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V.) MASS SPECTROMETRY OF LIPID ANTIGENS

A.) Recognition of Lipid Antigens by Natural Killer T Cells

A.1.) **Immunologic glycosphingolipidomics and NKT cell development in mouse thymus.** Immunologic glycosphingolipidomics and NKT cell development in mouse thymus. Invariant NKT cells are a hybrid cell type of Natural Killer cells and T cells, whose development is dependent on thymic positive selection mediated by double positive thymocytes through their recognition of natural ligands presented by CD1d, a nonpolymorphic, non-MHC, MHC-like antigen presenting molecule. Genetic evidence suggested that β-glucosylceramide derived glycosphingolipids (GSLs) are natural ligands for NKT cells. N-butyldeoxygalactonojirimycin (NB-DGJ), a drug that specifically inhibits the glucosylceramide synthase, inhibits the endogenous ligands for NKT cells. Furthermore, a β-linked glycosphingolipid, isoglobotriaosylceramide (iGb3), has been found to be a stimulatory NKT ligand. iGb3 synthase knockout mice have a normal NKT development and function, indicating that other ligands exist and remain to be identified. Here a glycosphingolipidomics study of mouse thymus was performed, and mutants were studied that are deficient in β-hexosaminidase b or α-galactosidase A, which are two glycosidases that are up- and downstream agents of iGb3 turnover, respectively. A first database for glycosphingolipids expressed in mouse thymus that are specifically regulated by rate-limiting glycosidases was generated by mass spectrometric analyses. Among identified thymic glycosphingolipids, only iGb3 is a stimulatory ligand for NKT cells, suggesting that large-scale fractionation, enrichment and characterization of minor species of glycosphingolipids are necessary for identifying additional ligands for NKT cells. These results provide insights into cellular lipidomics with a specific focus on glycosphingolipid immunological functions. (*J. Proteome Research* 2009; 8: 2740–2751).
Figure 2. Expression of iGb3 and iGb4 in mouse thymus.

Neutral GSLs from wild-type, Hexb KO, and Fabry mice were extracted, permethylated, and analyzed by ion trap mass spectrometry. MS\(^1\) ions representing regioisomers of iGb3/Gb3 were selected for MS\(^4\) analysis. MS\(^1\) ions representing regioisomers of iGb4/Gb4 were selected for MS\(^5\) analysis. Percentages of iGb3 in iGb3/Gb3 mixtures were calculated with reference to a standard curve.

(See figure on next page)

Figure 3. Increased ratio of iGb3 in Gb3 synthase KO mice.

Neutral GSLs from wild type, Gb3 synthase\(^{+/−}\) and Gb3 synthase\(^{−/−}\) mice were extracted, permethylated, and analyzed by ion trap mass spectrometry. MS\(^1\) ions representing regioisomers of iGb3 and Gb3 were selected for MS\(^4\) analysis. Percentages of iGb3 in iGb3 and Gb3 mixtures were calculated by reference to a standard curve.
Gb3 KO -/

iGb3/(Gb3+iGb3)
26.6%

Gb3 KO +/

iGb3/(Gb3+iGb3)
5.5%

Wild type

iGb3/(Gb3+iGb3)
1.7%
A.2.) Invariant natural killer T cells recognize lipid self antigen induced by microbial danger signals. Invariant natural killer T cells (iNKT cells) have a prominent role during infection and other inflammatory processes, and these cells can be activated through their T cell antigen receptors by microbial lipid antigens (2). Increasing evidence shows that they are also activated in situations in which foreign lipid antigens would not be present, which suggests a role for lipid self-antigen. We found that an abundant endogenous lipid, β-D-glucopyranosylceramide (β-GlcCer), is a potent iNKT cell self-antigen in mouse and human and that its activity depended on the composition of the N-acyl chain. Furthermore, β-GlcCer accumulates during infection and in response to Toll-like receptor agonists, contributing to iNKT cell activation. Thus, recognition of β-GlcCer by the invariant T cell antigen receptor is proposed to translate innate danger signals into iNKT cell activation (2). (Nat Immunol. 2011; 12: 1202-11)

Figure 4. β-GlcCer is present in primary lymphoid tissues and activates iNKT cells.
(a) TLC analysis of polar lipids from mouse thymus, spleen, whole liver and BMDCs with GSL standards and bovine milk β-GlcCer dose titration. Arrow indicates mobility of β-GlcCer. (b) ELISA of IL-2 production by the iNKT hybridoma DN32 cultured together with RAW cells or CD1d-transfected RAW cells as APCs, with lipid fractions from mouse thymus and spleen, or bovine milk β-GlcCer (fivefold dose titration to a top concentration of 20 µg/ml). (c,d) ESI-MS analysis of β-GalCer purified from thymus (c) and spleen (d), assessed in the electrospray-positive mode and presented relative to the most abundant species, set as 100 (m/z, mass/charge). Major β-GlcCer ions are presented with a lithium adduct; fatty acid composition (determined by collision-induced dissociation tandem mass spectrometry) is in parentheses. (e) Structures of two abundant β-GlcCer forms detected by ESI-MS.
A.3.) Innate and cytokine-driven signals, rather than microbial antigens, dominate in natural killer T cell activation during microbial infection. Invariant natural killer T cells (iNKT cells) are critical for host defense against various microbial pathogens, but the central question of how iNKT cells are activated by microbes is not fully understood (3). The example of adaptive MHC-restricted T cells, studies using synthetic pharmacological α-galactosylceramides, and the recent discovery of microbial iNKT cell ligands have all suggested that recognition of foreign lipid antigens is the main driver for iNKT cell activation during infection, but when the role of microbial antigens versus innate cytokine-driven mechanisms were compared, it was found that iNKT cell interferon-γ production after in vitro stimulation or infection with diverse bacteria overwhelmingly depended on toll-like receptor-driven IL-12. Importantly, activation of iNKT cells in vivo during infection with Sphingomonas yanoikuyae or Streptococcus pneumoniae, pathogens which are known to express iNKT cell antigens and which require iNKT cells for effective protection, also predominantly depended on IL-12. Constitutive expression of high levels of IL-12 receptor by iNKT cells enabled instant IL-12-induced STAT4 activation, demonstrating that among T cells, iNKT cells are uniquely equipped for immediate, cytokine-driven activation. These findings reveal that innate and cytokine-driven signals, rather than cognate microbial antigen, dominate in iNKT cell activation during microbial infections (3). (*J. Exp. Med.* 2011; 208: 1163-77.)

![Fig. 5. Detection of microbial lipid antigens expressed by bacteria.](image)

Lipids were extracted from bacteria and analyzed by ESI-MS. (A–C) [M-H]⁻ adducts of the GSL-1 (GlcAGSL) antigen in Sphingomonas capsulata, N. aromaticivorans, & S. yanoikuyae. (D) [M+CH₃COO]⁻ adducts of the GalDAG (BbGL-II) antigen in Borrelia burgdorferi. (E) [M+Na]⁺ adducts of GlcDAG and GalGlcDAG antigens in Streptococcus pneumoniae.

