAG and the elevation of TAG mass observed upon supplementation with 1 mM palmitic acid were attenuated in desipramine-treated cells, confirming that the stimulation of de novo synthesis of sphingolipids by palmitate was paralleled by decreased utilization of the fatty acid for TAG synthesis. Taken together, these data suggest that (i) ASMase activity negatively regulates the rate of sphingolipid biosynthesis, and (ii) increased sphingolipid biosynthesis may influence TAG synthesis and storage in the liver. (This work was supported by the National Institute of Aging grant RO1 AG019223)

Resistance to Dietary-Induced Obesity in Acid Sphingomyelinase Deficient Mice

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Obesity is one of the most prevalent public health problems in the United States, and a well-recognized risk for many chronic disease including cardiovascular diseases and type II diabetes. Here we report that mice deficient in acid sphingomyelinase do not accumulate body fat when fed a diet with an elevated content of saturated fats and cholesterol (Western diet).

Two month old ASMase(-/-)LDLR(-/-) mice and their litter-matched controls, ASMase(+/+)LDLR(-/-), were placed on either a Western or chow diet for 10 weeks. As expected, ASMase(+/+)LDLR(-/-) mice on Western diet showed a progressive increase in body weight which was accompanied by the accumulation of fat in adipose and non-adipose tissues. In contrast, ASMase(-/-)LDLR(-/-) mice remained lean, and histological analyses of the fat pads showed no increase in adipocyte size, nor accumulation of TAG in the liver. Consistent with these findings was the striking difference in the serum level of leptin, a hormone secreted by the adipose tissue, that increased substantially in ASMase(+/+)LDLR(-/-) mice on Western diet but not in ASMase(-/-)LDLR(-/-) mice. Measurement of food intake using metabolic cages showed no differences in the food and water intake ruling out the possibility that the differential body weight gain was due to abnormal eating behavior. This was further confirmed by analyses of the lipoprotein profile, which showed similar increases in VLDL and LDL cholesterol in all mice fed the high fat diet regardless of their genotype.

Despite the lack of triacylglycerol accumulation in the liver, there was a substantial increase in liver size in ASMase(-/-)LDLR(-/-) mice on a high fat diet as compared to ASMase(+/+)LDLR(-/-). Apparently, this was due to specific increases in the levels of the major phospholipid classes, namely sphingomyelin, phosphatidylcholine, phosphatidylserine and phosphatidylethanolamine. This data suggests that the lack of acid sphingomyelinase perhaps causes a shift in AcylCoA utilization from TAG to phospholipid synthesis. (This work was supported by the National Institute of Aging grant RO1 AG019223.)

Session IV: The roles of bioactive lipids in cell signaling (focus on glycerolipids)

Age-dependent heart failure in mice with combined deficiency of LPA1 and 2

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Lysophosphatidic acid (LPA) is a bioactive lipid molecule in serum that acts, at least in part, by binding to four G protein coupled receptors, termed LPA 1 - 4. Relatively little is known about LPA effects on the heart. In this study, we investigated the role of two LPA signaling pathways in cardiac function heart by use of LPA1/2 double knock out (DKO) mice. By echocardiography, systolic left ventricular function in the LPA1/2 DKO mice declined with age [7% ±0.7 fractional shortening (FS) at age 50-57 wk], but not in either wild-type (28% ±5.8 FS at 54-57 wk), LPA1 (27% ±5.1 FS at 50-61 wk), or LPA2 (26% ±3.3 FS at 56-61 wk) single knock out mice. The first manifestation of heart failure appeared to be enlargement of the right ventricle in the LPA1/2 DKO. Direct pressure measurements indicated higher systolic right ventricular pressures in aged LPA1/2 DKO mice (68 ±14.1 mmHg vs. 24 ±5.9 mmHg in wild-type), while the systolic blood pressure (130 ±23 mmHg) and heart rate (629 ±53 bpm) in the DKO mice were not different from wild-type (130 ±20 mmHg and 612 ±37 bpm). Examination of the lung failed to reveal extensive pulmonary vascular remodeling, although calcification and airway hemorrhage were observed. Thus, deficiency of LPA receptors 1 and 2 results in right ventricular hypertrophy and age-dependent left ventricular failure, suggesting a role for these receptors in normal heart physiology.

Regulation of a phospholipid flippase activity by phosphoinositides

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Phospholipid translocases (PLTs) flip fluorescent or spin-labeled derivatives of phospholipids from the exoplasmic leaflet of a membrane bilayer to the cytosolic leaflet, and are thought to establish asymmetry of endogenous phospholipid in biological membranes. The best candidates for PLTs are the Drs2p/ATPase II (ATP8A1) subfamily of P-type ATPases (P4 ATPases). Drs2p from budding yeast localizes to the trans-Golgi network (TGN) and is required for a flippase activity measured with purified TGN membranes using a 7-nitro-2-1,3-benzoxadiazol-4-yl (NBD) phosphatidylserine (PS) substrate (Natarajan et al., 2004, PNAS 101: 10614-10619). Drs2p is required for the formation of one of the two classes of exocytic vesicles targeted to the plasma membrane (dense vesicles) and this pathway also requires Pik1p, a phosphatidylinositol 4-kinase localized to the TGN. In addition, pik1ts is synthetically lethal with drs2, suggesting a functional relationship between Pik1p and Drs2p. Critical effectors of phosphatidylinositol 4-phosphate (PI4P) in the yeast system are unclear and so we tested if this lipid regulates Drs2p activity. Addition of ATP to purified TGN membranes induced production of both PI4P and PI3P and stimulated the Drs2p-dependent NBD-PS flippase activity in these membranes. Further addition of the Sac1p (phosphoinositide phosphatase) domain of Inp53p to destroy phosphoinositides markedly reduced Drs2p flippase activity. In contrast, wortmannin, a PI 3-kinase inhibitor, had no effect. Moreover, Drs2p activity is substantially reduced in TGN membranes from pik1ts incubated at the nonpermissive temperature (37°), but is unaffected by vps34ts (PI 3-kinase). These results indicate that PI4P stimulates Drs2p-dependent NBD-PS translocase activity in TGN membranes. Therefore, we propose that Drs2p is a critical effector of PI4P at the yeast TGN.

Regulation of PAFAH Expression by PAF and Oxidized Lipids

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Supported by DE13102 and DE15980 from NIH/NIDCR

Platelet Activating Factor Acetylhydrolase (PAFAH) is a monocyte-derived phospholipase A_2 (PLA_2) that associates with low density lipoproteins (LDL). PAFAH catabolyzes both platelet-activating factor (PAF) and oxidized phospholipids (ox-PL) such as those found in oxidized LDL (oxLDL). PAFAH is implicated in atherosclerosis although there is debate about whether this enzyme is pro- or anti-atherogenic. Both pro-inflammatory PAF and pro-atherogenic oxLDL accumulate in atherosclerotic lesions. We hypothesized that these lipid mediators would regulate PAFAH expression in monocytes. To test this hypothesis, primary monocytes were treated with oxLDL, ox-PL or PAF. Real time PCR was used to measure effects on PAFAH mRNA, effects on PAFAH transcription were quantified with a luciferase reporter assay, and a radiometric assay was used to measure PAFAH activity. In contrast to previous reports in transfected human embryonic kidney cells, PAF suppressed PAFAH transcription and expression approximately 2-fold in monocytes, a more physiologically relevant model. Conversely, both oxLDL and ox-PL induced PAFAH expression by more than 3-fold. These data suggest that although PAF and oxLDL both regulate PAFAH expression, the lipid mediators act through distinct receptors/ signaling mechanisms. Importantly, effects of PAF and oxLDL are only observed in monocytes with low levels of endogenous PAFAH activity, suggesting that PAF-like lipids are essential for both responses. Our current efforts are focused upon identifying receptors, signaling pathways, and transcriptional mechanisms by which PAF and oxLDL regulate PAFAH expression.

The role of LPA production in the survival signaling of $SKOV_3$ ovarian cancer cells.

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$SKOV_3$ ovarian cancer cells constitutively produce lysophosphatidic acid (LPA) which may act as a growth and survival factor for ovarian cancer cells both *in vitro* and *in vivo*. We found that the $SKOV_3$ cells were capable of growth in serum free medium and that this growth was blocked by the LPA receptor antagonist VPC32183. In contrast, VPC32183 did not affect $SKOV_3$ growth in serum containing medium. These data indicate that LPA is an endogenously produced growth factor for $SKOV_3$ cells. Further, we found that $SKOV_3$ cells had constitutively activated Akt, ERK, and NF-κB signaling pathways. VPC32183 blocked Akt and NF-κB responses indicating that LPA stimulates multiple pathways of signaling. Recently, reactive oxygen species (ROS) generated by NADPH oxidase have been implicated in proliferative signaling pathways. Diphenyleneiodinium chloride (DPI) is an inhibitor of NADPH oxidase and other flavin containing enzymes. We find that DPI blocks LPA mediated activation of Akt and ERK in $SKOV_3$ cells. Thus, ROS are implicated in the signaling pathway in response to LPA.

Statins Induce Endosomal Sequestration of LPA_1 Lysophosphatidic Acid Receptors

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Lysophosphatidic acid (LPA) is a multi-functional lipid growth factor that promotes angiogenesis, neurogenesis, atherosclerosis, and cancer progression by activating one of five closely-related G protein-coupled receptors (GPCRs) termed LPA_1, LPA_2, LPA_3, LPA_4, and LPA_5. Here we investigated the effects of HMG-CoA reductase inhibitors (statins) on the regulation of the ubiquitously-expressed LPA_1 receptor. Statins are the number one selling class of prescription drugs and have traditionally been used to lower blood cholesterol levels but are gaining increasing attention as effective anti-cancer agents. Our data indicate that statins inhibit basal and LPA-stimulated growth of HeLa cells expressing transfected LPA_1 and PC-3 prostate cancer cells, which express endogenous LPA_1. Interestingly, we observed that statins induce the redistribution of LPA_1, which is normally present on the surface of unstimulated cells, into intracellular, transferrin positive, recycling endosomes in a time and dose-dependent manner. This led to a significant reduction in the amount of surface LPA_1 and decreased the kinetics of LPA_1-induced phosphoinositide hydrolysis. This reduction of surface LPA_1 is likely to contribute to the anti-proliferative effects of statins by perturbing normal LPA_1 function.

Induction of Cox-2 Expression by Lysophosphatidic Acid in Ovarian Carcinoma Cells Involves a Permissive Signaling Input from a Receptor Tyrosine Kinase and Activation of Histone Deacetylases

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Cyclooxygenase-2 (Cox-2) is a key enzyme in the biosynthesis of prostaglandin (PGE) and thus functions as a critical mediator of inflammation. In addition to this well-established role, Cox-2 is implicated in the pathogenesis of human malignancies including colon, breast and skin cancers. The role of Cox-2 and the mechanism for its regulation in ovarian cancer are poorly understood. In the current study, we demonstrated that lysophosphatidic acid (LPA), a previously identified lipid mediator of ovarian cancer, induced expression of Cox-2 in ovarian cancer cell lines. Treatment of cells with LPA resulted in a rapid and robust accumulation of PGE_2 in culture supernatants, indicating that LPA-induced Cox-2 expression leads to PGE_2 synthesis and release. Although epidermal growth factor (EGF) itself was not able to induce Cox-2 expression, LPA-stimulated Cox-2 expression required the activity of EGF receptor, suggesting a permissive role for a receptor tyrosine kinase in LPA-mediated Cox-2 induction. Interestingly, when EGF receptor was suppressed with AG1478, LPA was fully capable of stimulating Cox-2 expression if hepatocyte growth factor (HGF), an agonist of another receptor tyrosine kinase was present. Independent of the input from receptor tyrosine kinase, LPA activated histone deacetylases (HDAC), leading to global deacetylation of histones and other cellular proteins in ovarian cancer cells. Inhibition of HDAC with pharmacological inhibitors such as sodium butyrate prevented Cox-2 upregulation in response to LPA. These results identified two distinct signaling processes that converge to up-regulate Cox-2 expression in response to LPA.

Inhibition of calcium–independent phospholipase A_2 suppresses proliferation, survival and tumorigenicity of ovarian carcinoma cells

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Phospholipase A_2 (PLA_2) enzymes play critical roles in membrane phospholipid homeostasis and in generation of lysophospholipid growth factors. In the current study, we show that the activity of the calcium-independent cytosolic PLA_2 (iPLA2), but not that of the calcium-dependent cytosolic PLA_2 (cPLA2) or the secreted PLA_2 (sPLA2) is required for growth factor-independent, autonomous replication of ovarian carcinoma cells. Cell cycle analysis reveals that blocking $iPLA_2$ activity with the pharmacological inhibitor bromoenol lactone (BEL) induces cell cycle arrest in S and G2/M phases independently of the status of the p53 tumor suppressor. BEL treatment also leads to weak to modest increases in apoptosis. The S- and G2/M-phase accumulation is accompanied by increased levels of the cell cycle regulators cyclin A, B and E. Interestingly, the S phase arrest is released by supplementing growth factors such as lysophosphatidic acid (LPA) or epidermal growth factor (EGF). However, inhibition of $iPLA_2$ activity with BEL remains effective in repressing growth factor- or serum-stimulated proliferation of ovarian cancer cells through G2/M phase arrest. Downregulation of $iPLA_2$ expression with lentivirus-mediated RNA interference inhibited cell proliferation in culture and tumorigenicity of ovarian cancer cell lines in nude mice. These results indicate an essential role for $iPLA_2$ in cell cycle progression, survival and tumorigenesis of ovarian carcinoma cells.

Posters:

Poster # 1

LIPIDOMICS CORE

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The diversity of bioactive lipids and their interconnected metabolism provides a network of pathways regulating intra- and inter-cellular signaling and function. Dysfunctions in these pathways contribute to the pathobiology of specific diseases such as cancer progression and metastasis, accelerated aging, inflammation, and fungal pathogenesis. This emphasized needs for developing lipid chemistry and analysis.

The Lipidomics Core was created based on unique expertise of the key personnel in lipid chemistry, analysis and biology and has evolved into an institutional resource that serves the needs of the research community in the field of Sphingolipids.

The core provides conceptual and practical training in various aspects of lipidology, qualitative and quantitative analysis of lipid components from different biological materials (cells, tissue, biological fluids), synthetic molecular tools to study lipid metabolism (functionalized and fluorescent ceramides, site-specific radioactive sphingolipids), diversified synthetic lipids and analogs for cellular, *in vitro*, and *in vivo* studies (organelle-targeting sphingolipids and organelle-targeting inhibitors of sphingolipid metabolizing enzymes), and assists investigators in experimental design, selection of lipid of interest and interpretation of the analytical results.