![Fig. 6. Innate & cytokine-driven iNKT cell activation during microbial infection.](image)

iNKT cell activation during microbial infection is dominantly driven by innate TLR-mediated signals and IL-12, which is released by dendritic cells after stimulation with microbial products. TCR-mediated stimulation also contributes to iNKT cell activation, but the TCR-mediated signal alone, provided either by recognition of CD1d-presented self- or microbial antigens, is not sufficient to result in iNKT cell IFN-γ production in the absence of IL-12. Constitutive expression of high levels of IL-12 receptor endows iNKT cells with ability to respond rapidly to cytokine-mediated stimulation and ensures immediate iNKT cell activation in response to virtually any infectious agent that induces IL-12 production, irrespective of expression of microbial lipid antigens. This allows iNKT cells to overcome their restricted TCR specificity.
A.4.) Structural elucidation of diglycosyl diacylglycerol and monoglycosyl diacylglycerol from *Streptococcus pneumoniae* by multiple-stage linear ion-trap mass spectrometry with electrospray ionization. The cell wall of the pathogenic bacterium *Streptococcus pneumoniae* contains glucopyranosyl diacylglycerol (GlcDAG) and galactoglucofuranosyl diacylglycerol (GalGlcDAG). GlcDAG species with sn-2 vaccenic acid substituents were recently demonstrated to be recognized by invariant natural killer T-cells and thus represent glycolipid antigens. Here, we describe an electrospray ionization (ESI) linear ion-trap (LIT) multiple-stage mass spectrometric (MS^n) approach for structural characterization of GalGlcDAG and GlcDAG molecular species (4). MS^n (n = 2, 3) of [M+Li]^+ adduct ions yields product ions that permit identification of the fatty acid substituents and their positions on the glycerol backbone and location(s) of double bond(s) in the fatty acyl chain. Assignments of the identities and positions of the fatty acid substituents was confirmed by MS^n (n = 2, 3) of [M+NH4]^+ ions. The GalGlcDAG and GlcDAG species isolated from *S. pneumoniae* were found to consist of major species with...
16:1- or 18:1-fatty acid substituents mainly at the sn-2 position with an ν-7 (n-7) double bond. More than one isomer was observed for each m/z value in the family. This approach provides a simple method for structural characterization of this important lipid family that would be difficult to achieve with conventional techniques (4). *(J. Mass. Spectrom. 2012; 47: 115–123.)*

![Fig. 7. Proposed fragmentation pathways for structural characterization of 16:0/Δ11 18:1-DGDG by CAD of [M+Li]+ (*masses observed for deuterium labeled analogs).*](image)

![Fig. 8. Electrospray ionization mass spectra of the [M+Li] ions of monoglycosyl diacylglycerol (a) and diglycosyl diacylglycerol (b) isolated from Streptococcus pneumoniae.](image)
A.5.) Recognition of microbial and mammalian phospholipid antigens by NKT cells with diverse TCRs. CD1d-restricted natural killer T (NKT) cells include two major subgroups. The most widely studied are Vα14Jα18+ invariant NKT (iNKT) cells that recognize the prototypical α-galactosylceramide antigen, whereas the other major group uses diverse T-cell receptor (TCR) α- and β-chains, does not recognize α-galactosylceramide, and is referred to as diverse NKT (dNKT) cells. dNKT cells play important roles during infection and autoimmunity, but the antigens they recognize remain poorly understood. Here, we identified phosphatidylglycerol (PG), diphosphatidylglycerol (DPG, or cardiolipin), and phosphatidylinositol from Mycobacterium tuberculosis or Corynebacterium glutamicum as microbial antigens that stimulated various dNKT, but not iNKT, hybridomas. dNKT hybridomas showed distinct reactivities for diverse antigens (5).

Stimulation of dNKT hybridomas by microbial PG was independent of Toll-like receptor-mediated signaling by antigen-presenting cells and required lipid uptake and/or processing. Furthermore, microbial PG bound to CD1d molecules and plate-bound PG/CD1d complexes stimulated dNKT hybridomas, indicating direct recognition by the dNKT cell TCR. Interestingly, despite structural differences in acyl chain composition between microbial and mammalian PG and DPG, lipids from both sources stimulated dNKT hybridomas, suggesting that presentation of microbial lipids and enhanced availability of stimulatory self-lipids may both contribute to dNKT cell activation during infection (5). (Proc. Nat’l. Acad. Sci. U.S.A. 2013; 110: 1827–1832).

(See Figure on Next Page). Figure 10. Isolation of stimulatory Mtb lipids. (A) Using DEAE cellulose columns, Mtb polar lipids were separated into 10 semi-preps (SP) that were tested for stimulation of 14S.10 cells. (B) Lipids from fraction 4.3 were further separated by preparative 1D & 2D TLC, resulting in the stimulatory 4.3.2 Mtb lipid. (C & D) MS analyses revealed lipid 4.3.2 (C) to be various molecular species of diradyl-glycerophosphoglycerol (GPG) lipids (D).
Fig. 11. Antigen specificities of Vα14+ iNKT, Vα10+ NKT, and dNKT cells. GSL-1 is found in *Sphingomonas* spp., Bb DAG in *Borrelia burgdorferi*, and Ms α-GlcA DAG in *Mycobacterium smegmatis*. 
Figure 12. Cg lipids stimulate dNKT hybridomas. (A) Polar or apolar Cg lipids were tested for reactivity of dNKT hybridomas as in Fig. 1B. (B) Cg polar lipids were separated by 2D TLC and analyzed for reactivity with 14S.10 cells. (C–E) MS analysis revealed (C) the stimulatory lipid p6 to be 18:1/16:0-GPG (C) and the active lipid p5 (D) to be cardiolipin (CL) (E). (F) Stimulation of 14S.10 cells in the presence of RAW.CD1d or RAW.uit cells and purified Mtb or Cg GPG.
VI.) MASS SPECTROMETRY OF PRODUCTS OF LIPID METABOLIZING ENZYMES INVOLVED IN METABOLIC REGULATION

A.) Fatty Acid Synthase

A.1.) New hepatic fat activates PPARα to maintain glucose, lipid, and cholesterol homeostasis. De novo lipogenesis is an energy-expensive process whose role in adult mammals is poorly understood. Here, mice were generated with liver-specific inactivation of fatty-acid synthase (FAS), a key lipogenic enzyme. On a zero-fat diet, FASKOL (FAS knockout in liver) mice developed hypoglycemia and fatty liver, which were reversed with dietary fat (6). These phenotypes were also observed after prolonged fasting, similarly to fasted PPARα-deficiency mice. Hypoglycemia, fatty liver, and defects in expression of PPARα target genes in FASKOL mice were corrected with a PPARα agonist. On either zero-fat or chow diet, FASKOL mice had low serum and hepatic cholesterol levels with elevated SREBP-2, decreased HMG-CoA reductase expression, and decreased cholesterol biosynthesis; these were also corrected with a PPARα agonist. These results suggest that products of the FAS reaction regulate glucose, lipid, and cholesterol metabolism by serving as endogenous activators of distinct physiological pools of PPARα in adult liver (6). (Cell Metabolism 2005; 1: 309-322).

Fig. 13. Targeting the fatty-acid synthase gene. A) Wild-type, targeting vector, and fatty-acid synthase (FAS) lox allele before & after recombination. Post-Cre allele depicts deletion of exons 4–8 (gray shaded boxes). NEO, neomycin positive-selection cassette; open boxes, exons preserved; black arrowheads, loxP sites; open arrowheads, genotyping primers. B) Southern blot DNA analyses of control (W, lox+/− Cre+; F+, lox+/− Cre−) and FASKOL (F, lox+/− Cre+) mice. Genomic DNA (from liver, lung, heart, brain, kidney, and skeletal muscle) was digested with NdeI and HindIII and detected with probe C to yield expected 7.3 kb (allele without loxP), 8.9 kb (floxed allele), and 5.3 kb (post-Cre allele) fragments. C) PCR analyses. DNA from FASKOL livers (lox+/− Cre+) produced a 317 bp product (lane 3) indicating appropriate deletion at the FAS gene. The product was absent from livers of wt mice of various genotypes and from hearts of FASKOL mice (lane 7). D) Assessing FAS activity (upper panel) and malonyl-CoA content (lower panel). Liver homogenates from overnight-fasted 12 hr chow-refed male FASKOL (black bar) and littermate control mice of various genotypes aged 16–20 weeks were assayed for FAS activity and malonyl-CoA content. Inset of (D) represents FAS activity in lung of FASKOL (black bar) and wt (lox+/+ Cre−, white bar) mice. E) GC-MS analyses of hepatic palmitate (C16:0) and palmitoleate (C16:1) from chow and zero-fat diet (2FD) fed wt (lox+/+ Cre−) and FASKOL mice. Peak identities were verified by full EI-MS scans.
FIG. 14. Effects of PPARα activation on glucose and lipid homeostasis in FASKOL and wt (lox+/+ Cre−) mice A–J. Measurements of serum glucose (A), insulin (B), glucagon (C), insulin to glucagon ratio (D), liver glycogen (E), liver triglycerides (F), serum triglycerides (G), serum nonesterified fatty acids (H), serum total cholesterol (I), and liver cholesterol content (J) were obtained from 4 hr fasted wt and FASKOL mice on chow or zero-fat diet for 10 d (ZFD), or PPARα agonist Wy14,643 added to chow (C+Wy) or ZFD (Z+Wy) for 10 d. p < 0.05 for the following: *, compared to the corresponding wt mice; #, compared to the corresponding chow-fed mice; **, compared to the corresponding ZFD-fed mice.
A.2.) **Identification of a physiologically relevant endogenous ligand for PPARα in liver.** The nuclear receptor PPARα is activated by drugs to treat human disorders of lipid metabolism. Its endogenous ligand is unknown. PPARα-dependent gene expression is impaired with inactivation of fatty acid synthase (FAS), suggesting that FAS is involved in generation of a PPARα ligand. Here the FAS-dependent presence of a phospholipid bound to PPARα isolated from mouse liver is demonstrated (7). Binding was increased under conditions that induce FAS activity and displaced by systemic injection of a PPARα agonist. Mass spectrometry identified the species as 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphocholine (16:0/18:1-GPC). Knockdown of the enzyme Cept1, which is required for phosphatidylcholine synthesis, suppressed PPARα-dependent gene expression. Interaction of 16:0/18:1-GPC with the PPARα ligand-binding domain and coactivator peptide motifs was comparable to PPARα agonists, but interactions with PPARγ were weak and none were detected with PPARγ. Portal vein infusion of 16:0/18:1-GPC induced PPARα-dependent gene expression and decreased hepatic steatosis. These data suggest that 16:0/18:1-GPC is a physiologically relevant endogenous PPARα ligand (7). *(Cell 2009; 138: 476–488).*

**Figure 15. Model for differential effects of hepatic lipid.** Fat absorbed from the diet or synthesized de novo via FAS in the liver constitutes “new” fat, capable of activating PPARα to ensure normal glucose and lipid homeostasis. Fat derived from peripheral mobilization of adipose stores constitutes a different hepatic compartment (“old” fat) that does not appear to activate PPARα as effectively as new fat, leading to fatty liver. In contrast to de novo synthesized fat, dietary fat is inadequate for the maintenance of cholesterol homeostasis, suggesting different PPARα pools.

[(See Figure on Next Page). Figure 16. Generation of Liver-Specific FAS Knockout Mice on a PPARα-Null Background and Reconstitution of Liver PPARα Expression. (A) PCR analysis. Liver DNA was amplified using primer sets for the FAS floxed allele (top), PPARα (middle), and Cre (bottom). (B) Immunoblot analysis of liver lysates for wild-type (WT) and FASKOL mice on a PPARα null background via FAS (top) and actin (bottom) antibodies. (C and D) FAS activity (C) and malonyl-CoA content (D). Liver homogenates from overnight-fasted 12 hr chow-refed male WT and FASKOL mice on a PPARα null background were assayed. *p < 0.05. (E) Diagram for isolation of FLAG-tagged PPARα. (F) Immunoprecipitation (IP) and immunoblot (IB) analysis in livers of WT and FASKOL mice on a PPARα null background infected with adenoviruses encoding GFP alone (AdGFP) or FLAG-tagged PPARα (AdFLAG-PPARα). Nuclear fractions were immunoprecipitated with FLAG antibodies and immunoblotted with either FLAG antibody (top) or PPARα antibody (middle). Crude liver lysates were immunoblotted with actin antibody (bottom).]
Positive ion ESI/MS analyses of lithiated adducts of hepatic nuclear phospholipids were performed to monitor neutral loss of 189 [LiPO₄(CH₂)₆N(CH₃)₃], which identifies parent [MLi⁺] ions that contain the phosphocholine head-group in lipid mixtures. (A–D) Representative profiles of glycerophosphocholine (GPC) species in chow-fed WT and FASKOL mice on a PPARα null background infected with AdGFP (A and C) or AdFLAG-PPARα (B and D). (E–H) Representative profiles of GPC species in zero-fat diet (ZFD)-fed WT and FASKOL mice on a PPARα null background infected with AdGFP (E and G) or AdFLAG-PPARα (F and H). Insets in (B), (D), (F), and (H) depict the fragment ion at mass-to-charge ratio (m/z) 766 as the specific GPC species that is both PPARα and FAS dependent. (I) Quantification of the relative abundance of the m/z 766 ion with respect to genotype (W/P, WT on PPARα null background; F/P, FASKOL on PPARα null background) and diet (chow and ZFD). Each bar represents mean ± SEM from 3 independent experiments with 4–6 mice in each group per experiment. *p < 0.05 versus corresponding W/P control; **p < 0.05.