Analytical approach is based on High Performance Liquid Chromatography-Tandem Mass Spectrometry (LC-MS) technology. Currently, we provide simultaneous analysis of sphingoid bases and their phosphates, ceramide species and their phosphates, sphingomyelin species and diacyl-glycerol species. Our goal is to reach metabolomic profile of lipids.

The core has been instrumental to the success of the COBRE, two Program Project Grants and the award of a competitive shared instrument grant from NIH, which supported the second MS instrument. The core is engaged in several collaborative projects and provides also a paid fee-for-service.

Poster # 2

Non-Aqueous Reverse Phase HPLC with Tandem MS for the Acquisition of Lipid Profiles

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Profiling the endogenous metabolites present in the tissues or secretions of test subjects is an increasingly important complement to more traditional drug metabolism, genetics, proteomics and protein biomarker research. Disease symptoms, metabolic responses to xenobiotics and genetic changes all alter metabolite profiles. These differences in the molecular makeup of organisms are the primary response that produces any phenotype that we may wish to study.

Ideally, profiling the metabolome involves obtaining the relative concentration of every molecule found in the test subjects. Reaching this ideal may be impractical. A more workable compromise would be to extract an interesting subset of the metabolome using minimal sample handling. Then, to utilize an analytical methodology that differentiates most of the molecular species present while measuring their relative concentrations in a reproducible way.

Lipids are an interesting subset of the metabolome that can be extracted fairly easily from tissues and fluids. Lipids determine the structure and function of many types of membranes within organisms. Also, the richly inter-related metabolic pathways of the various lipid types influence a vast number of life's regulatory functions.

We present a reverse phase HPLC-MS method which differentiates and determines a quantitative response for a vast range of lipid molecules. The method uses common C18 columns and well known methanol and ethyl acetate mobile phases. A single injection shows individual signals for lipids ranging from very polar precursors, glyco- and phosphor- lipids out to very non-polar ceramides and triacylglycerides. These standardized data sets are converted to peak tables which are compared using statistics based procedures which identify differences in the observed lipidome. Utilization of modern tandem MS instrumentation allows the automatic acquisition of MS/MS structural information to help identify those lipid species which are shown to be of interest by experimental design and statistical analysis.

Poster # 3

Development and characterization of a novel anti-ceramide antibody

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Ceramide is emerging as a key sphingolipid that regulates a variety of cellular processes that include differentiation, apoptosis and stress response. To facilitate the study of ceramide localization and its interaction with other cellular proteins we have developed a novel antibody against ceramide. Our results indicate that the antibody (rabbit IgG fraction) specifically recognizes ceramide in a lipid overlay assay, when tested against a variety of sphingolipids and sphingolipid derivatives. The rabbit IgG fraction recognizes ceramide with different fatty acid chain lengths that include C2, C16, C18, C20 and C24 ceramide. The newly developed antibody was compared with the commercially available anti-ceramide antibody (polyclonal mouse IgM) in immunocytochemistry experiments to study the localization of ceramide in F11 neuroblastoma cells. While both antibodies stain the same regions on the membrane, the rabbit IgG staining reveals the distribution of ceramide in greater detail. Incubation of the cells with myriocin (an inhibitor of *de novo* ceramide biosynthesis) reduces the staining intensity, while incubation with NB-dNJ (an inhibitor of glucosylceramide synthase) or with bacterial sphingomyelinase increases the signal, thus verifying the specificity of the antibody. In addition to staining of ceramide in protrusions of the plasma membrane, the rabbit IgG also stains a peri-nuclear compartment, identified as the Golgi apparatus. This is consistent with the intracellular site of glycosphingolipid biosynthesis, utilizing ceramide as the substrate. These results indicate that the rabbit IgG is a suitable antibody to determine localization of ceramide and its interaction with other proteins by immunocytochemistry.

Poster # 4

Crystal structures of a close Sec14 homolog, Sfh1p, bound to Phosphatidylinositol and Phosphatidylcholine Reveal the Structural Basis for Sec14 – Phospholipid Recognition

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Sec14p is the major phosphatidylinositol (PtdIns)/phosphatidylcholine (PtdCho) transfer protein in yeast and the founding member of a large eukaryotic protein superfamily containing over 550 members. The protein catalyzes the exchange of either PtdIns or PtdCho between membrane bilayers *in vitro* in an energy independent fashion and is critical for cell viability. The structure of a detergent-bound apo structure of Sec14p was reported; however, it remains unclear how Sec14 and its homologs bind and exchange their various phospholipid substrates. Here we report the X-ray crystal structures of yeast Sfh1p, the closest homolog of Sec14p, in complex with phosphatidyethanolamine, phosphatidylcholine and phosphatidylinositol at 1.9 Å, 1.8 Å and 2.0 Å resolution respectively. Unexpectedly, these phospholipids bind deep within core of the protein, which accompanied by a ~20 Å conformational shift relative to the apo-Sec14 structure, shields the bound lipids from solvent. These structures facilitated the rational design of Sec14 mutants that either abolish overall phospholipid binding or exhibit specific defects in either PtdIns or PtdCho binding and *in vitro* transfer activity allowing us to further trace out the unique roles of both PtdIns and PtdCho bound Sec14. This study provides insights on how Sec14 regulates membrane trafficking at the interphase of lipid metabolism and Golgi secretory function.

Poster # 5

Associative and Predictive Biomarkers of Changing Cognitive Status in HIV-1 Infected Patients

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Infection with HIV can result in a debilitating CNS disorder known as HIV-associated cognitive-motor impairment or HIV-dementia (HIV-D). Since the advent of highly active antiretroviral therapy (HAART), the incidence of HIV-D has declined but the prevalence continues to increase. In general, the cognitive manifestations of HIV-D are less severe and the course of HIV-D appears to be more variable. Traditional biomarkers such as CSF viral load and MCP-1 levels are less likely to be associated with dementia in patients on HAART, and other biomarkers that can predict HIV-D have not yet been identified. In order to identify associative and predictive biomarkers of HIV-D we differentially grouped HIV-infected patients from the North Eastern AIDS Dementia (NEAD) cohort based on changes in cognitive status over a 1 year time period and analyzed sphingolipid, sterol, triglyceride, antioxidant and lipid peroxidation levels in CSF. We found that increases of the antioxidant vitamin E and triglyceride C52 predicted the onset or worsening of dementia while the catabolism of sphingomyelin to ceramide and the accumulation of 4-hydroxynonenals were associated with an actively progressing dementia. We interpret these findings to indicate that early in the pathogenesis of HIV-D there is an up-regulation of endogenous antioxidant defenses in brain. The failure of this attempted neuroprotective mechanism leads to the accumulation of sphingomyelin and moderate neuronal dysfunction that manifests as an inactive dementia. The breakdown of this enlarged pool of sphingomyelin to ceramide and the accumulation of highly reactive aldehydes is associated with neuronal degeneration and an active dementia. Thus, increased levels of endogenous antioxidants in CSF may identify HIV-infected patients who are at higher risk for the development or progression of dementia.

Poster # 6

p38 MAPK regulates neutral sphingomyelinase 2 translocation to the plasma membrane in TNF-α-stimulated A549 cells

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Neutral sphingomyelinases (N-SMases) are major candidates for stress-induced ceramide production but there is little information on the physiological regulation of the cloned N-SMase enzyme – nSMase2. Using a V5-tagged nSMase2 construct and confocal microscopy, the role of MAPKs in regulating TNF-stimulated nSMase2 translocation in A549 epithelial cells was investigated. In unstimulated A549 cells, V5-nSMase2 localised predominantly to the Golgi and was found to translocate acutely to the plasma membrane in response to TNF-α in a time and dose-dependent manner. The earliest translocation was detected by 10 min and V5-nSMase2 was found at the plasma membrane for up to 12 hours. This translocation was prevented by pharmacological inhibition of p38 MAPK, but not ERK or JNK and this was further confirmed by use of siRNA specific to p38-α MAPK. In addition, anisomycin, previously shown to strongly activate p38 MAPK in epithelial cells also induced nSMase2 translocation to the plasma membrane. Finally, nSMase2 did not co-immunoprecipitate with p38 MAPK in basal or stimulated conditions suggesting that p38 MAPK does not regulate nSMase2 translocation by direct interaction. In summary, these data reveal a role for p38 MAPK as an upstream regulator of nSMase2 translocation to the plasma membrane in TNF-α-stimulated A549 cells.

Poster # 7

Direct redox regulation of sphingolipid signaling by NADH and the anticancer effect to tNOX inhibitors

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Cancer may be viewed as the unregulated proliferation of malignant tumor cells and is characterized by a resistance to normal, regulated cell death (apoptosis). We have implicated expression of a drug-responsive, tumor-associated hydroquinone (NADH) oxidase with protein disulfide-thiol interchange activity (tNOX) in this deregulation. The central hypothesis under investigation is that tNOX inhibition leads to apoptosis via accumulation of NADH at the inner leaflet of the plasma membrane. This is accompanied by an increase in the ceramide/sphingosine-1-phosphate ratio through direct NADH stimulation of sphongomyelinase and a direct inhibition by NADH of sphingosine kinase. As one test of this hypothesis, the time course of intracellular NADH was determined from the maximal excitation and emission wavelengths at 340 and 460 nm respectively of living HeLa cells. In control experiments, the NADH of unextracted cells remains nearly unchanged within the time frame of the experiments. However, following treatment with the tNOX inhibitors phenoxodiol or (-)-epigallocatechin-3-gallate (EGCg), the NADH concentration increased markedly for the first 2 h of treatment after which it continuously declined often to values lower than the original values as apoptosis was initiated. These findings are part of a larger study to establish a direct signaling role for NADH within sphingolipid regulatory pathways leading to growth arrest and apoptosis that might effectively link redox regulation and cancer cell survival.

Poster # 8

p38δ is required for interleukin-6 production in acid-β-glucosidase-depleted cells: linking impaired ceramide formation to inflammation

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Ceramide, a product of sphingolipid metabolism, has emerged as a pleiotropic lipid mediator implicated in various cellular processes including growth arrest, senescence, apoptosis, stress responses and inflammation. Previously, our study demonstrated involvement of protein kinase C (PKC) in ceramide synthesis through the salvage pathway in human breast cancer cells, MCF-7 cells. Therefore, we employed phorbol myristate acetate (PMA), an activator of PKCs which stimulates inflammation, to explore the involvement of ceramide in inflammatory responses including production of interleukin-6 (IL-6).

Acid-β-glucosidase (GCase) cleaves glucosylceramide to form ceramide. The results from knock-down of GCase by RNA interference (RNAi) demonstrated that GCase partly contributes to ceramide synthesis in PMA-stimulated MCF-7 cells. To determine roles of ceramide in IL-6 generation, effects of silencing GCase were evaluated. Knock-down of GCase enhanced IL-6 generation induced by PMA, suggesting ceramide down-regulates IL-6 generation. Interestingly, clinical studies have demonstrated elevated blood levels of IL-6 in patients with Gaucher disease caused by defective GCase. In order to determine the mechanism by which impaired ceramide formation leads to exaggerated IL-6 production, we evaluated involvement of p38. Inhibition of p38 isoforms α, β and γ, but not the δ isoform, by SB202190 had no effects on enhancement of IL-6 generation, but knock-down of p38δ by RNAi significantly abrogated the stimulatory effect of impairing ceramide synthesis by GCase knock-down on IL-6 generation. Therefore, GCase is likely to negatively modulate activation of p38δ through ceramide signaling. Ongoing work displays that ceramide-activated protein phosphatases mediate ceramide signaling and play a central role in dephosphorylation/inactivation of p38 in PMA-stimulated MCF-7 cells. Thus, knockdown of GCase eliminates ceramide synthesis, leading to impaired ceramide signaling. Diminished ceramide production leads to decreased activation of ceramide-activated protein phosphatases resulting in hyperphosphorylation of p38 and subsequent IL-6 elaboration. Here we propose that impairment of ceramide signaling is implicated in inflammatory responses.

Poster # 9

Sphingosine-1-phosphate signaling is a critical determinant of renal morphogenesis

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Branching morphogenesis of the embryonic kidney occurs by reciprocal inductive interactions between the ureteric bud and metanephric mesenchyme mediated by a complex interplay of many factors. Sphingosine-1-phosphate (S1P) is a potent bioactive lipid that functions as both an intracellular second messenger and a high affinity ligand for S1P receptors to influence cellular proliferation, apoptosis, migration, and differentiation. This study examined: 1) the influence of sphingosine kinase activity and S1P receptor signaling on branching morphogenesis in cultured mouse kidneys; and 2) expression of sphingosine kinases and S1P receptors during embryonic kidney development. Treatment with SK inhibitors including DMS, DHS, F-123509A, and B-5354c resulted in a dose-dependent reduction of ureteric bud tip numbers. Treatment with FTY720, a potent agonist known to cause internalization and degradation of S1P receptors, also inhibited branching morphogenesis suggesting autocrine or paracrine signaling through S1P receptors. Expression of sphingosine kinases (SK1, SK2) and S1P receptors ($S1P_{1-5}$) all exhibited dynamic regulation during kidney development. Expression of both kinases was lower in embryonic kidneys than in adult tissue, and expression of SK2 was consistently higher than SK1 except at gestational age E14.5 when SK1 expression increased while SK2 expression decreased. Expression of $S1P_1$ increased throughout kidney development and was most highly expressed in the adult kidney. Expression of $S1P_2$, $S1P_3$, and $S1P_5$ were all greatest during early stages of development, although S1P5 expression was considerably lower than other S1PRs. Together, these results demonstrate that sphingosine kinase and S1P receptor activity are essential for branching morphogenesis of the developing kidney and suggest that $S1P_2$, $S1P_3$, and $S1P_5$ may play a critical role during early stages of renal morphogenesis.