**Figure 18. Tandem MS Identifies the GPC Species as 1-Palmitoyl-2-Oleoyl-sn-Glycerol-3-Phosphocholine**

(A) Fragmentation pattern upon collisionally activated dissociation of the ion of m/z 766, which corresponds to the lithiated adduct [MLi⁺] of 16:0/18:1-GPC. (B) Expansion of the mass spectrum in (A) from m/z 400 to m/z 540 to illustrate relative abundances of ions that represent losses of fatty acid substituents. The data indicate that palmitate and oleate are the sn-1 and sn-2 substituents, respectively. (C) Structure of the putative PPARα ligand.

**Figure 19. In vivo displacement of the endogenous PPARα ligand with a PPARα agonist.** Li⁺ adducts of GPC species in excess FLAG-eluted hepatic nuclear extracts obtained from Wy14,643 (Wy)-treated mice were analyzed by positive ion ESI/MS/MS scans for neutral loss of 189, reflecting elimination of lithiated phosphocholine from [MLi⁺]. (A–D) ESI/MS/MS scans of GPC species at baseline (time 0) (A), 10 min (B), 30 min (C), and 60 min (D) after an intraperitoneal injection of 50 mg/g Wy14,643 in chow-fed WT mice on a PPARα null background injected with AdFLAG-PPARα adenovirus. (E–H) ESI/MS/MS scans of GPC species at baseline (time 0) (E), 10 min (F), 30 min (G), and 60 min (H) after the same treatment in ZFD-fed mice. Insets in (A)–(H) depict the ion m/z 766 (16:0/18:1-GPC) that is displaced in a time-dependent manner by Wy14,643. (I) Quantitation of relative abundance of m/z 766 ion in response to Wy14,643.
Figure 20. Portal vein infusion of 16:0/18:1-GPC rescues hepatic steatosis in a PPARα-dependent manner.

(A) Operative field depicting the portal vein (PV) cannulated with a catheter (pv-cath) positioned at the entry site into the liver (lvr). The catheter is intentionally marked in black ink at its proximal tip to enhance visualization. Labels indicate gall bladder (gb), bile duct (bd), inferior vena cava (ivc), and pancreas (pan).

(B) Intraportal 16:0/18:1-GPC treatment protocol. After insertion of the portal vein catheter, C57BL/6 mice (wild-type for FAS and either wild-type or null for PPARα) were allowed to recover. On day 4, chow was changed to a zero-fat diet (ZFD) and mice received 3 intraportal injections/day of 10 mg/kg 16:0/18:1-GPC sonicated in normal saline/0.5% ethanol/0.5% fatty acid-free BSA or vehicle alone. On the last day before the end of treatment (day 9), mice were fasted for 24 hr.

(C) Liver sections were stained with oil red O at the end of treatment to visualize neutral lipids from wild-type (C57/BL6) and PPARα−/− mice treated with 16:0/18:1-GPC or vehicle (Veh).

(D) Quantification of hepatic triglyceride content per unit mass of tissue from vehicle and 16:0/18:1-GPC treated C57/BL6 and PPARα−/− mice. *p < 0.05 vs. corresponding Veh. #p < 0.05 vs. C57/BL6 controls.

(E) Expression of hepatic Acox1 (ACO; top) and Cpt1a (CPT-1; bottom) mRNA by RT-PCR normalized to control L32 ribosomal mRNA after the 16:0/18:1-GPC injections. *p < 0.05 vs. corresponding Veh. #p < 0.05 vs. C57/BL6 controls.

(F) Proposed model for generation of the endogenous PPARα ligand in liver. FAS yields palmitate (C16:0), and 16:0/18:1-GPC is likely generated through the diacylglycerol (DAG) intermediate and the enzymatic activity of CEPT1 either in the ER or the nucleus. Binding of 16:0/18:1-GPC to PPARα in the nucleus activates transcription machinery (TM) turning on PPARα-dependent genes and affecting hepatic lipid metabolism. ACC, acetyl CoA carboxylase; ER, endoplasmic reticulum.
Macrophage fatty acid synthase deficiency decreases diet-induced atherosclerosis. Fatty acid metabolism is perturbed in atherosclerotic lesions, but whether it affects lesion formation is unknown. Here fatty acid synthase (FAS) was inactivated in macrophages of apoE-deficient mice to determine whether fatty acid synthesis affects atherosclerosis. Serum lipids, body weight, and glucose metabolism were the same in FAS knock-out in macrophages (FASKOM) and control mice, but blood pressure was lower in FASKOM animals (8). Atherosclerotic extent was decreased 20–40% in different aortic regions of FASKOM as compared with control mice on Western diets. Foam cell formation was diminished in FASKOM as compared with wild type macrophages due to increased apoAI-specific cholesterol efflux and decreased uptake of oxidized low density lipoprotein. Expression of the anti-atherogenic nuclear receptor liver X receptor-α (LXRα; Nr1h3) and its downstream targets, including Abca1, were increased in FASKOM macrophages, whereas expression of the potentially pro-atherogenic type B scavenger receptor CD36 was decreased. Peroxisome proliferator-activated receptor-α (PPARα) target gene expression was decreased in FASKOM macrophages. PPARα agonist treatment of FASKOM and wild type macrophages normalized PPARα target gene expression as well as Nr1h3 (LXRα). Atherosclerotic lesions were more extensive when apoE null mice were transplanted with LXRα-deficient/FAS-deficient bone marrow as compared with LXRα-replete/FAS-deficient marrow, consistent with antiatherogenic effects of LXRα in the context of FAS deficiency. These results show that macrophage FAS deficiency decreases atherosclerosis through induction of LXRα and suggest that FAS, which is induced by LXRα, may generate regulatory lipids that cause feedback inhibition of LXRα in macrophages (8). (J. Biol. Chem. 2010; 285: 23398–23409).
Figure 21. Targeting FAS in macrophages. A, top shows the targeted allele (FAS locus with loxP-flanked exons 4–8 (with the loxP sites indicated as triangles)) and the post-Cre allele. P1 and P2 represent primers used to detect a 317-bp fragment indicating Cre excision at the FAS locus. The bottom shows a simplified breeding scheme (omitting intermediate progeny) to produce FASKOM mice. B, PCR analyses of DNA from FASKOM and control mice using P1 and P2. DNA sources for both FASKOM and WT were as follows: lane 1, liver; lane 2, spleen; lane 3, lung; lane 4, kidney; lane 5, heart; lane 6, aorta; lane 7, adipose tissue; lane 8, peritoneal macrophages; and lane 9, negative control. C, FAS mRNA expression in peritoneal macrophages by quantitative RT-PCR. Data are expressed relative to L32 mRNA. *, p<0.05. D, FAS enzyme activity in peritoneal macrophages. *, p<0.001. The inset shows FAS enzyme activity in liver.
**Figure 22. Cholesterol metabolism in peritoneal macrophages.** A, Oil Red O staining of peritoneal macrophages (MØ). B, Lipid content of peritoneal MØ. *, p < 0.05. C, Electrospray ionization-mass spectrometric analysis of fatty acids from WT and FASKOM peritoneal MØ. The y-axis represents relative abundance and the x-axis m/z values. The m/z 311.3 peak is an internal standard. D, ApoAI-specific cholesterol efflux from MØ of each genotype. Cells were loaded with acetylated LDL in the presence of [3H]cholesterol and then treated with apoAI for 48 h. *, p<0.05. E, Western blot of proteins extracted from WT and FASKOM MØ and probed with an anti-ABCA1 antibody (top panel) and an anti-actin antibody (bottom panel). F, cholesterol uptake at 4 and 16 h after exposure to fluorescently labeled oxidized LDL using MØ of each genotype. *, p<0.05.

**Figure 23. Lipid content in atherosclerotic lesions.** WT and FASKOM mice were fed a high fat diet for 8 wk; mice were visually selected for differences in atherosclerosis and then the aortic arch was isolated without fixation, cleaned, and subjected to lipid extraction. For these mice, serum cholesterol at the time of sacrifice was 1747±217 mg/dl for WT and 1842±170 mg/dl for FASKO. A, cholesterol content in the aortic arch for each genotype. *, p<0.05. B, total lipid phosphorus in aortic arches. C, total triglyceride content in aortic arches. D, ESI/MS analysis of triacylglycerol species extracted from aortic arches of WT and FASKOM mice. The x-axis represents m/z values and the y-axis relative abundance in arbitrary units. The internal standard is not shown to simplify data presentation.
A.4. Inhibiting adipose tissue lipogenesis reprograms thermogenesis and PPARγ activation to decrease diet-induced obesity. *De novo* lipogenesis in adipocytes, especially with high fat feeding, is poorly understood. Here, an adipocyte lipogenic pathway encompassing fatty acid synthase (FAS) and PexRAP (peroxisomal reductase activating PPARγ) is demonstrated to modulate endogenous PPARγ activation and adiposity (9). Mice lacking FAS in adult adipose tissue manifested increased energy expenditure, increased brown fatlike adipocytes in subcutaneous adipose tissue, and resistance to diet-induced obesity. FAS knockdown in embryonic fibroblasts decreased PPARγ transcriptional activity and adipogenesis. FAS-dependent alkyl ether phosphatidylcholine species were associated with PPARγ and treatment of 3T3-L1 cells with one such ether lipid increased PPARγ transcriptional activity. PexRAP, a protein required for alkyl ether lipid synthesis, was associated with peroxisomes and induced during adipogenesis. PexRAP knockdown in cells decreased PPARγ transcriptional activity and adipogenesis. PexRAP knockdown in mice decreased expression of PPARγ dependent genes and reduced diet-induced adiposity. These findings suggest that inhibiting PexRAP or related lipogenic enzymes could treat obesity and diabetes (9). *(Cell Metab. 2012; 16: 189-201.)*
Figure 25. Cloning and characterization of the terminal component in the mammalian peroxisomal ether lipid synthetic pathway.

(A) The peroxisomal acyl-DHAP pathway of lipid synthesis. FAS, fatty acid synthase; ACS, acyl CoA synthase; G3PDH, glycerol 3-phosphate dehydrogenase; DHAP, dihydroxyacetone phosphate; DHAPAT, DHAP acyltransferase; FAR1, fatty acyl CoA reductase 1; ADHAPS, alkyl DHAP synthase; ADHAP Reductase, acyl/alkyl DHAP reductase activity; LPA, lysophosphatidic acid; AGP, 1-O-alkyl glycerol 3-phosphate. (B) Mouse DHRS7b is homologous to yeast acyl DHAP reductase, Ayr1p. TMD, transmembrane domain; Adh_short, short chain dehydrogenase/reductase domain. (C) PexRAP (peroxisomal reductase activating PPARγ, detected using anti-DHRS7b antibody) is enriched in peroxisomal fractions isolated from 3T3-L1 adipocytes. S, supernatant; P, pellet after sedimentation. (D) Pex19 coimmunoprecipitates with Myc-tagged PexRAP. WCL, whole cell lysates. (E) Pex19 interacts with PexRAP in GST pull-down experiments using 3T3-L1 adipocytes. (F) RT-PCR analysis of PexRAP expression with PexRAP knockdown in MEFs. **p = 0.0084. (G) Mass spectrometric analyses of [M+H]+ ions of GPC lipids in MEFs after PexRAP knockdown. Quantification of the 1-O-alkyl ether GPC lipid peak at m/z 746 [M+H]+ (identical to the lithium adduct at m/z 752) is shown in the inset. **p = 0.0009. (H) Mouse tissue distribution of PexRAP protein by western blotting. (I) Protein abundances of PexRAP and FAS increase prior to increases in C/EBPα and aP2 during differentiation of 3T3-L1 adipocytes.
B.1. Effects of stable suppression of Group VIA phospholipase A₂ (iPLA₂β) expression on phospholipid content and composition, insulin secretion, and proliferation of INS-1 insulinoma cells. Studies involving pharmacologic inhibition or transient reduction of Group VIA phospholipase A₂ (iPLA₂β) expression have suggested that it is a housekeeping enzyme that regulates cell 2-lysophosphatidylcholine (LPC) levels, rates of arachidonate incorporation into phospholipids, and degradation of excess phosphatidylcholine (PC). In insulin-secreting islet β-cells and some other cells, in contrast, iPLA₂β signaling functions have been proposed. Using retroviral vectors, we prepared clonal INS-1 β-cell lines in which iPLA₂β expression is stably suppressed by small interfering RNA (10). Two such iPLA₂β knockdown (iPLA₂β-KD) cell lines express less than 20% of the iPLA₂β of control INS-1 cell lines. The iPLA₂β-KD INS-1 cells exhibit impaired insulin secretory responses and reduced proliferation rates. Electrospray ionization mass spectrometric analyses of PC and LPC species that
accumulate in INS-1 cells cultured
with arachidonic acid suggest that
18:0/20:4-glycerophosphocholine
(GPC) synthesis involves sn-2
remodeling to yield 16:0/20:4
GPC and then sn-1 remodeling via a
lyso/20:4-GPC intermediate.