Poster # 10

Mechanism of inhibition of sequestration of protein kinase C α/ßII by ceramide: roles of ceramide activated protein phosphatases and phosphorylation/dephosphorylation of PKCα/ßII on threonine 638/641

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Sustained activation of protein kinase C (PKC) isoenzymes α and ßII leads to their translocation to a pericentriolar region and to the formation of the pericentrion, a PKC-dependent subset of recycling endosomes. In MCF-7 breast cancer cells, the action of the PKC activator 4β-phorbol-12-myristate-13-acetate (PMA) also evokes ceramide formation which in turn prevents PKCα/βII translocation to the pericentrion. In this study, we investigated the mechanisms by which ceramide negatively regulates this translocation of PKCα/βII. Upon PMA treatment, HEK-293 cells displayed dual phosphorylation of PKCα/βII at carboxyl-terminal sites (Thr638/641 and Ser657/660), whereas in MCF-7 cells PKCα/βII were phosphorylated at Ser657/660, but not Thr638/641. Inhibition of ceramide synthesis by fumonisin B1 overcame the defect in PKC phosphorylation and restored translocation of PKCßII to the pericentrion. To determine the involvement of ceramide-activated protein phosphatases in PKC regulation, we employed small interference RNA to knock-down individual serine/threonine protein phosphatases. Knock-down of isoforms α or β of the catalytic subunits of protein phosphatase 1 (PP1) not only increased phosphorylation of PKCα/βII at Thr638/641, but also restored PKCα translocation to the pericentrion. Mutagenesis approaches in HEK-293 cells revealed that mutation of either Thr641 or Ser660 to Ala in PKCβII abolished translocation of PKC, implying the indispensable roles of phosphorylation of PKCα/βII at those sites for their translocation to the pericentrion. Reciprocally, point mutation of Ser641 to Glu, which mimics phosphorylation, in PKCβII, overcame the inhibitory effects of ceramide on PKC translocation in PMA-stimulated MCF-7 cells. Therefore, the results demonstrate a novel role for carboxyl-terminal phosphorylation of PKCα/βII in the translocation of PKC to the pericentrion, and they disclose specific regulation of PKC autophosphorylation by ceramide through the activation of specific isoforms of PP1.

Poster # 11

Ceramide kinase utilizes ceramide provided by ceramide transport protein: localization to subcellular compartments of eicosanoid synthesis.

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Ceramide kinase (CERK) is a critical mediator of eicosanoid synthesis, and its product, ceramide-1-phosphate (C1P), is required for the production of prostaglandins in response to inflammatory agonists. In this study, mass spectrometry analysis disclosed that the main form of C1P in cells was C_{16} C1P. Interestingly, the C_{16} C1P was also the main form elevated in response to the inflammatory agonists, ATP and A23187, suggesting CERK utilized ceramide transported to the *trans*-golgi apparatus by ceramide transport protein (CERT). To this end, downregulation of the *CERT* by RNA interference technology (RNAi) dramatically reduced the levels of newly synthesized C1P as well as significantly reduced the total mass levels of C1P in cells. Confocal microscopy and surface plasmon resonance analysis localized CERK to the *trans*-Golgi/*trans*-Golgi network placing the generation of C1P in the proper intracellular location in regards to CERT and for recruitment of activated cPLA$_2\alpha$. Ceramide kinase was also localized to the mitochondria and endosomal/exosomal compartments. In conclusion, these results demonstrate that CERK localizes to areas of eicosanoid synthesis and utilizes a ceramide "pool" transported in an active manner via CERT.

Poster # 12

ATP-induced apoptosis of thymocytes is mediated by activation of P2X7 receptor and involves de novo ceramide synthesis and mitochondria

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Thymocytes were reported to undergo apoptosis in the presence of extracellular ATP through the activation of the purinergic receptors P2X1R, P2X7R or both. We investigated the identity of the P2XR and the signaling pathways involved in ATP-mediated apoptosis. Apoptosis elicited by ATP was prevented by inhibition of P2X7R, or in thymocytes bearing a mutated P2X7R, and reproduced with a P2X7R agonist, but not with a P2X1R agonist. Stimulation of thymocytes with either ATP or a P2X7R agonist was found to stimulate a late de novo ceramide synthesis and mitochondrial alterations. Inhibition of either processes attenuated apoptosis. Interestingly, stimulation with either ATP or a P2X1R agonist induced an early ceramide accumulation and a weak caspases-3/7 activation that did not lead to apoptosis. In conclusion, de novo ceramide generation and mitochondrial alterations, both resulting from P2X7R activation, were implicated in ATP-induced thymocyte apoptosis.

Poster # 13

Filamin A links sphingosine kinase 1 and $S1P_1$ at the leading edge

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Sphingosine kinase 1 (SphK1) is a key enzyme that catalyzes the phosphorylation of sphingosine to produce sphingosine-1-phosphate (S1P), a potent lipid mediator that regulates diverse biological processes including cell motility, angiogenesis and cell growth. S1P exerts many of its functions through binding to five G protein coupled receptors, named $S1P_{1-5}$, but it also can act intracellularly. Though the regulatory mechanisms governing SphK1 activation have not been fully characterized, many chemoattractants stimulate SphK1, which in turn plays a critical role in cell motility. We have now identified filamin A (FlnA), an actin crosslinking protein involved in motility, as an SphK1-interacting protein by a yeast two-hybrid screen. Importantly, we demonstrated that endogenous SphK1 co-immunoprecipitates endogenous FlnA. To examine the functionality of this interaction, we used established matched melanoma cell lines, the FlnA-deficient M2 cells and FlnA-reconstituted subline A7. Modified Boyden chamber cell migration assays demonstrated that FlnA is required for cell migration as only FlnA-containing A7 cells migrated towards the growth factor heregulin (Hrg) and serum. Hrg also stimulated SphK1 activity in a FlnA-dependent manner. Moreover, Hrg induced the translocation and colocalization of SphK1 and FlnA at membrane ruffles. Additionally, siRNA specific for SphK1 inhibited Hrg-induced cell migration, demonstrating that both SphK1 and FlnA are required for motility. Intriguingly, it has been reported that the substrate for SphK1, sphingosine, can induce the activation of the pro-motility Rac-effector PAK, which phosphorylates and activates FlnA. However, SphK1-specific siRNA blocked Hrg- and sphingosine-induced activation of PAK and phosphorylation of FlnA. Indeed, SphK1-specific siRNA also blocked membrane ruffling in response to Hrg and sphingosine, suggesting that the SphK1 product, S1P, not its precursor sphingosine, promotes motility. Consistent with this notion, Hrg induced colocalization to membrane ruffles of FlnA, SphK1, and $S1P_1$, a pro-motility S1P receptor, but not $S1P_2$, which inhibits motility. A requirement for $S1P_1$ was further suggested by the finding that the $S1P_1$ antagonist VPC 23019 inhibited Hrg- and sphingosine-induced membrane ruffling. Together, our data suggest that FlnA links SphK1 and $S1P_1$ at the plasma membrane to promote cell motility.

Poster # 14

Role and implication of Isc1p in G2/M phase and in replication stress

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Isc1p a neutral sphingomyelinase homolog is a regulator enzyme involved in the production of ceramide in *Saccharomyces cerevisiae*. This protein localizes in the ER during log phase but trans-locates to mitochondria when cells shift to post-diauxic phase. Deletion of *ISC1* gene renders cells defective in growth on non-fermentable carbon sources such as glycerol. In this study we found that isc1Δ strain is also sensitive to hydroxyurea (HU), a ribonucleotide reductase inhibitor. Treatment with HU blocks the cells in G1 phase, the S phase checkpoint activation makes cells progress slowly through S phase before they complete the cell cycle. In cells treated with HU we found that *isc1*Δ strains accumulate in the G2/M phase compared to the wild type cells. However when we used an expression plasmid containing *cdc28Y19F* witch mimics an active state of the cyclin dependent kinase Cdc28p, the G2/M block was overcome. However, overexpression of *cdc28Y19F* rendered the *isc1*Δ cells more sensitive for growth on fermentable and non-fermentable carbon sources. In this study we investigated the interconnection of Isc1p and Cdc28p and the role of Isc1p in the regulation of G2/M phase progression.

Poster # 15

SAP155 Binds to Ceramide-Responsive RNA *Cis*-Element 1 and Regulates the Alternative 5' Splice Site Selection of Bcl-x Pre-mRNA

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Two splice variants are derived from the *BCL-x* gene, pro-apoptotic Bcl-x(s) and anti-apoptotic Bcl-x(L), via alternative 5' splice site selection. In previous studies, our laboratory identified an RNA *cis*-element within exon 2 of Bcl-x pre mRNA that is ceramide responsive termed CRCE 1. In this study, mass spectrometric analysis identified the splicing factor, SAP155, as an RNA *trans*-acting factor binding to the purine-rich CRCE 1. The interaction of SAP155 with CRCE 1 was confirmed by the addition of an anti-SAP155 antibody to electromobility shift assays (EMSA) decreasing the mobility of a protein:CRCE 1 complex (supershift). Furthermore, the downregulation of SAP155 in A549 cells by RNA interference (RNAi) technology resulted in the loss of a 155 kDa protein complexed with CRCE 1. Moreover, this downregulation of SAP155 induced an increase in the Bcl-x(s) with a concomitant decrease in the Bcl-x(L) splice variants and immunoreactive protein levels, thereby decreasing the Bcl-x(L)/Bcl-x(s) ratio. Specific downregulation of SAP155 also inhibited the ability of exogenous ceramide treatment to further induce the activation of the Bcl-x(s) 5' splice site. Additionally, the specific downregulation of SAP155 sensitized cells to undergo apoptosis in response to daunorubicin in a manner similar to ceramide. Therefore, we have identified SAP155 as an RNA *trans*-acting factor that binds to CRCE 1, functions to regulate the alternative 5' splice site selection of Bcl-x pre-mRNA, and is required for ceramide to induce the activation of the Bcl-x(s) 5' splice site. Furthermore, we have demonstrated that activation of the Bcl-x(s) 5' splice site can increase the effectiveness of chemotherapeutic drug treatment, thus establishing a role for the alternative splicing mechanism of Bcl-x in chemotherapeutic sensitivity.

Poster # 16

The Role of Sphingosine-1-Phosphate receptor 3 (S1P3) in the alveolar macrophage-*Cryptococcus neoformans* interaction

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Cryptococcus neoformans (*Cn*) is an opportunistic pathogen causing the most common form of fungal meningoencephalitis worldwide. Upon inhalation, *Cn* enters into alveolar spaces where the fungus is first confronted by alveolar macrophages (aMø), which internalize fungal cells for their removal by the host degradative pathway. The phagocytic receptors currently known that are involved in the internalization process are the mannose, beta-glucan, complement (CR) and immunoglobulin Fc (FcR) receptors. In recent years, host sphingolipids have been found to have intricate roles in the infection of several intracellular pathogens including *Chlamydia trachomatis*, *Leishmania* species, and *Mycobacterium.* Sphingosine-1-phosphate (S1P) initiates extracellular signaling cascades by stimulation of five members of the endothelial cell differentiation gene family of G protein coupled receptors. As all host immune cells express a distinctive profile of S1P1-5 receptors, we wondering whether S1P3 receptor would have a role in the aMø-*Cn* interaction. When infected with *Cn*, primary aMø from S1P3-/- mice have significantly decreased ability to internalize the fungus compared to S1P+/+ aMø*.* Identical results were also obtained from *in vitro* co-incubation experiments. Interestingly, the intracellular growth of internalized fungi was not affected by the S1P3 deficiency, suggesting that S1P signaling affects the internalization but not intracellular fungal survival. Pretreatment of S1P3-/- aMø with 10 micromolar S1P *in vitro* enhanced attachment and internalization of *Cn*. These studies identify a new host receptor (S1P3) on the surface of aMø that is involved in the phagocytosis of Cn, with important implications for the understanding of S1P signaling at the host-pathogen interface and the potential development of new therapeutic strategies.

Poster # 17

Plasma Membrane Translocation of Protein Phosphatase-1: ceramide-mediated localization and activation.

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The generation of ceramide is now well established as a strategic intermediate in cellular responses to inflammation, proliferation, differentiation, cell cycle arrest, senescence, and cell death. The identification of direct targets of ceramide continues to be a major objective in defining the role of this bioactive sphingolipid in these responses and its mechanisms of action.

It was previously observed that neutral sphingomyelinase 2 (nSMase2) translocated from intracellular sites to the plasma membrane as monolayer cultures of MCF7 breast carcinoma cells became more confluent. Concomitant with this translocation, cellular levels of the very long chain ceramides (C24:1, C24:0) increased, as did the fraction of cells growth arrested in the G0/G1 phase of the cell cycle. Furthermore, confluence arrest was associated with the induction of the hypophosphorylation of the retinoblastoma protein (Rb). Because the type I serine/threonine protein phosphatase (PP1) has been previously shown to dephosphorylate Rb in a ceramide-dependent manner, and because PP1 has been proposed as a direct cellular target of ceramide, we investigated the role of ceramide in the activation and localization of GFP-PP1 in MCF7 cells.

The results in this study show a confluence-dependent GFP-PP1 plasma membrane translocation which was partially inhibited by down regulation of nSMase2. In addition, we show that exogenous ceramide induces the translocation of PP1 and the dephosphorylation of ?-catenin; thus mimicking the effects of confluence.

In conclusion this study defines a role of ceramide in regulating the localization and activity of PP1 in cells and will further establish PP1 as a lipid regulated protein phosphatase.

Poster # 18

Poster18a: **The hunt for the CLN9 gene**

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The neuronal ceroid lipofuscinoses are a group of neurodegenerative disorders characterized by retinitis pigmentosa, seizures, cognitive and motor decline, and early death. CLN9 is a novel clinical NCL variant identified in 2 families. Two Serbian sisters and two German brothers are affected with CLN9. We have recently shown that CLN9 is a positive modulator of Dihydroceramide synthase. In a family with both unaffected and affected offsprings, regions of homozygosity are random between different family members but similar between affected individuals. To identify the locus, genome-wide single nucleotide polymorphism (SNP) analysis with over 100,000 SNP loci was used to locate genetic variations. SNP genotypes were generated for parents, siblings and affecteds. These SNPs allow localization of the disease locus to areas where only affected individuals are homozygous for alleles. This approach has been recently used by Strauss, et al to characterize a disease in an Old Order Mennonite population. Candidate genes in those regions are screened for mutations. Homozygosity mapping in this family consisting of two parents, two unaffected and two affected children allowed localization of homozygous regions on chromosomes 2, 8, 11, 13, 15, and 17. 550 candidate genes were found in these regions using the Ensembl program. We were able to generate a preferential list of 73 genes by focusing on gene function (i.e. genes involved in lipid biosynthesis, cell adhesion and oncogenesis). These genes are currently being investigated. Analysis is almost complete for the following genes: NME2, ALG9, DGKE, and LOH11CR2A.

Poster 18b: **CLN8 impacts sphingolipid metabolism**

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The neuronal ceroid lipofuscinoses (NCLs) are inherited neurodegenerative disorders characterized by retinitis pigmentosa, seizures, cognitive and motor decline, and early death. In a subgroup of patients with Turkish late infantile variant, the CLN8 gene is mutated.
CLN8 is a transmembrane ER resident protein harboring a TLC domain and a Lag 1 motif. CLN8-deficient fibroblasts exhibit increased rates of growth, lower levels of ceramide and dihydroceramide, and higher levels of sphingomyelin. They also exhibit decreased activities of dihydroceramide synthase and normal activities of glucosylceramide synthase and sphingomyelin synthase. All species of dihydroceramide are decreased in CLN8-deficient fibroblasts, most notably C24 and C24:1 dihydroceramide. This suggests that CLN8 may impact a step in the *de-novo* dihydroceramide synthetic pathway, affecting C24 and C24:1 species synthesis specifically. Alternatively, most sphingomyelin species are increased in CLN8-deficient fibroblasts compared to normal. Again this affects the C24 and C24:1 sphingomyelin species. Since sphingomyelin synthase activity is normal in those fibroblasts, CLN8 may be a positive regulator of sphingomyelinase or an inhibitor of sphingomyelin synthase. These results suggest that CLN8 may impact sphingolipid metabolism by regulating two key enzymes: sphingomyelin synthase and/or sphingomyelinase in a C24/C24:1 fatty acid chain-specific manner. Dysregulation of sphingolipid pathways invokes a theme common to CLN2, CLN3, CLN6, CLN8, and CLN9-deficient cells.

Poster # 19

Ceramide and sphingosine-1-phosphate levels accompanying G1 arrest and apoptosis may respond to NAD+/NADH and/or CoQ/CoQH2 ratios from plasma membrane electron transport

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To elucidate possible biochemical links between growth arrest from antiproliferative chemotherapeutic agents and apoptosis, our work has focused on agents (EGCg, capsaicin, cis-platinum, adriamycin, anti-tumor sulfonylureas, phenoxodiol) that target a cancer-specific cell surface hydroquinone (NADH) oxidase with protein disulfide-thiol interchange activity (tNOX) that functions in cancer cells as the terminal oxidase for plasma membrane electron transport. When tNOX is active, coenzyme Q10 (ubiquinone) of the plasma membrane is oxidized and NADH is oxidized at the cytosolic surface of the plasma membrane. However, when tNOX is inhibited and plasma membrane electron transport is diminished, both reduced coenzyme Q10 (ubiquinol) and NADH are expected to accumulate. To relate inhibition of plasma membrane redox to increased ceramide levels and arrest of cell proliferation in G1 and apoptosis, neutral sphingomyelinase, a major contributor to plasma membrane ceramide, was shown to be inhibited by both reduced glutathione and by ubiquinone. Ubiquinol was without effect or stimulated. In contrast, sphingosine kinase, which generates anti-apoptotic sphingosine-1-phosphate, was stimulated by ubiquinone but inhibited by ubiquinol and NADH. The hypothesis under investigation is that pyridine nucleotide products of plasma membrane redox, NAD+ and NADH, as well as the quinone products, ubiquinone and ubiquinol, may directly modulate in a reciprocal manner two key plasma membrane enzymes, sphingomyelinase and sphingosine kinase. Such modulation might then lead to G1 arrest (increase in ceramide) and apoptosis (loss of sphingosine-1-phosphate) to link NADH and coenzyme Q10-mediated plasma membrane electron transport and the anticancer action of several clinically-relevant anticancer agents.

Poster # 20

The alternative splicing of caspase 9 regulates the chemotherapy sensitivity of lung cancer cells.

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Alternative splicing of the caspase 9 gene produces two splice variants, caspase 9a (pro-apoptotic) and caspase 9b (anti-apoptotic). In the current study, we found that the transformed lung epithelial cells and lung adenocarcinoma tumors show a highly dysregulated or reduced caspase 9a/9b ratio as compared to the non-transformed lung epithelial cells. A biological relevance for this dysregulation was also demonstrated using antisense RNA oligonucleotides and RNA interference technology to directly manipulate the caspase 9a/9b ratio. An antisense RNA oligonucleotide redirected caspase 9 pre-mRNA splicing in favor of caspase 9b expression resulting in an increased IC_{50} of chemotherapeutic drugs (e.g. daunorubicin). Specific silencing of caspase 9b was also achieved using RNAi technology resulting in a decrease in the IC_{50} of chemotherapy drugs. Utilizing these technologies, we also demonstrate that the sensitization of A549 cells to daunorubicin by co-treatment with ceramide is via increasing the ratio of caspase 9a/9b. Thus, we demonstrate for the first time, that the alternative splicing of caspase 9 is an important mechanism regulating the sensitivity of non-small cell lung cancers to chemotherapy. Furthermore, the direct manipulation of the alternative splicing of caspase 9 is a potential therapeutic target for treating lung adenocarcinomas.

Poster # 21

The Immunosuppressant FTY720 Interacts with the CB1 Cannabinoid Receptor Independently of its Phosphorylation by Sphk2

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The immunosuppressant FTY720, a sphingosine analogue, has recently received much attention since in contrast to other immunosuppressive agents, it acts to specifically induce sequestration of lymphocytes without causing cytotoxicity. FTY720 is now in clinical trials for prevention of kidney graft rejection and treatment of multiple sclerosis. We previously showed that sphingosine kinase type 2 (SphK2) can phosphorylate FTY720 and studies with SphK2 knockout mice have demonstrated that it is the relevant SphK isoenzyme that is responsible for in vivo phosphorylation of FTY720. FTY720-P interacts with four of the five sphingosine-1-phosphate (S1P) receptors which has important implications for its functions. Because the CB1 and CB2 cannabinoid receptors (which bind the active components of marijuana and endocannabinoids) are closely related to the S1P receptor family, we examined their interaction with S1P and FTY720-P. Although neither S1P nor FTY720-P interacted potently with CB1 or CB2, we found that their precursors, sphingosine and FTY720, compete for binding of [^{3}H]SR141716A, an antagonist that specifically targets CB1 receptors, as well as the CB1 agonist CP 55,940. Moreover, both FTY720 and sphingosine inhibited maximal stimulation of [^{35}S]GTPgammaS binding by the high efficacy agonist WIN 55,212-2 in a dose-dependent manner. In agreement, they also drastically reduced activation of ERK1/2 and Akt induced by binding of WIN 55,212-2 to CB1. Because FTY720 is concentrated to high levels in the CNS, our results suggest that the pro-drug FTY720 may directly target CB1 in the CNS, independently of any effects mediated through S1P receptors.

Poster # 22

Sphingosine-1-Phosphate in Development and Regulation of Human Mast Cells

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Mast cells (MC) play a critical role in both acute and chronic inflammation by secreting a plethora of mediators following antigen triggering. *In vivo*, MC mature in peripheral tissues from bone marrow-derived progenitors that circulate in the blood as immature precursors. Serum-born factors, including the bioactive metabolite sphingosine-1-phosphate (S1P), are present during MC maturation, yet the role of these factors in MC development remains unknown. Previous studies from our lab have begun to define novel functions of S1P and the enzyme that produce it, sphingosine kinase (SphK), in regulation of inflammatory responses and mast cell functions. Moreover, human stem cell factor (SCF), an important growth factor required for mast cell survival and differentiation, also activates SphK, further emphasizes the important role of activation of SphKs and S1P generation in mast cell physiology. To examine the effect of S1P development of MC, we cultured cord blood-derived progenitor cells with SCF in the absence and presence of various concentrations of S1P. Remarkably, culturing in the presence of SCF and S1P increased the number of mast cells and strikingly increased chymase expression to skin-like MC_{TC}. This is the first demonstration that any factor in addition to IL-6 can induce differentiation of CB-MC. Treatment with S1P induced release of IL-6, yet no significant secretion of IL-6 could be detected in the medium of purified cord blood derived MC (CB-MC) cultured in the presence of S1P. We also found that these CB-MC_{TC} have functional FcεRI and similar to skin MC_{TC}, are also activated by C5a. Thus, these cells express CD88, the receptor for anaphylatoxin C5a, another phenotype of Sk-MC_{TC}. In addition, S1P triggered degranulation of purified CB-MC and subsequent secretion of MCP-1. Taken together, these data suggest crucial roles for S1P in regulating development of human mast cells and their functions. (Supported by NIH/AI50094 to SS.)

Poster # 23

Caveolin-1 scaffolding domain peptide inhibits mouse primary keratinocyte differentiation in response to an elevated extracellular calcium concentration.

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Phospholipase D2 (PLD2) has been found functionally to interact with caveolin in low-density membrane microdomains[1], and the activity of this caveolar PLD2 can be regulated by caveolin-1 scaffolding domain peptide in the HaCaT human keratinocyte cell line [2]. A previous study in our lab has suggested the possibility that PLD2 and aquaporin 3 (AQP3) work together to regulate keratinocyte proliferation and differentiation. We hypothesized that caveolin-1 scaffolding domain peptide interacts with lipid rafts and disrupts their function, thereby disturbing the association of AQP3 and PLD2 and resulting in less phosphatidylglycerol (PG) synthesis. We proposed that PG acts as a second messenger to inhibit keratinocyte proliferation and promote differentiation. We treated mouse primary keratinocytes with 3 μM cell-permeable caveolin-1 scaffolding domain peptide and stimulated cell differentiation using 125μM $CaCl_2$. Then cell proliferation and differentiation were measured by the incorporation of [^{3}H]thymidine into DNA and a transglutaminase (TGase) assay, respectively. Total PLD (PLD1/PLD2) activity and PG synthesis (PLD2 activity) were measured using a PLD activity assay in the presence of ethanol (PA/PEt) and [^{14}C]PG production from [^{14}C]glycerol, respectively. AQP3 activity was monitored as [^{3}H]glycerol uptake. The caveolin-1 scaffolding domain peptide had no effect itself on AQP3 activity, total PLD activity, TGase and proliferation but prevented the changes induced by a moderate $CaCl_2$ concentration; whereas a peptide control did not. However, the caveolin-1 scaffolding domain peptide inhibited PG synthesis with or without moderate $CaCl_2$ stimulation. We conclude that the caveolin-1 scaffolding domain peptide regulates both $CaCl_2$-inhibited proliferation and –stimulated differentiation, at least in part, through effects on PG production. We propose that reduced AQP3 and PLD2 interaction resulting from disruption of lipid rafts by the caveolin-1 scaffolding domain peptide could be a reason for decreased PG synthesis and the inhibition of $CaCl_2$–induced keratinocyte differentiation.

Poster # 24

Altered NCL lipid raft morphology and sphingolipids

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Increased apoptosis, trafficking defects and endosomal inclusions are present in all NCL types. The primary defects in nine currently known variants of NCL are unrelated, but lead to similar pathological changes. Juvenile neuronal ceroid lipofuscinosis (JNCL) is caused by mutations in the CLN3 gene. CLN3 recycles from Golgi to lipid rafts via Rab 4- and Rab11- positive recycling endosomes. CLN3 has a structural galactosylceramide (GalCer) lipid raft binding domain. In the absence of CLN3, GalCer and mutant CLN3p are barely visible at the cell surface and are retained in the Golgi. The reintroduction of CLN3 rescues the GalCer deficit at lipid rafts. We show that wild type of CLN3 directly binds to GalCer and sulfatide, the sulfated form of GalCer and another component of lipid rafts, and mutant CLN3 fails to bind to GalCer or sulfatide. Sphingolipid composition of lipid rafts, Golgi and endoplasmic reticulum (ER) is altered in JNCL cells: GalCer was decreased in lipid rafts, but increased in Golgi, supporting the hypothesis that CLN3p is involved in transport of GalCer from Golgi to lipid rafts. Furthermore, sphingolipid composition of lipid rafts, Golgi, and ER in CLN1-, CLN2-, CLN6-, CLN8- and CLN9-deficient cells differed significantly from normal. In addition, JNCL lipid rafts, as well as lipid rafts of CLN1-, CLN2-, CLN6-, CLN8- and CLN9-deficient cells had smaller vesicular structures than normal rafts by electron microscopy. These structural differences may reflect the altered sphingolipid composition we documented in all NCL lipid rafts. Binding of lipids to NCL proteins was evaluated using the lipid protein overlay assay, suggesting NCL proteins other than just CLN3 may be involved in the regulation of sphingolipid trafficking to lipid rafts. Abnormal lipid raft function may be a common theme for all the NCLs.

Poster # 25

Alterations of Human Longevity Assurance Gene 1 (LASS1)/Sphingosine Kinase-1-Dependent Ceramide Generation and Metabolism Involve in the Regulation of Imatinib-Induced Apoptosis and Resistance in K562 Human Chronic Myeloid Leukemia (CML) Cells

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In this study, mechanisms of resistance to imatinib-induced apoptosis in human K562 chronic myeloid leukemia (CML) cells were examined. Continuous exposure of cells to step-wise increasing concentrations of imatinib resulted in the selection of K562/IMA-0.2 and -1 cells, which expressed about 2.3- and 19-fold resistance, respectively. Measurement of endogenous ceramide levels by high performance liquid chromatography/mass spectroscopy (LC/MS) showed that treatment with imatinib increased the generation of C_{18}-ceramide significantly, which is mainly synthesized by the human longevity assurance gene 1 (hLASS1), in sensitive, but not in resistant cells. Inhibition of hLASS1 by small interfering RNA (siRNA) partially prevented imatinib-induced cell death in sensitive K562 cells. In reciprocal experiments, over-expression of hLASS1 in drug resistant cells caused a marked increase in imatinib-induced C_{18}-ceramide generation, and enhanced mitochondrial dysfunction and caspase-3 activation. Mechanistically, analysis of mRNA and enzyme activity levels of hLASS1 in the absence or presence of imatinib did not show any significant differences in these resistant cells when compared to its sensitive counterparts, suggesting that accumulation and/or metabolism, but not the synthesis of ceramide, might be altered in resistant cells. Indeed, further data demonstrated that expression levels, and enzyme activity of sphingosine kinase-1 (SK-1), was increased significantly in resistant K562/IMA-0.2 and -1 cells. Moreover, in resistant cells, partial inhibition of SK-1 expression by siRNA increased sensitivity to imatinib-induced apoptosis. On the other hand, over-expression of SK-1 in sensitive K562 cells increased the ratio between total S1P/C_{18}-ceramide levels about 6-fold, and prevented apoptosis significantly in response to imatinib when compared to vector transfected controls. In conclusion, these data show, for the first time, a role for endogenous ceramide synthesis via hLASS1 in imatinib-induced mitochondrial apoptosis, and that alterations of the balance between the levels of pro-apoptotic ceramide and pro-survival S1P by over-expression of SK-1 result in resistance to imatinib in K562 cells.

Poster # 26

The protooncogene Akt2 regulates the alternative splicing of caspase 9 in a prosurvival manner.