Electrospray ionization mass
spectrometric analyses also indicate
that the PC and LPC content and
composition of iPLAβ-KD and
control INS-1 cells are nearly identical, as are the rates of arachidonate
incorporation into PC and the composition and remodeling of other
phospholipid classes. These findings indicate that iPLAβ plays signaling
or effector roles in β-cell secretion and proliferation but that stable
suppression of its expression does not affect β-cell GPC lipid content or
composition even under conditions in which LPC is being actively
consumed by conversion to PC (10). This calls into question the generality

Fig. 27. Suppression of iPLAβ expression in iPLAβ knockdown INS-1 cells. INS-1 cell lines were prepared with retroviral vectors containing an insert encoding scrambled RNA (control) or siRNA against iPLAβ mRNA to generate iPLAβ knockdown cell lines, and iPLAβ mRNA was analyzed by Northern blots (A, lane 1, vector control; lane 2, iPLAβ-KD1; lane 3, iPLAβ-KD2; lane 4, parental cells) and real time PCR (B). Activity of iPLAβ (C) was measured without Ca2+ in the presence of EGTA and without (open bars) or with 1 mM ATP alone (cross-hatched bars) or with ATP and 10 µM BEL (solid bars). The leftmost bar (B) or set of bars reflects control cells, and the center and rightmost bar or set of bars reflects iPLAβ-KD1 and iPLAβ-KD2 cells, respectively.

Fig. 28. Effects of glucose and the adenylyl cyclase activator forskolin on insulin secretion from iPLAβ knockdown and control INS-1 cells. Insulin secretion by control, iPLAβ-KD1, and iPLAβ-KD2 INS-1 cells was measured after a 1-h incubation in medium containing 3 or 20mM glucose without or with 2.5 µM forskolin. Mean values ± S.E. (n = 6) normalized to cell protein content are displayed. Values for iPLAβ-KD INS-1 cells were significantly lower (p < 0.05) than control under all conditions.

Fig. 29. Rates of proliferation of control and iPLAβ knockdown INS-1 cells. INS-1 cells (0.3 × 10⁶ cells/well) were cultured (37°C) for various intervals and then detached with trypsin/EGTA solution, and cell number was determined from fluorescence enhancement upon association of CyQuant indicator withDNA (A) or from BrdUrd incorporation (B). Mean values ± S.E. are indicated (n = 6). Values for iPLAβ-KD1 and iPLAβ-KD2 cells were significantly lower (p < 0.05) than control at 1 and 3 days.
of proposed housekeeping functions for iPLA$_2$β in PC homeostasis and remodeling. J. Biol. Chem. 2006; 281: 187–198.)
FIG. 33. ESI/MS analyses of INS-1 cell lysophosphatidylcholine species. Extracted lipids from control (A) or iPLA$_{2}$β-KD1 INS-1 cells (B) at time 0 or after 24 h (D) of incubation with arachidonic acid were analyzed as Li$^+$ adducts by positive ion ESI/MS/MS for neutral loss of 59. In C, the ion m/z 528 from ESI/MS analyses in A or B was subjected to CAD, and product ions were analyzed. D, the ESI/MS/MS constant neutral loss of 59 scan for LPC from INS-1 cells incubated (24 h) with arachidonic acid.

Fig. 34. Tandem MS of arachidonate-containing lysophosphatidylcholine species from INS-1 cells cultured with arachidonic acid and standard (1-arachidonoyl, 2-lyso)-sn-glycero-phosphocholine. Extracted lipids from control or iPLA$_{2}$β-KD1 INS-1 cells incubated for 24 h with arachidonic acid were analyzed as Li$^+$ adducts by positive ion ESI/MS/MS; the ion m/z 550 was subjected to CAD, and its product ions were analyzed. A, the spectrum obtained with fresh extracts; B, the spectrum obtained after the extract had been stored for 2 weeks. C, the tandem spectrum of standard 1-arachidonoyl, 2-lyso-GPC prepared from 20:4/20:4-GPC with N. naja phospholipase A$_{2}$.