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Two splice variants are derived from the Caspase 9 gene, pro-apoptotic caspase 9a and anti-apoptotic caspase 9b. They are produced via the post-transcriptional processing of caspase 9 pre-mRNA by the inclusion (9a) or exclusion (9b) of a four exon cassette (exons 3, 4, 5, and 6). In previous studies, our laboratory has demonstrated that the alternative splicing of Caspase 9 is regulated by *de novo* ceramide in a PP1-dependent and pro-apoptotic manner. In this study, we now demonstrate that pro-survival signaling pathway that regulates the alternative splicing of caspase 9 in an anti-apoptotic manner. Specifically, we show that the PI_3 Kinase/Akt2 pathway regulates the alternative splicing of caspase 9 in a pro-survival manner. To this end, treatment of A549 lung adenocarcinoma cells with the phosphatidylinositol 3-kinase (PI_3K) inhibitor LY294002 (50 μM) or the Akt1/2 inhibitor Akt Inhibitor VIII (25 μM) dramatically increased the caspase 9a/caspase 9b mRNA ratio. Blockade of the protooncogene, Akt2, but not Akt1 using small interfering RNA (siRNA) also resulted in an increased caspase 9a/b ratio. Therefore, we conclude that the PI_3K/Akt2 pathway, a pathway important for cancer cell survival and transformation of epithelial cells, regulates the alternative splicing of caspase 9 to produce an anti-apoptotic phenotype.

Poster # 27

Docking Interactions Regulate the Phosphorylation of Sphingosine Kinase 1

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Sphingosine kinase 1 catalyzes the formation of sphingosine-1-phosphate and plays an important role in cell signaling, proliferation, inhibition of apoptosis and inflammatory responses. It is activated by extracellular signal-regulated kinase 1/2 during agonist-induced stimulation. Our hypothesis is that, like many other MAPK activated proteins, a docking interaction between sphingosine kinase 1 and ERK1/2 may be the key to regulate the efficiency and fidelity of this activation. However, the location and molecular mechanism of such a docking interaction remains unknown. Our preliminary data indicates that the phosphorylation of sphingosine kinase 1 shows a dose-dependent reduction by small molecule inhibitors specifically targeting the ERK2 docking groove. Site-directed mutagenesis also reveals that mutations of several residues of the putative docking domain on sphigosine kinase 1 abolish or reduce the phosphorylation. Taken together, these studies suggest the docking interaction may be crucial in regulating sphingosine kinase 1/sphingosine-1-phosphate signaling pathways.

Poster # 28

LAG1 and the Chronological Lifespan of Yeast

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The chronological lifespan (CLS) of yeast, assessed by the ability of a stationary culture to form colonies after plating, has been proposed as a model of mammalian postmitotic cellular aging/senescence, e.g. neurons (Chen, Ding et al. 2005). Little is known about either the components or the mechanisms of CLS, but a high-throughput study recently ranked the strains of the yeast deletion library for chronological lifespan (Powers, Kaeberlein et al. 2006). The ranks of genes encoding sphingolipid metabolic enzymes ranged from very low (short CLS) to high (long CLS), and these rankings were used to begin studies to determine the role of sphingolipids in CLS. The effect of caloric restriction (CR) was also assessed since it is known to increase lifespan in numerous eukaryotic species including yeast (both replicative and chronological lifespans). Specifically, the CLS of Δ*lag1* and its homolog Δ*lac1*, components of ceramide synthase, were assayed under normal and CR conditions. Lipid profiles corresponding to CLS time points were determined via mass spectrometry. The results suggest that LAG1 deletion has a prolonged CLS in the BY4741 background strain and additionally, may be involved in the CR response pathway.

Poster # 29

Metabolic Fate of Sphingosine-1-phosphate is determined by the intracellular localization of sphingosine kinase

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Sphingosine-1-phosphate (S1P) is a potent signaling lipid that plays critical roles in a variety of cellular processes such as proliferation, anti-apoptotic responses, and migration. Sphingosine kinase-1 (SK1) is the enzyme that produces S1P. The high basal activity of this enzyme has been linked to a housekeeping role which serves to regulate levels of the upstream signaling lipids, sphingosine and ceramide. Additionally, SK1 is an agonist-stimulated signaling enzyme that promotes signaling events through formation of S1P. We propose that one mechanism of regulating the dual roles of SK1 involves the intracellular localization of this enzyme. We further propose that this localization of SK1 leads to spatially distinct pools of S1P. Here we present evidence that localization of SK1 to specific cellular membranes produces pools of S1P that are more susceptible to degradation by lipid-specific lyases and/or phosphatases. Using a unique whole-cell system that measures the immediate metabolism of S1P, we show that localization of SK1 to distinct intracellular locations alters the amount of S1P available for degradation. Combining this system with pharmacological inhibitors targeting the degradative enzymes, we are beginning to discern the effects of SK1 localization on the relative contributions made by each degradative pathway. We propose that the housekeeping role of SK1 is served by localizing the enzyme to the ER, where the degradative enzymes reside, thereby keeping the levels of S1P below signaling levels. Conversely, the signaling role of SK1 is served by moving the enzyme away from the ER, and localizing it to the plasma membrane where signaling levels of S1P can be achieved.

Poster # 30

Ceramide-1-phosphate binds group IVA cytosolic phospholipase $A_2\alpha$ via a novel binding site.

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C1P is a new addition to the growing group of bioactive sphingolipids, which includes ceramide and sphingosine-1-phosphate. C1P was shown to enhance the activity of $cPLA_2\alpha$ in a specific manner thereby acting as a positive allosteric activator of $cPLA_2\alpha$. In this study, we have identified three basic amino acids adjacent to the CBR II region of the CaLB domain of $cPLA_2\alpha$ (R57,K58,R59) required for interaction with C1P. To examine the role of these amino acids in the interaction of cPLA2α and C1P, we generated triple (R57A,K58A,R59A), double (R57A,K58A), (K58A,R59A), (R57A,R59A) and single (R57A), (K58A), (R59A) mutants of $cPLA_2\alpha$ using site-directed mutagenesis. Using surface plasmon resonance (SPR), the C1P/$cPLA_2\alpha$ mutant demonstrated the same binding affinity to PC vesicles as compared to wild type $cPLA_2\alpha$. Also, the mutants bound PC: PIP2 vesicles with similar affinity as wild-type suggesting that the mutants do not have any structural effects. However, the $cPLA_2\alpha$ triple and double mutant failed to demonstrate enhanced binding affinity for PC/C1P vesicles as compared to the wild type $cPLA_2\alpha$. No significant loss of binding was observed with the single mutants. Using the Triton X-100/PC mixed micelle assay, the $cPLA_2\alpha$ mutant was examined in parallel with wild type $cPLA_2\alpha$ for activation by C1P. The $cPLA_2\alpha$ double mutant failed to induce an increase in the activity in the presence of C1P in contrast to wild type $cPLA_2\alpha$. We cloned these mutants in adenovirus and over-expressed in mammalian cells. As shown before AdWild-type GFP$cPLA_2\alpha$ translocated to the peri-nuclear region in response to Ca^{+2} ionophore. This translocation in response to Ca^{+2} ionophore was impaired with the over-expression of the triple mutants (R57A,K58A,R59A), and double mutant (R57A,K58A). These data demonstrate that the amino acids (R57,K58,R59) are critical for the C1P/$cPLA_2\alpha$ interaction confirming the role of C1P as a specific allosteric activator of $cPLA_2\alpha$.

Poster # 31

Mechanisms of the Regulation of Human Telomerase Reverse Transcriptase (hTERT) Promoter Activity by LASS1/C18-Ceramide Signaling: Repressor Role of Sp3 Deacetylation by Histone Deacetylase 1 (HDAC1)

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In this study, molecular mechanisms involved in ceramide-mediated regulation of the hTERT promoter via Sp3 were examined. The data suggested that acetylation or deacetylation status of Sp3 at lysine 551 (K551) plays a role in Sp3-dependent transactivation or repression of the promoter, respectively. Importantly, the results presented here also demonstrated that the generation of C18-ceramide by LASS1/UOG1 inhibits the expression of hTERT mRNA, and promoter activity via mediating the deacetylation of Sp3. Ceramide-induced deacetylation of Sp3 appears to involve increased association of HDAC1 and Sp3 in the expanse of HDAC1-histone H3 interaction.

Poster # 32

Phospholipid Profiling Of Macrophages – Arachidonate Deficiency In Raw264.7 Cells And Its Implications

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Arachidonate containing glycerophospholipids serve as precursors for biologically active lipid mediators (prostaglandins, thromboxanes, leukotrienes, lipoxins, endocannabinoids), most of which are produced by macrophages. We applied mass spectrometry based lipid profiling technology to evaluate the glycerophospholipid composition of two macrophage populations, resident peritoneal macrophages (RPM) and RAW264.7 cells. The results demonstrated a difference in the distribution of lipid species including a deficiency in 20:4 (AA) containing phospholipids in RAW264.7 (10±1 mole %) compared to peritoneal macrophages (26±1 mole %). Enrichment of RAW264.7 with arachidonic acid (AA) increased the fatty acid to 20±1 mole % but the distribution of the incorporated 20:4 was different from that of primary macrophages. Moreover, treatment of RAW264.7 with granulocyte-macrophage colony stimulating factor (GM-CSF) followed by lipopolysaccharide (LPS) and interferon γ (IFN γ) mobilized similar quantities and produced similar amounts of prostaglandins as peritoneal macrophages treated with LPS alone. AA-enriched RAW264.7 cells lost 88% of the incorporated AA during LPS treatment without additional prostaglandin synthesis. Our data supports a hypothesis that there are fundamental differences in metabolic pathways between RAW264.7 and RPM demonstrated in the different distribution of fatty acids in their phospholipids. AA enrichment of RAW264.7 cells results in incorporation of 20:4 in different phospholipid species as well as a rapid mobilization of the phospholipid 20:4 after LPS/ IFN γ treatment. RPM maintain high 20:4 content after isolation and overnight treatment with LPS.

Poster # 33

Mechanism of the Ceramide Synthase Reaction – A Combined Kinetic and Mathematical Approach

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Ceramide is a second messenger involved in the regulation of important cellular processes such as senescence and apoptosis. In cells, ceramide is generated by three pathways: (1) *de novo* synthesis from serine and fatty acid CoA; (2) degradation of complex sphingolipids and (3) recycling of sphingoid bases. Ceramide synthase is the key enzyme involved in pathways (1) and (3). In mammals, isoforms of this important enzyme are encoded by six *LASS* homologues (which correspond to longevity assurance homologues of yeast *LAG1*). Despite recent progress in cloning the genes for ceramide synthase very little is known about the mechanism of the reaction and regulation of the enzyme. For the present study, we used the mouse *LASS1* homologue and employed a combined kinetic-mathematical approach to characterize the mechanism of the ceramide synthase reaction. The experimental data revealed that the ceramide synthase reaction cannot be explained by simple Michaelis-Menten kinetics. Instead the data appeared to be consistent with two alternative reaction mechanisms, in which dihydrosphingosine and fatty acid co-enzyme A compete in the formation of enzyme complexes: 1) a non-rapid equilibrium random bi-substrate reaction system; or 2) an ordered bi-substrate “ping-pong” reaction. To reveal the more likely mechanism, we developed and analyzed in parallel mathematical models for both mechanisms. These models were designed from first principles of mass action kinetics, and computational optimization techniques were used for system identification and the estimation of rate constant parameters, based on quality of fit. Even though the enzyme levels should theoretically have been the same for all data sets, the kinetic data were obtained with microsomes as an enzyme source. In the optimization process, we accounted for this uncertainty by permitting some variation in enzyme concentrations between experiments. Under these conditions, the ping-pong model performed dramatically better than the random model. Specific validation experiments are presently being performed to confirm, amend, or refute this mathematical result. (*SS and GG contributed equally to this work)

Poster # 34

Neutral Sphingomyelinase Modulates the Trafficking of NMDA Receptors

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The trafficking of ionotropic glutamate receptors modifies synaptic integrity by controlling the number of receptors at the surface of dendritic spines. While the protein-protein interactions involved in glutamate-receptor trafficking are an active area of research, the roles of protein-lipid interactions in receptor trafficking have been more elusive to identify. To study how local lipid domains are modified during NMDA receptor trafficking we used a model system where NMDA receptors are rapidly inserted to the cell surface following a brief stimulation with the cytokine TNFα. In cultured rat cortical cells exposed to TNFα for two minutes, there was nearly twice the number of NMDA receptors per square micron at the cell surface (1.820+0.296 microns^2) as compared with untreated cultures (0.973+ 0.164 microns^2). When we pre-incubated neurons with an inhibitor of vesicle fusion (0.05% sodium azide for 30 minutes) prior to TNFα application, the number of surface expressed NMDA receptors was similar to untreated cultures (0.908+ 0.365 microns^2), suggesting that TNFα mobilized a pool of vesicle bound receptors to fuse with the plasma membrane. Consistent with increased numbers of functional receptors at the cell surface, NMDA-evoked large focal calcium bursts were increased in neurons treated with TNFα. To better understand the change in local lipid content that is required for this fusion event we stimulated neurons for 2 minutes with TNFα, rapidly harvested and sonicated the cells, and isolated the NR1 subunits of NMDA receptors on an affinity column. The lipids bound to NR1 were then chloroform extracted and analyzed by electrospay tandem mass spectroscopy. The NR1-bound lipid domains isolated after TNFα exposure were enriched in the phosolipids phosphatidylserine and phosphatidylinositol, the sphingolipid ceramide C24:0 and the fatty acid cholesterol derivative linoleate C18:2. Using a capacitor model we calculated that the change in sphingomyelin, ceramide and phospholipid content following a 2 min treatment with TNFα would shift the relative dielectric constant (κ) of the local membrane domain to κ = 1.213. This value is in close agreement with our experimental findings that TNFα increased the NMDA-evoked capacitative current from 32 pA to 36.8 pA (n=8, $p<0.05$, at +50mV). This increase in current is equal to a membrane capacitance change of 6.4 x 10^{-10}F to 7.36 x 10^{-10}F and in our modeling, is equivalent to the addition of a dielectric with a uniform coefficient of κ = 1.15. These calculations suggest that nSmase can modulate plasticity by controlling the trafficking of NMDA receptors into ceramide-rich lipid rafts with distinct local membrane capacitance. Pharmacological inhibition of neutral sphingomyelinase prevented TNFα-induced increases in the number of NMDA receptor clusters, NMDA-evoked EPSPs and large focal calcium transients suggesting an important role for ceramide in NMDA receptor trafficking. Together our findings suggest that the local composition of lipids bound to the NMDA receptor are modified during trafficking of the receptor to the cell surface, and that the lipid microdomain surrounding the NMDA receptor modify the kinetics of channel function. (Supported by AG023471 & MH068388 to NJH.)

Poster # 35

Localization and sphingomyelin transfer determinants of the essential mammalian phosphatidylinositol transfer protein PITPbeta

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PITPs are important molecules with roles in regulating lipid metabolism and membrane trafficking. Mammals have five PITPs; two of these, PITPα and PITPβ, share 77% identity (94% similarity) yet have distinct cellular and physiological roles. PITPα is not essential for cell survival or growth, though PITPα knockout mice exhibit intestinal and hepatic steatosis, hypoglycemia, and neurodegeneration. PITPβ, in contrast, seems to be an essential housekeeping protein. We are interested in identifying what contributes to the unique functions of these molecules in order to gain a greater understanding of the cellular roles of PITPs. One possibility for the different functions is localization; PITPα localizes to the nucleus and cytoplasm, while PITPβ localizes to the trans-Golgi network (TGN). Another important difference is that, while both PITPα and PITPβ can transfer phosphatidylinositol and phosphatidylcholine, PITPβ alone can transfer sphingomyelin (SM). In this study, we identify mutants defective in TGN localization and SM transfer. Two separate regions of PITPβ contribute to its localization: its unique C-terminal tail and a putative membrane interaction domain common to both PITPα and PITPβ. Together these regions are sufficient to target a reporter to the membrane. The PITPβ-specific SM transfer activity can be abolished by mutation of a single residue within the phospholipid binding pocket. Interestingly, the critical sphingomyelin transfer residue is not conserved in human PITPβ (rather this residue is the same as PITPα), but human PITPβ retains sphingomyelin transfer ability, suggesting that sphingomyelin transfer is an important feature of the molecule. Future studies will test whether the mutants deficient in SM transfer or localization are capable of rescuing loss of PITPβ function.

Poster # 36

Analysis of membrane topology and post-translational modification of neutral sphingomyelinase-2.

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The neutral sphingomyelinases (SMases) are considered major candidates for mediating the stress-induced production of ceramide. Neutral SMase-2, which has two putative transmembrane domains at its N-terminus, has been reported to be located at the plasma membrane and play important roles in ceramide-mediated signaling. In this study, we investigated the membrane topology and posttranslational modification of neutral SMase-2. When a double-tagged neutral SMase-2, with V5 at the N-terminus and GFP at the C-terminus, was overexpressed in MCF-7 cells, the signals from both tags were detected in the inner leaflet of the plasma membrane. Furthermore, the topological analysis by GFP-fused deletion mutants of neutral SMase-2 revealed that whole region of the protein was located on the cytosolic face of the membranes. We also found that neutral SMase-2 is palmitoylated via thioester bonds. Site-directed mutagenesis of Cys residues to Ala indicated that two Cys clusters of the enzyme are palmitoylated. The loss of palmitoylation on neutral SMase-2 caused mislocalization of the protein from the plasma membrane and its lysosomal degradation. The palmitoylation had no effect on the enzyme activity, but affected membrane-association properties of the protein. In summary we firstly showed the molecular evidence for the presence of inner plasma membrane-localized neutral SMase and the role of palmitoylation in its localization.

Poster # 37

The N-Terminal Membrane Occupation And Recognition Nexus (Morn) Domain Of Arabidopsis Phosphatidylinositol Phosphate Kinase 1 Regulates Enzyme Activity

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*At*PIPK1 (At1g21980) is a member of the type I B family of phosphatidylinositol phosphate kinases (PIPKs) from Arabidopsis that contain a characteristic region of Membrane Occupation and Recognition Nexus (MORN) motifs at the N-terminus. The MORN motifs are not found in PIPKs from mammals, *C. elegans* or yeast. To understand the impact of the unique N-terminal domain on enzyme activity and subcellular distribution, we designed constructs encoding truncated and full-length proteins. Deletion of the N-terminal MORN domain (amino acids #1-251) increased the Vmax of the remaining C-terminal peptide (ΔMORN) >4 fold and eliminated activation by phosphatidic acid (PtdOH) without affecting the substrate specificity. Adding back the MORN peptide to ΔMORN increased activity of ΔMORN 2-fold; while adding back the MORN peptide to the full-length protein had no effect on activity unless PtdOH was added first to activate the enzyme. These data suggest that PtdOH activation was mediated in part through a change in protein conformation. Consistent with this hypothesis, a Pro #396 to Ala (P396A) substitution in the predicted linker region between the MORN and the kinase homology domains eliminated PtdOH activation of the enzyme. Our results reveal a new mechanism for regulating *At*PIPK activity in which a PtdOH-mediated conformational change of the linker region increases the accessibility of the active site.

Poster # 38

Modulation of DGKθ Activity by α-Thrombin and Phospholipids

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Diacylglycerol kinase modulates the levels of diacylglycerol and phosphatidic acid, two critical lipid second messengers, yet little is known about the effects of cellular stimulation on the kinetic behavior of this enzyme. We examined the effects of α-thrombin and activating phospholipids on the activity and substrate affinity of a soluble diacylglycerol kinase, DGKθ. Our data demonstrate that the apparent binding parameters of DGKθ increase following thrombin stimulation, suggesting that α-thrombin antagonizes DGKθ activity. Interestingly, this effect is obscured in the presence of high bulk substrate concentrations. Given the known stimulatory effects of phosphatidylserine on many diacylglycerol kinases, we examined the effects of various phospholipids on DGKθ and found that phosphatidic acid is a more effective activator than phosphatidylserine. Phosphatidic acid decreased the apparent surface K_M ($K_{M(surf)}^{app}$) of DGKθ for dioleoylglycerol (DOG) and promoted binding to vesicles in a dose-dependent manner. Phosphatidylserine also lowered the $K_{M(surf)}^{app}$ of DGKθ, though higher concentrations were required to achieve the same effect. Interestingly, PS promoted binding to vesicles only when present at levels beyond that required to saturate enzyme activity, suggesting that PS and PA activate DGKθ through different mechanisms.

Poster # 39

Fatty Acid Induced Gene Expression in Humans

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A large body of recent research suggests that ingestion of certain dietary fats affects inflammatory diseases such as rheumatoid arthritis and atherosclerosis, but the mechanism of action of these fatty acids has yet to be elucidated. A central hypothesis of Project 3 of the Wake Forest and Brigham and Women's Center for Botanical Lipids is that dietary fatty acids alter gene expression in humans, leading to a decrease in the expression of inflammatory genes. As a proof-of-principle study, we initially examined the effect of a central omega-6 fatty acid, arachidonic acid (AA), on *in vitro* gene expression in HCT-116 cells. Treatment of cells with either exogenous AA or inhibitors of AA metabolism led to accumulation of unesterified AA within the cells. Additionally, these reagents induced changes in the growth characteristics of the cells in a transcriptionally-dependent manner. Gene array analysis revealed that both exogenous AA and inhibitors that raise intracellular AA levels alter the expression of similar genes. More specifically, the majority of genes altered play a critical role in controlling cell growth and apoptosis. These *in vitro* studies reveal that generation of intracellular AA and its subsequent impact on gene expression may represent a critical step that regulates the biology of AA.

These *in vitro* studies set the stage for carefully controlled *in vivo* studies in humans. Humans were placed on background diets to control fat and other nutrient intake for.5 weeks. The volunteers were given daily borage and fish oil supplements for 4 weeks. Data examining fatty acid content revealed that supplementation caused marked alteration in circulating levels of key polyunsaturated fatty acids that we have hypothesized will regulate *in vivo* gene expression. Complete blood counts with differential leukocytes were done each week to ensure the stability of the blood cell populations. RNA was isolated from the peripheral blood mononuclear cells and microarray analysis was performed. Preliminary data in a small number of volunteers suggest that the fatty acid combination used in this supplement regulates a number of key pro-inflammatory and pro-allergic genes. Of particular interest was the down-regulation of several interleukins, including tumor necrosis factor, and the histamine receptors HRH1, HRH2, and HRH3, as well as the pancreatic group 1B phospholipase A_2. Taken together, both the *in vitro* and *in vivo* data suggest that altering fatty acid levels may influence inflammatory disease processes via their ability to control expression of genes involved in cell growth, apoptosis, and inflammation.

Poster # 40

Expression, purification and circular dichroism spectral analysis of *Pseudomonas* ceramidase

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Ceramidases (CDases) hydrolyze ceramide to sphingosine and fatty acid. *Pseudomonas* CDase (pCDase) is a homologue of mammalian neutral ceramidases and may play roles in diseases, such as atopic dermatitis.

In this study, the full length CDase was cloned from *Pseudomonas* aeruginosa strain PAO1 and expressed in *E. coli*. The expressed recombinant pCDase was solubilized by optimizing the culture conditions, and several factors, including culture medium, the concentration of IPTG, temperature and time of induction, were identified to be critical for the optimal production of recombinant pCDase. The recombinant pCDase was purified using Ni-NTA affinity, phenyl-Sepharose, and Q-Sepharose column chromatography, which gave an overall yield of 0.45 mg purified protein per liter of starting culture. Biochemical characterization of the recombinant pCDase showed that the enzyme followed classical Michaelis-Menten kinetics, with an optimum activity in the neutral pH range. Both the hydrolytic and reverse activities of CDase were stimulated by calcium with K_{Dapp} of 1.5 μM. Kinetics studies showed that calcium caused a decrease of Km and an increase in Vmax of pCDase. Both calcium and D-*erythro*-sphingosine caused significant changes in the near UV (250-320 nm) but not the far UV CD spectra. This change was inhibited in the presence of EGTA. These results identify important interactions between calcium and pCDase which may play an essential role in the interaction of pCDase and its substrate.

Poster # 41

Characterization of a Novel Splice Variant of $iPLA_2$

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Group VI phospholipase A_2 ($iPLA_2$) is a homeostatic enzyme that has a variety of roles in basal lipid metabolism. The $iPLA_2$ pre-mRNA undergoes alternative splicing to generate a variety of splice variants, with varying levels of catalytic activity. An $iPLA_2$ specific inhibitor, BEL, inhibits proliferation of the ovarian cancer cell lines SKOV-3, OVCAR-3 and Dov-13, indicating that $iPLA_2$ activity is required for proliferation. We hypothesize that a novel, highly active isoform of the enzyme provides this essential $iPLA_2$ activity for ovarian cancer cells. SKOV-3 and Dov-13 cells exhibit high levels of $iPLA_2$ activity, but activity is more modest in OVCAR-3 cells. Interestingly, the well characterized ~85 kDa $iPLA_2$ is nearly absent in Dov-13 cells, but present in OVCAR-3 cells despite their low levels of $iPLA_2$ activity. In contrast, a larger (~120 kDa) protein is observed in SKOV-3 and Dov-13 but not OVCAR-3 cells. This protein cross reacts with two anti-$iPLA_2$ antibodies, suggesting that it is an isoform of $iPLA_2$. Together, these data suggest that the ~120kDa protein is derived from a novel splice variant and the $iPLA_2$ activity of ovarian cancer cell lines is largely dependent on this protein. At present, we are further characterizing the novel $iPLA_2$ protein and performing RACE experiments to determine if it is indeed encoded by a novel splice variant of the $iPLA_2$ pre-mRNA.

Poster # 42

Group V secretory PLA2 regulates TLR2-dependent eicosanoid generation in mouse mast cells through amplification of ERK activation and cPLA2 alpha phosphorylation

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Mouse bone marrow-derived mast cells (BMMC) may be activated through Toll-like receptor 2 (TLR2) for the dose- and time-dependent release of eicosanoids. However, the signaling mechanisms of TLR-dependent rapid eicosanoid generation are not known. We have previously reported that eicosanoid generation from mouse macrophages in response to zymosan, that signals in part through TLR2, is attenuated in mice lacking group V secretory phospholipase A2 (sPLA2). We therefore examined the mechanisms of rapid eicosanoid generation in BMMC in response to the TLR2 ligand, PAM3CSK4 with particular focus on the role of group V sPLA2.

The release of leukotriene (LT) C4 and prostaglandin (PG) D2 was maximal 60 minutes after stimulation of mouse BMMC with 3 microgram/ml PAM3CSK4 and were attenuated ~50% in group V sPLA2-null BMMC compared to wild type BMMC. LTC4 and PGD2 generation in response to Pam3CSK4 was absent in BMMC lacking cPLA2 alpha. Inhibition of MEK by UO126 blocked phosphorylation of ERK1/2, phosphorylation of cPLA2 alpha and the immediate phase of eicosanoid generation in response to Pam3CSK4. Furthermore, the phosphorylation of ERK1/2 and cPLA2 alpha and the generation of LTC4 and PGD2 were absent in response to Pam3CSK4 in TLR2-null BMMC and MyD88-null BMMC. In both wild type and group V sPLA2-null BMMC, the phosphorylation of ERK1/2 and of cPLA2 alpha was detectable within 1 min and 2 min, respectively. However, the further rise in phosphorylation and the sustained phosphorylation of ERK1/2 and of cPLA2 alpha were markedly attenuated in group V sPLA2-null BMMC.

These findings provide the first dissection of the mechanisms of TLR-dependent rapid eicosanoid generation, which is MyD88-dependent, requires cPLA2 alpha, and is amplified by group V sPLA2 through its ability to augment the sequential phosphorylation and activation of ERK1/2 and cPLA2 alpha.

Poster # 43

Cell wounding activates PLD in primary mouse keratinocytes.

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Skin is the largest organ in the body and is the first barrier of defense against environmental insults. Wounding of the keratinocytes, the major cell type of the epidermis of the skin, is a routine phenomenon but repair involves complex downstream signaling events. We hypothesized that wounding of primary mouse keratinocytes would activate phospholipase D (PLD), which is known to be involved in vesicle trafficking, and that this activation would be decreased by curcumin, a natural component of curry. Activated PLD catalyzes the hydrolysis of phospholipids to produce phosphatidic acid (PA), which can be dephosphorylated by lipid phosphate phosphatases to produce diacylglycerol (DAG). Both PA and DAG are known to modulate apoptosis and cell survival. Primary mouse keratinocytes were pre-labeled with [^{3}H] oleic acid for 20-24 hours. Wounding was induced by scraping the cells from the dish. As a control for loss of adhesion, cells were also treated with 100 µl of trypsin. Ethanol at a concentration of 0.5 % was added to take advantage of the transphosphatidylation reaction catalyzed by PLD as a means of measuring PLD activity. Lipids were extracted into chloroform/methanol after 15 minutes, separated by thin-layer chromatography, and radioactivity in phosphatidylethanol (PEt) was quantified using a scintillation counter. As we hypothesized, wounding of primary mouse keratinocytes led to activation of PLD and our preliminary results suggest that this activation was decreased by curcumin pre-treatment. We propose that wound repair may involve activation of PLD, and curcumin may reduce this activation thus decreasing injured cell survival.