Fig. 35. Negative ion ESI/MS analyses of anionic glycerophospholipids in iPLA$_{2}$β-KD and control INS-1 cells incubated with arachidonic acid. Lipid phosphorus was measured in extracts of iPLA$_{2}$β-KD (A & B) or control (C & D) INS-1 cells incubated with arachidonic acid for 0 h (A and C) or 24 h (B and D); internal standard 14:0/14:0-GPE was added; and the mixture was analyzed by negative ion ESI/MS.
B.2.) Insulin secretory responses and phospholipid composition of pancreatic islets from mice that do not express Group VIA Phospholipase A, and effects of metabolic stress on glucose homeostasis. Studies involving pharmacologic or molecular biologic manipulation of Group VIA Phospholipase A subunit (iPLAβ) activity in pancreatic islets and insulinoma cells suggest that iPLAβ participates in insulin secretion. It has also been suggested that iPLAβ is a housekeeping enzyme that regulates cell 2-lysophosphatidylcholine (LPC) levels and arachidonate incorporation into phosphatidylcholine (PC). We have generated iPLAβ-null mice by homologous recombination and have reported that they exhibit reduced male fertility and defective motility of spermatozoa. Here we report that pancreatic islets from iPLAβ-null mice have impaired insulin secretory responses to D-glucose and forskolin (11). Electrospray ionization mass spectrometric analyses indicate that the abundance of arachidonate-containing PC species of islets, brain, and other tissues from iPLAβ-null mice is virtually identical to that of wild-type mice, and no iPLAβ mRNA was observed in any tissue from iPLAβ-null mice at any age. Despite the insulin secretory abnormalities of isolated islets, fasting and fed blood glucose concentrations of iPLAβ-null and wild-type mice are essentially identical under normal circumstances, but iPLAβ-null mice develop more severe hyperglycemia than wild-type mice after administration of multiple low doses of the β-cell toxin streptozotocin, suggesting an impaired islet secretory reserve. A high fat diet also induces more severe glucose intolerance in iPLAβ-null mice than in wild-type mice, but PLAb-null mice have greater responsiveness to exogenous insulin than do wild-type mice fed a high...
These and previous findings thus indicate that iPLA$_2$β-null mice exhibit phenotypic abnormalities in pancreatic islets in addition to testes and macrophages. (J. Biol. Chem. 2006: 281: 20958-20973.)
B.3.) Glucose homeostasis, insulin secretion, and islet phospholipids in mice that overexpress \( \text{iPLA}_2 \beta \) in pancreatic \( \beta \)-cells and in \( \text{iPLA}_2 \beta \)-null mice. Studies with genetically modified insulinoma cells suggest that group VIA phospholipase \( \text{A}_2 \) (\( \text{iPLA}_2 \beta \)) participates in amplifying glucose-induced insulin secretion. INS-1 insulinoma cells that overexpress \( \text{iPLA}_2 \beta \), for example, exhibit amplified insulin-secretory responses to glucose and cAMP-elevating agents. To determine whether similar effects occur in whole animals, we prepared transgenic (TG) mice in which the rat insulin 1 promoter (RIP) drives \( \text{iPLA}_2 \beta \) overexpression, and two characterized TG mouse lines exhibit similar phenotypes. Their pancreatic islet \( \text{iPLA}_2 \beta \) expression is increased severalfold, as reflected by quantitative PCR of \( \text{iPLA}_2 \beta \) mRNA, immunoblotting of \( \text{iPLA}_2 \beta \) protein, and \( \text{iPLA}_2 \beta \) enzymatic activity. Immunofluorescence microscopic studies of pancreatic sections confirm \( \text{iPLA}_2 \beta \) overexpression in RIP\( \text{iPLA}_2 \beta \)-TG islet \( \beta \)-cells without obviously perturbed islet morphology. Male RIP-\( \text{iPLA}_2 \beta \)-TG mice exhibit lower blood glucose and higher plasma insulin concentrations than wild-type (WT) mice when fasting and develop lower blood glucose levels in glucose tolerance tests, but WT and TG blood glucose levels do not differ in insulin tolerance tests. Islets from male RIP-\( \text{iPLA}_2 \beta \)-TG mice exhibit greater amplification of glucose-induced insulin secretion by a cAMP-elevating agent than WT islets. In contrast, islets from male \( \text{iPLA}_2 \beta \)-null mice exhibit blunted insulin secretion, and those mice have impaired glucose tolerance. Arachidonate incorporation into and the phospholipid composition of RIP-\( \text{iPLA}_2 \beta \)-TG islets are normal, but they exhibit reduced \( \text{Kv2.1} \) delayed rectifier current and prolonged glucose-induced action potentials and elevations of cytosolic \( \text{Ca}^{2+} \) concentration that suggest a molecular mechanism for the physiological role of \( \text{iPLA}_2 \beta \) to amplify insulin secretion. (Am. J. Physiol. Endocrinol. Metab. 2008; 294: E217–E229.)
Figure 42. Overexpression of iPLA_2β in pancreatic islets of RIP-iPLA_2β-TG mice.
A: real time PCR analyses of iPLA_2β mRNA levels in islets from wild-type (WT) or RIP-iPLA_2β-TG mice of line TG1 or TG2. B: Western blot of immunoreactive iPLA_2β protein in pancreatic islets isolated from WT or RIP-iPLA_2β-TG mice of line TG1 or TG2. Immunoblots were also probed with actin antibodies as a loading control. C: iPLA_2β-specific enzymatic activity measurements in islets from WT or RIP-iPLA_2β-TG mice from line TG1. Black bars represent basal specific activity, and light gray bars represent activity in presence of ATP. Third (rightmost) bar in each set represents activity in presence of iPLA_2 suicide substrate (E)-6-(bromomethylene) tetrahydro-3-(1-naphthalenyl)-2Hpyran-2-one (BEL). Mean values SE (n = 6) are displayed in A and C. *P<0.05 for WT vs. RIP-iPLA_2β-TG.

Fig. 43. Intraperitoneal glucose and insulin tolerance tests with RIP-iPLA_2β-TG and WT male mice. In A, D-glucose (2 mg/g) and in B human regular insulin (0.75 U/kg) was administered by intraperitoneal injection to WT (■) or RIP-iPLA_2β-TG (▲) male mice 20–24 wk of age, and blood was collected at baseline and at 30, 60, and 120 min after injection to measure glucose concentration. *P 0.05 for WT vs. TG.
Figure 44. Upper Panels: Electrospray ionization tandem mass spectrometric analyses (ESI/MS/MS) of diradyl-GPC lipids from pancreatic islets of WT and RIP-iPLA_2β-TG mice. Phospholipids from pancreatic islets of WT (A) & RIP-iPLA_2β-TG (B) mice were mixed with internal standard 14:0/14:0-GPC and were analyzed as Li^+ adducts by positive ion ESI/MS/MS scanning for neutral loss of 183 (phosphocholine), and relative abundances of ion currents were plotted vs. m/z value. Lower Panels: ESI/MS/MS of the lysophosphatidylcholine (LPC) content of islets of WT & RIP-iPLA_2β-TG mice. Phospholipids were extracted from islets of WT (A) & RIP-iPLA_2β-TG (B) mice, mixed with internal standard 19:0-LPC, and analyzed as Li^+ adducts by positive ion ESI/MS/MS scanning for neutral loss of 59 to visualize LPC species.

Figure 45. RIP-iPLA_2β-TG mouse islets have increased glucose-induced action potential duration with decreased frequency and corresponding changes in glucose-induced elevation of cytosolic [Ca^{2+}] vs. WT islets. Electrical activity induced by 20 mM glucose is recorded for WT control (A) or RIP-iPLA_2β-TG (B) mouse β-cells. Insets display action potentials (APs) from segment of activity indicated by horizontal bars. Lowest tracings in each panel represent fast-acquisition [Ca^{2+}] traces recorded from same entire mouse islet during segment of activity indicated by horizontal bars, loaded with fluo 4, and imaged at a frequency of 10 kHz. Decrease in AP frequency of WT control vs. RIP-iPLA_2β-TG mouse islets (from 1.7/s to 0.96/s) taken at 5 min after glucose was significant (P< 0.035).
B.4. Effects of ER stress on Group VIA PLA$_2$ (iPLA$_2$-β) in beta cells include tyrosine phosphorylation and increased association with calnexin. The Group VIA phospholipase A$_2$ (iPLA$_2$-β) hydrolyzes glycerophospholipids at the sn-2-position to yield a free fatty acid and a 2-lysophospholipid, and iPLA$_2$-β has been reported to participate in apoptosis, phospholipid remodeling, insulin secretion, transcriptional regulation, and other processes. Induction of endoplasmic reticulum (ER) stress in β-cells and vascular myocytes with SERCA inhibitors activates iPLA$_2$-β, resulting in hydrolysis of arachidonic acid from membrane phospholipids, by a mechanism that is not well understood. Regulatory proteins interact with iPLA$_2$-β, including the