Poster # 44

Palmitic Acid Treatment Alters Lipid Profiles and Gene Expression in C2C12 Myotubes

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Elevated plasma free fatty acid (FFA) concentration is a key factor in developing insulin resistance (IR), an early step in the progression to a variety of pathologies, including hypertension, dyslipidemias, cardiovascular disease, and type 2 diabetes mellitus. However, the mechanism by which elevated FFA precipitate IR remains obscure. A major site of FFA-induced IR is skeletal muscle. Interestingly, recent data suggest that FFA oversupply perturbs cellular lipid metabolism and leads to the aberrant production of lipid mediators which interfere with appropriate insulin response in human and rodent skeletal muscle and tissue culture models of skeletal muscle including C2C12 myotubes.

Previous studies from our group have demonstrated the ability of sphingolipids to participate in gene regulation. Thus, we propose one mechanism of FFA-induced IR is the FFA-mediated production of sphingolipids, and this aberrant sphingolipid metabolism leads to alterations in gene regulation. Mouse C2C12 myoblasts were differentiated to contractile myotubes by standard methods. The myotubes were treated with palmitic acid conjugated to bovine serum albumin, with or without pretreatment with myriocin, an inhibitor of de novo sphingolipid synthesis. Over a 24 hour time course of treatment, samples were taken at various points for lipid extraction and RNA isolation for microarray hybridization. Lipid fractions were subjected to high-throughput quantative LC/MS to measure diverse lipid species. The results indicated that fatty acid treatment caused accumulation of ceramides, dihydroceramides, and diacylglycerols; However, sphingomyelin pools were not affected, indicating that ceramide formed upon fatty acid treatment is not incorporated into sphingomyelin. Importantly, the majority of the sphingolipids were inhibited by treatment with myriocin. Also of interest was that myriocin treatment augmented DAG synthesis at early time points, suggesting that DAG synthesis may initially compete with de novo sphingolipid synthesis for fatty acid pools.

Initial characterization of sphingolipid-dependent gene misregulation reveals global changes in gene transcription involving broad categories including lipid metabolism and insulin and cytokine signaling. Novel computational methods are currently in use to determine correlations between lipid levels and gene expression and thereby delineate sphingolipid-specific effects on gene regulation. Moreover, biological outcomes are being assayed to determine the impact of sphingolipid-mediated gene misregulation on skeletal muscle function.

Poster # 45

Involvement of PLD2 in Lysophosphatidic Acid Production by Ovarian Carcinoma Cells

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Lysophosphatidic acid (LPA) refers to a family of small lipid mediators involved in diverse cellular processes. LPA binds to G-protein coupled receptors to induce cellular effects that include proliferation, angiogenesis, and migration and metastasis of tumor cells. Levels of LPA have been shown to be increased in the acites fluid surrounding ovarian tumors. Several enzymes are potentially involved in the production and metabolism of LPA, including phospholipase D (PLD), autotaxin (ATX), and acylglycerol kinase (AGK). However, the pathways and mechanisms that regulate LPA production are poorly understood. We have shown that epidermal growth factor (EGF) can stimulate LPA production. This study addresses the hypothesis that EGF stimulates PLD2 to increase LPA production in ovarian cancer cells. We found that LPA levels in the medium increased within 30 minutes after addition of EGF to OVCAR3 cells. OVCAR3 cells have basal and EGF-stimulated PLD activity. Over-expression of PLD2 increases PLD activity, as does EGF, in whole cell and membrane preparations. PLD2 over-expression also increases basal levels of LPA production. Small-interfering RNA's were used to down-regulate PLD2. These siRNAs downregulated membrane PLD activity and decreased EGF-stimulated LPA production in OVCAR3 cells. Together, these results suggest that PLD2 plays a critical role in EGF-stimulated LPA production (Supported by DAMD17-01-1-0730).

Poster # 46

Lysophosphatidic Acid Stimulates Differentiation and Survival in Rat Growth Plate Chondrocytes

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Lysophosphatidic acid (LPA) is a bioactive lipid that promotes cell growth, survival, and migration by activating cell surface G protein-coupled receptors. Growth plate chondrocytes respond to 24,25-dihydroxyvitamin D3 [24R,25$(OH)_2D_3$] which increases phospholipase D activity. Currently, little is known about the role of LPA signaling in chondrogenesis. To investigate the potential role of LPA in this process, we used rat growth plate chondrocytes as a model system. These cells were shown to express the LPA receptors LPA3, LPA4, and LPA5. Additionally, 24R,25$(OH)_2D_3$ increased the abundance LPA secreted by the cells. LPA treatment also promoted an increase in two markers of chondrocyte differentiation: alkaline phosphatase activity and proteoglycan sulfation. Lastly, treatment of the cartilage cells with LPA decreased abundance of p53, a protein that has been implicated in the promotion of cancer cell survival. Collectively, these results indicate a role for LPA in chondrogenesis by stimulating the differentiation and survival of growth plate chondrocytes.

Poster # 47

Agonist Activation of G-Protein Coupled Receptors (GPCRs) Induces formation of the Protein Kinase C (PKC)-dependent Pericentrion.

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The PKC family of isoenzymes has been shown to regulate a variety of cellular processes, including receptor desensitization and internalization. Recent studies have also identified a novel translocation of PKC to a subset of Rab11-positive recycling endosomes, named the pericentrion. It has been demonstrated that sustained activation of PKC (longer than 30 minutes) with 4-phorbol-12-myristate-13-acetate (PMA) globally affected endocytic trafficking; the observed sequestration involved not only PKC itself but also various plasma membrane lipids and proteins. In this study we demonstrate that agonist-induced internalization of GPCRs involves pericentrion formation. We used C6 glioma cells expressing endogenous 5HT2A receptors and HEK293 cells overexpressing YFP-5HT2A. We demonstrated that agonist treatment (1μM serotonin) results in the sequestration of the 5HT2A receptors into Rab11-positive endosomes. This sequestration required sustained activation and autophosphorylation of classical PKC isoform. Importantly, treatment with GPCR agonists induced sequestration of various recycling molecules e.g. CD59 and transferrin, as well as PKC to the same compartment. Finally, using pharmacological inhibitors we showed that signaling downstream of phospholipase D (PLD) is involved in the formation of pericentrion. Taken together, these studies point to the possibility that pericentrion formation is of critical importance to GPCR trafficking.

Poster # 48

Acylglycerol kinase mediates glioma cell motility and survival through activation of PI3K/Akt and downregulation of stress signalling

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Glioblastomas are the leading cause of CNS tumor-related death with short life expectancy, despite the wide range of treatments, including surgical removal, and radiation and chemotherapy. Gliomas are refractory to the treatment in part due to their diffuse infiltration into surrounding brain and increased pro-survival signaling. Acylglycerol kinase (AGK) is a novel lipid kinase that can produce lysophosphatidic acid (LPA) and phosphatidic acid, potent lipid mediators. LPA is known to stimulate glioma cell proliferation, migration and invasion. We have now found that downregulation of AGK with siRNA in U373MG (PTEN null, p53 null) glioblastoma cells caused a significant decrease in survival. This was partially reversed by LPA, the product of AGK, and by serum, which contains large amounts of LPA. Furthermore, downregulation of AGK profoundly decreased U373MG cell motility, affecting both the number of migrating cells and their rate of migration. Decreased migration correlated with drastically reduced lamellipodia length with concomitant appearance of multiple smaller lamellipodia throughout cell periphery rather than a single lamellipodia at the leading edge of the cell, together with fewer stress fibers. Investigation of the signaling pathways modulated by downregulation of AGK revealed that the PI3K/Akt pathway was primarily affected. Interestingly, AGK downregulation had smaller effects on survival, migration, and PI3K/Akt in PTEN-positive LN229 glioblastoma cells. PTEN disruption leads to accumulation of PI3K products and constitutive activation of Akt. Loss of AGK markedly reduced Akt phosphorylation, and increased p38 and JNK phosphorylation, without affecting ERK activation in U373MG cells. These results suggest that AGK may play an important role in glioblastoma cell survival, motility, cytoskeletal rearrangements, and basal activation of the PI3K/Akt axis. This work was supported by NIH grant 37 CA61774.

Poster # 49

Lipids isolated from bone induce migration and metastasis of breast cancer cells

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Bone is the most common site to which breast cancer cells metastasize. We found that osteoblast-like MG63 cells and human bone tissue contain the bile acid salt sodium deoxycholate (DC). MG63 cells take up and accumulate DC from the medium suggesting that the bone-derived DC originates from serum. DC released from MG63 cells or bone tissue promotes cell survival and induces migration of metastatic human breast cancer MDA-MB-231 cells. The bile acid receptor (FXR) antagonist Z-guggulsterone prevents migration of these cells and induces apoptosis. DC elevates the gene expression of FXR and induces its translocation to the nucleus of MDA-MB-231 cells. Nuclear translocation of FXR is concurrent with elevation of uPA (urokinase-type plasminogen activator) and formation of F-actin, two factors critical for the migration of breast cancer cells. In a metastasis mouse model (fluorescent 4T1 cells), systemic administration of DC increases the number of breast cancer cells recovered from the bone marrow in a dose-dependent manner. Our results suggest a novel mechanism by which DC-induced elevation of uPA and binding to the uPA receptor of the same breast cancer cell self-propels its migration and metastasis to the bone. This work is funded by the Susan Komen Breast Cancer Foundation.

Poster # 50

The Sac1p phosphoinositide phosphatase is required for maintenance of Golgi organization

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Phosphoinositides are ubiquitous and essential regulators of an array of signal transduction events in eukaryotic cells. These phospholipids are degraded by various enzymes, including phosphoinositide phosphatases. The biological functions of such phosphatases, with the exception of PTEN, are poorly characterized, however. Herein, we demonstrate that depletion of an integral membrane phosphoinositide phosphatase, Sac1p, results in reduced cellular proliferative capacity and viability in human cell lines. These phenotypes are accompanied by a remarkable disorganization of Golgi membranes that is apparent at all levels of the Golgi stack.

Poster # 51

Coenzyme Q and lipid oxidation in aging and cardiovascular disease

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Organisms produce reactive oxygen species (ROS) at all stages of their development. As organisms age, ROS production appears to increase as several important antioxidant defenses decrease. The enhanced ROS production leads to oxidation of biological macromolecules including proteins and lipids as important targets. The oxidized materials related to aging would be expected to accumulate as organisms age and the rate of such accumulation should increase with time. One such material that accumulates as individuals age and increases with time is oxidized low density lipoproteins (oxidized LDLs). They are gradually engulfed by macrophages which then accumulate in the subendothelial spaces (intima) of blood vessels where they lead to endothelial damage, development of atherosclerotic plaques, occlusion of vessels and ultimately myocardial infarction. Our work has focused on hyperactivity of a plasma membrane electron transport system resulting in cell surface generation of reactive oxygen species. The terminal oxidases are age-related NADH oxidases (arNOX), ca. 22 kDa proteins distinct from the PHOX-NOX proteins of host defense. The arNOX proteins are associated with lymphocytes and other circulating cells of patients of age 50 y or older and are capable of directly reducing ferric cytochrome c through generation of superoxide. The arNOX proteins are located at the external surface of the cell (ECTO-NOX proteins) and, as such, are shed into the circulation. Both the cell surface location and the existence of a circulating form of the activity serve as opportunities to propagate the aging cascade to cells adjacent to aging cells and to oxidize circulating lipoproteins. These arNOX proteins have been postulated, as well, to link the accumulation of lesions in mitochondrial DNA to cell surface accumulation of reactive oxygen species as one consequence of their roles as terminal oxidases in plasma membrane electron transport. Cells with functionally-deficient mitochondria become characterized by an anaerobic metabolism. NADH accumulates from the glycolytic production of ATP and an elevated plasma membrane electron transport activity becomes necessary to maintain the NAD^+/NADH homeostasis essential for survival. A feature of the aging isoform of the ECTO-NOX proteins is that the generation of superoxide by this protein and the attendant oxidation of lipoproteins leading to atherogenesis are inhibited by coenzyme Q_{10}. These findings provide a rational basis for the anti-aging activity of circulating coenzyme Q_{10} in the prevention of atherosclerosis and of the oxidative changes in cell membrane thiols. An ongoing clinical trial has provided data linking coenzyme Q_{10} supplementation with significant ablation of arNOX activity in elderly volunteers.

Poster # 52

Optimization of growth conditions for rhodomonas salina as a source of stearidonic acid

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The cardiovascular benefits of fish oil enriched in omega-3 polyunsaturated fatty acids (ω-3 PUFAs) are well-documented. Despite overwhelming evidence and strong recommendations supporting the health benefits of ω-3 PUFAs, the consumption of ω-3 PUFAs remains low in the American population, due mainly to unpleasant odor and taste of fish oil and persistent questions about environmental contaminations. A natural alternative to fish oil that could supplement the intake of ω-3 PUFA would be predicted to reduce the overall incidence in CVD. Stearidonic acid has been identified recently as the most promising alternative to fish oil for preventing cardiovascular diseases. However, the natural source of SDA is limited only in a few plants. The feasibility of growing mass quantities of these plants is questionable and the toxicology profile of these plants remains to be a concern. To solve this problem, we employed an innovative approach: exploiting SDA-rich microalgae as an alternative source of natural SDA for human consumption. We first identified four SDA-rich microalgal species with SDA contents ranging from 20% to 40% of total fatty acids. By screening the growth characteristics of these four species, we selected *Rhodomonas salina* for further studies because of its non-toxic nature and fast growth rate. To establish the optimal conditions for cell growth, comparative studies on the light intensity, growth temperature and culture media composition were conducted. The results indicated that *Rhodomonas salina* is suitable for cultivation using artificial media under controlled light intensity and growth temperature. Further studies are under way to determine whether higher SDA concentration can be achieved when cells are subjected to stress conditions, such as nitrogen deficiency.

Poster # 53

Lysophosphatidic Acid (LPA) and Angiogenesis

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The role of Lysophosphatidic Acid (LPA) in angiogenesis is uncertain. Thus goal of our study was to determine whether LPA, acting via the LPA receptors (LPA_{1-3}), evokes an angiogenic response. We used the chicken chorio-allantoic membrane (CAM) assay to evaluate LPA and LPA analogs selective for individual LPA receptors. We found that LPA elicited a significant increase in vessel number. LPA-induced angiogenesis is blocked by VPC32183, an antagonist for LPA_1 and LPA_3 receptors. Further, the LPA_3 selective agonist, S-OMPT, induced angiogenesis. An invertebrate lysophospholipase D (produces high amounts of LPA) likewise evoked angiogenesis in the CAM assay and this response was blocked by VPC32183. A catalytically inactive mutant form of the enzyme did not induce vessel growth. We conclude that LPA is angiogenic *in vivo* and that its response proceeds via activation of the LPA_1 or LPA_3 receptors, or both. Further *in vivo* and *in vitro* angiogenesis studies using mammalian systems are in progress. (Supported by R01GM052722 and 1 F31 HL79881-01)

Poster # 54

Acetyl-CoA Carboxylase and Control of Fatty Acid Synthesis in Trypanosomes

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Trypanosoma brucei is a eukaryotic parasite that causes African sleeping sickness. *T. brucei* lacks a type I fatty acid synthase and instead, relies primarily (~90%) on an elongation system (ELO) in the ER as well as a type II system in the mitochondrion (~10%). Fatty acid synthesis is initiated by acetyl-CoA carboxylase (ACC), which makes malonyl-CoA from acetyl-CoA. *T. brucei* ACC is structurally similar to the large yeast/mammalian ACCs. To examine the function of ACC, we knocked down ACC mRNA by RNA interference (RNAi). RNAi of TbACC resulted in 75-85% reduction of mRNA and an 88-98% reduction in protein, which reduced assayable ACC activity to background levels. In turn, the loss of TbACC activity reduced *in vivo* ELO activity, presumably through the loss of its substrate, malonyl-CoA. Interestingly, the loss of ACC activity and reduction in ELO activity had no effect upon growth in culture. One possible explanation may be that TbACC is not essential and/or that *T. brucei* has an alternative metabolic source of malonyl-CoA. We are addressing this possibility by constructing a conditional KO strain for TbACC. Another explanation is that *T. brucei* can metabolically compensate enough for the low level of residual ACC activity to allow for normal growth in culture. One enzyme that could function in this metabolic compensation is malonyl-CoA decarboxylase (MCD), which degrades malonyl-CoA to acetyl-CoA and CO_2. We have just begun a genetic and biochemical characterization of TbMCD and how its action may coordinate with ACC in controlling cellular malonyl-CoA levels and thus, fatty acid synthesis.

Poster # 55

Polyunsaturated Fatty Acids Downregulate MHC Class I Antigen Presentation: A Potential Drawback for Immunotherapy

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Polyunsaturated fatty acids (PUFAs) with immunosuppressive effects are recognized as potential therapeutic agents for treating symptoms associated with inflammatory and/or autoimmune disorders. However, it is unclear if adjuvant PUFA immunotherapy can also modify susceptibility to infection. Here we show that PUFAs downregulate immune responses of antigen presenting cells (APCs) and consequent T cell responses by altering MHC class I antigen display and APC-T cell conjugate formation. Relative to controls, treatment of APCs with the ω-6 PUFA arachidonic acid (AA) or the ω-3 PUFA docosahexaenoic acid (DHA) significantly lowers susceptibility of PUFA-modified cells to lysis by alloreactive cytotoxic $CD8^+$ T lymphocytes. MHC class I surface expression is lowered by ~15% upon PUFA modification due to a reduction in the rate of forward trafficking of newly synthesized MHC class I molecules from the ER to Golgi; however, calibration experiments show that a reduction in surface expression is not sufficient to completely account for the change in lysis. PUFA treatment reduced the rate of APC-T cell conjugate formation by ~30%, sufficient to account for the reduction in lysis. Our data show for the first time that a ω-6 and a ω-3 PUFA alter MHC class I mediated antigen presentation which suggest that elimination of pathogen derived peptides by APCs could be compromised by using PUFAs as immunosuppressants. Additionally, PUFA induced changes in ER-Golgi trafficking of MHC class I molecules point to a new area of lipid modulation of immune responses.

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Poster # 56

Angiotensin II activates protein kinase D in primary adrenal glomerulosa cells: Is there a role for this enzyme in aldosterone secretion

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Aldosterone (Aldo) is a steroid hormone responsible for salt balance, and therefore is necessary for regulation of blood volume and blood pressure. The lipid-dependent signaling pathways involved in Aldo secretion remain unclear. Currently under study in our laboratory is the role of diacylglycerol-responsive protein kinase D (PKD) in Aldo secretion. In order to determine the effects of various Aldo secretagogues on PKD activation, we treated primary fetal bovine adrenal glomerulosa cells (AG cells) with angiotensin II (Ang II; 10nM), potassium (K^+; 15mM), 12-O-tetradecanoylphorbol-13-acetate (TPA; 100nM), and adreno- corticotrophic hormone (ACTH; 10nM). We observed that each of these compounds stimulated aldosterone secretion significantly. Interestingly, Ang II, and TPA acutely (5, 10 and 30 minutes) increased phosphorylation of serine 916, an indirect measure of PKD activation. Conversely, both the K^+ and the ACTH had no effect on PKD serine 916 phosphorylation. These observations were confirmed using an *in vitro* PKD kinase activity assay. In order to further understand Ang II's action on PKD activation, we ablated either Ang II AT-1 receptor signaling with candesartan (Cand; 10μM) or AT-2 receptor signaling with PD-123319 (PD; 10μM). We observed that Cand, but not PD, diminished Ang II-stimulated aldosterone secretion and serine 916 phosphorylation. Thus, PKD is activated by the aldo secretagogue Ang II through an AT-1 receptor-mediated signaling mechanism. In an effort to further elucidate PKD's role in a primary biological system, we have inserted wild type PKD, a non-activatable PKD mutant and a consititutively active PKD mutant DNA constructs into an adenovirus vector. We will then introduce these constructs into AG cells and monitor Ang II-induced aldosterone secretion. Because of PKD's activation by Ang II, we hypothesize that PKD has a role in aldosterone secretion.

Poster # 57

Aberrant expression of lysophosphatidic acid (LPA) receptors in human colorectal cancer

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Lysophosphatidic acid (LPA) is a simple bioactive phospholipid with diverse effects on various cells, that interacts with three G protein-coupled transmembrane receptors, LPA1, LPA2, and LPA3. The expression pattern and functions of these LPA receptors in various tumors have not been fully examined, except in ovarian cancer. To evaluate the LPA receptor expression profile in human colorectal cancer and in normal mucosa, we used real-time RT-PCR and measured the expression levels of LPA1, LPA2, and LPA3 mRNA in 26 colorectal cancers and 16 corresponding normal tissue samples. Normal epithelium expressed both LPA1 and LPA2 mRNA at similar levels. In comparison, colorectal cancers expressed LPA1 mRNA at a significantly lower level (0.3-fold; $p<0.05$), and LPA2 mRNA at a significantly higher level (3-fold; $p<0.05$), as compared with normal tissues. Thus, the ratio of LPA2 / LPA1 increased markedly during malignant transformation (18-fold increase). LPA3 mRNA was expressed at only a low level in both normal and cancer tissues. We also assessed LPA2 expression immunohistochemically using a rat anti-LPA2 monoclonal antibody, and confirmed high expression of LPA2 in colorectal cancer at the protein level. As for LPA1, we examined western blot analysis for 16 matched normal and cancer tissues. It revealed a significant decrease in the expression of LPA1 protein in cancer tissues compared to normal mucosa in 9 of 16 cases, and in the remaining 7 cases the expression levels was much the same. These results suggested that alteration of LPA receptor expression might be an important event in the development of colorectal cancer, and therefore, LPA and its receptors could be a chemopreventive target against colorectal cancer.

Poster # 58

Activation of sphingosine kinase type 2 by phosphorylation

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Sphingosine 1-phosphate, a potent lipid mediator, is a ligand for a family of five G protein-coupled receptors ($S1P_{1-5}$) that have been shown to regulate a variety of biological responses important for cancer progression. The cellular level of S1P is low and tightly regulated in a spatio-temporal manner through its synthesis catalyzed by two sphingosine kinases, denoted SphK1 and SphK2. SphK1 plays an important role in cancer while SphK2 has been implicated in lymphocyte trafficking. Many stimuli activate and translocate SphK1 to the plasma membrane by mechanisms that are dependent on its phosphorylation. Much less is known about activation of SphK2. We have now demonstrated that EGF as well as the protein kinase C activator, phorbol ester, induce rapid phosphorylation of hSphK2 which was markedly reduced by inhibition of MEK1/ERK pathway. Recombinant ERK1 was phosphorylated by hSphK2 *in vitro* and increased its enzymatic activity. ERK1 also was found to be in a complex with hSphK2 *in vivo*. Site-directed mutagenesis indicated that hSphK2 is phosphorylated on Ser351 and Thr578 by ERK1. These studies provide the first clues to the mechanism of agonist-mediated SphK2 activation and enhance understanding of the regulation of SphK2 activity by phosphorylation.

Poster # 59

A Role for the Cytoplasmic Tail of LPA_1 Lysophosphatidic Acid Receptors in the regulation of Signaling and Endocytosis

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Lysophosphatidic acid stimulates heterotrimeric G protein signaling by activating three closely related GPCRs, termed LPA_1, LPA_2, and LPA_3. The cytoplasmic tails of many GPCRs have been shown to be important in their regulation. Beta-arrestins are cytosolic proteins known to interact with the tails of many GPCRs leading to signal attenuation and localization to clathrin-coated pits for endocytosis. The cytoplasmic tail of LPA1 contains several domains, including a serine cluster, a di-cysteine motif, a di-leucine motif and a PDZ binding domain, which could regulate signaling and trafficking of LPA1. Here we show that the serine cluster in the cytoplasmic tail of LPA_1 is essential for its association with beta-arrestins, which in turn leads to signal attenuation and clathrin-dependent endocytosis of LPA_1. Mutant LPA1 receptors lacking the various domains in the tail were generated by site-directed mutagenesis. Deletion of the serine cluster region, in particular, inhibited LPA-dependent beta-arrestin binding and subsequent endocytosis. Additionally, this mutant failed to attenuate LPA-dependent phosphoinositide signaling, consistent with the lack of beta-arrestin binding.

A mutant LPA_1 receptor that lacks the PDZ binding domain at the C-terminal end failed to activate Rho-GTPases, in response to LPA stimulation, but displayed normal endocytosis, MAPK phosphorylation and phosphoinositide signaling, comparable to the wild type LPA_1 receptor. These results collectively highlight the important role of the cytoplasmic tail of LPA_1 in its regulation.

Poster # 60

Role for lysophosphatidic acid as an autocrine mediator in human breast cancer cells

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Lysophosphatidic acid (LPA) is a lipid mediator that may play an important role in growth and survival of carcinoma. In this study, LPA production and response were characterized in two human breast cancer cell lines: MCF-7 and MDA-MB-231. Both cell lines express mRNA for LPA_1, LPA_2, and LPA_3, G protein-coupled receptors for LPA. At 10μM, 18:1 LPA potentiates both directed (chemotactic) and random (chemokinetic) migration of MDA-MB-231 cells but has no effect on migration of MCF-7 cells. Both MCF-7 and MDA-MB-231 cells generate LPA; LPA levels in the medium are increased by exogenous 18:1 LPA. Both MCF-7 and MDA-MB-231 cells proliferate in response to 18:1 LPA. 18:1 LPA stimulates the activation of Erk and Akt kinases in both cell lines. LPA-induced activations of Erk and Akt kinases, as well as proliferation, are inhibited by Ki16425 and VPC32183, antagonists for LPA_1/LPA_3. These results demonstrate that 18:1 LPA can act as an autocrine mediator in breast cancer cells, binding to G-protein-coupled receptors to elicit numerous biological responses, such as migration and growth. (Supported by DAMD17-01-1-0730).

Poster # 61

HIV Protease inhibitors disrupt hepatic lipid metabolism

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HIV-protease inhibitors (PIs) have been successfully used in highly active anti-retroviral therapy (HAART) for HIV-1 infection. However, disorders of lipid metabolism associated with HAART therapy have become a major concern in clinic since the introduction of HIV PIs. The exact mechanisms underlying HIV-PI-induced dysregulation of lipid metabolism are still unclear. In exploring the direct effects of HIV-PIs on lipid metabolism, it has become apparent that individual HIV-PIs have distinct effects on lipid metabolism through different mechanisms. Liver is the major organ responsible for maintaining lipid homeostasis in the body. In the current study, we examined the effects of three HIV PIs, including amprenavir, atazanavir, and ritonavir, on the UPR activation and the expression of key genes involved in lipid metabolism in primary rodent hepatocytes. ***Methods:*** Studies were conducted in rat primary hepatocytes. Individual HIV-PI at various concentrations (5 to 100 μM) was used, and total RNA was isolated at different times after treatments. Stead-state mRNA levels encoding key enzymes in cholesterol biosynthesis (HMG CoA reductase, HMG-CoAR), transport (low density lipoprotein receptor, LDLR), and bile acid biosynthesis (cholesterol 7α-hydroxylase, CYP7A1) were measured by RNase protection assays and real time RT-PCR. Bile acid synthesis was measured by using [^{14}C]-labeled cholesterol. ***Results:*** Exposure of primary hepatocytes to the individual HIV-PI had different effects on mRNA levels of CYP7A1 and LDLR. Both atazanavir and ritonavir activated the UPR, induced apoptosis, and increased nuclear SREBP levels, but amprenavir had no significant effect at the same concentrations. In rat primary hepatocytes, cholesterol 7α-hydroxylase (CYP7A1) mRNA levels were significantly decreased by atazanavir (38%) and ritonavir (56%) but increased by amprenavir (90%); 3-hydroxy-3-methyl-glutaryl-Coenzyme A reductase (HMG-CoA-R) mRNA levels were increased by amprenavir (23%), but not by ritonavir and atazanavir; low density lipoprotein receptor (LDLR) mRNA was increased by atazanavir (20%), but not by amprenavir and ritonavir. Similar results were obtained in mouse primary hepatocytes. Atazanavir and ritonavir also decreased CYP7A1 protein levels and bile acid biosynthesis, while amprenavir had no significant effect. ***Conclusions:*** These results indicate that treatment with various HIV-PIs disrupts cellular cholesterol homeostasis by decreasing LDLR and CYP7A1 expression and inhibiting bile acid synthesis in hepatocytes and provide critical information for a better understanding of the cellular mechanisms of HIV-PI-associated dyslipidemia.

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