Figure 46. Delayed rectifier currents in pancreatic islet β-cells from WT control and RIP-iPLA$_2$β-TG mice. Kv current traces recorded from islet β-cells from WT control (A) or RIP-iPLA$_2$β-TG mice (B) incubated in medium without glucose (left tracings) or with 20 mM glucose (right tracings) and subjected to 500-ms depolarization in 10 mV and 5 mV increments from 80 mV to 30 mV are shown. In C, fold increase in Kv inactivation percentage is expressed as a function of depolarizing voltage for WT control (black bars) or RIP-iPLA$_2$β-TG islet β-cells (gray bars) at 10 min after switching medium glucose concentration from 0 to 20 mM.
Ca\textsuperscript{2+}/calmodulin-dependent protein kinase II\(\beta\), and we have characterized the iPLA\(\beta\) interactome further using affinity capture and LC/electrospray ionization/MS/MS. An iPLA\(\beta\)-FLAG fusion protein was expressed in an INS-1 insulinoma cell line and then adsorbed to an anti-FLAG matrix after cell lysis (13). iPLA\(\beta\) and any associated proteins were then displaced with FLAG peptide and analyzed by SDS-PAGE. Gel sections were digested with trypsin, and the resultant peptide mixtures were analyzed by LC/MS/MS with database searching. This identified 37 proteins that associate with iPLA\(\beta\), and nearly half of them reside in ER or mitochondria. They include the ER chaperone calnexin, whose association with iPLA\(\beta\) increases upon induction of ER stress. Phosphorylation of iPLA\(\beta\) at Tyr616 also occurs upon induction of ER stress, and the phosphoprotein associates with calnexin. iPLA\(\beta\) activity \textit{in vitro} increases upon co-incubation with calnexin, and overexpression of calnexin in INS-1 cells results in augmentation of ER stress-induced, iPLA\(\beta\)-catalyzed hydrolysis of arachidonic acid from membrane phospholipids, reflecting the interaction’s functional significance. Similar results were obtained with mouse pancreatic islets (13). (\textit{J. Biol. Chem.} 2010; 285: 33843-33857).
Fig. 50. Induction of ER stress with thapsigargin increases the association of calnexin with iPLA₂β in INS-1 cells that overexpress FLAG-iPLA₂β. INS-1 cells stably transfected to overexpress FLAG-iPLA₂β were incubated for various intervals with 1 µM thapsigargin and then lysed. Lysates were processed to capture FLAG-iPLA₂β and associated proteins, which were then analyzed by SDS-PAGE. (A) Immunoblotting was performed with antibodies against calnexin (Ab) or SERCA (B).
Fig. 53. Effects of calnexin overexpression on ER stress-induced release of [1]H]arachidonic acid from prelabeled cells. INS-1 cells stably transfected with a lentivirus vector construct that causes overexpression of His-calnexin (CNX-OE; dark bars) and cells transfected with empty vector (VECTOR, light bars) were prelabeled by incubation with [1]H]arachidonic acid. To remove unincorporated radiolabel, the cells were incubated in serum-free medium and washed 3 times with glucose-free RPMI 1640 medium. Labeled cells were incubated in RPMI 1640 medium containing BEL (10 µM) or DMSO vehicle. After removal of medium, cells were placed in RPMI 1640 with 0.5% BSA that contained thapsigargin (THAPS; 1 µM) or vehicle (CONTROL or CON) and incubated. Cells were collected by centrifugation, and the supernatant [1]H content was measured by liquid scintillation spectrometry. Amounts of released [1]H were expressed as % incorporated [1]H and normalized to the value for the vector control.

Fig. 54. Co-immunoprecipitation of iPLAβ and calnexin from pancreatic islets isolated from mice. (A) Islets were isolated from C57BL/6J WT mice and incubated with thapsigargin. A lysate was then prepared in immunoprecipitation (IP) buffer containing 2% CHAPS and divided into 2 aliquots. One was incubated with Protein A-agarose and fetal bovine serum as a control (CON). The other was incubated with Protein A-agarose and a rabbit antibody (Ab) against iPLAβ to effect IP. The resultant immunoprecipitate was analyzed by SDS-PAGE, transferred to PVDF membrane, and probed with rabbit Ab against calnexin (upper panel). After stripping, the blot was probed with goat Ab against iPLAβ (middle panel). The lower panel represents a loading CON probed with anti-calnexin Ab. (B) Islets were isolated from RIP-iPLAβ-transgenic mice and lysed. The lysate was divided into 2 aliquots. One was incubated with Protein A-agarose and fetal bovine serum as CON. The other was incubated with Protein A-agarose and rabbit anti-calnexin Ab. The resultant immunoprecipitate was analyzed by SDS-PAGE, transferred to a PVDF membrane, and probed with T-14 iPLAβ antibody (upper panel). After stripping, the blot was probed with mouse anti-calnexin Ab (middle panel). The lower panel is a loading CON probed with T-14 iPLAβ Ab.

**Fig. 52. Identification of a phosphotyrosine residue in iPLAβ after thapsigargin treatment of His-calnexin-INS1 cells.** INS-1 cells were stably transfected to overexpress His-calnexin and incubated with thapsigargin or vehicle. The cells were then lysed, and the lysates were passed over cobalt affinity columns to capture and then elute His-calnexin and associated proteins, including iPLAβ. Eluates were processed by SDS-PAGE and tryptic digestion, and digests were analyzed by LC/MS/MS. A, tandem spectrum of a tryptic peptide ([**O**FLDGGGLANPTLDAMTEIHEYNQDMIRM**]) from the iPLAβ sequence in which Tyr616 is phosphorylated that was obtained from materials in a thapsigargin-treated cell lysate. B, reconstructed ion chromatogram for the [M+3H]** ion (m/z 1091–1092) of that peptide from LC/MS analyses of tryptic digests. Solid line, thapsigargin-treated cells; dashed line, vehicle-treated cells. C, immunoblots from SDS-PAGE analyses of cobalt column eluates obtained from thapsigargin-treated cells. The eluates in lanes 1 and 3 (CONTROL) were not treated with phosphatase. The eluates in lanes 2 and 4 were treated with λ-phosphatase (λ-PPase) and protein phosphatase-1 (PP-1), respectively, before SDS-PAGE analyses. The blots were probed with antibody directed against iPLAβ.

**Fig. 55. Effects of calnexin overexpression on ER stress-induced release of [1]H]arachidonic acid from prelabeled cells.** INS-1 cells stably transfected with a lentivirus vector construct that causes overexpression of His-calnexin (CNX-OE; dark bars) and cells transfected with empty vector (VECTOR, light bars) were prelabeled by incubation with [1]H]arachidonic acid. To remove unincorporated radiolabel, the cells were incubated in serum-free medium and washed 3 times with glucose-free RPMI 1640 medium. Labeled cells were incubated in RPMI 1640 medium containing BEL (10 µM) or DMSO vehicle. After removal of medium, cells were placed in RPMI 1640 with 0.5% BSA that contained thapsigargin (THAPS; 1 µM) or vehicle (CONTROL or CON) and incubated. Cells were collected by centrifugation, and the supernatant [1]H content was measured by liquid scintillation spectrometry. Amounts of released [1]H were expressed as % incorporated [1]H and normalized to the value for the vector control.
Group VIA phospholipase A₂ (iPLA₂β) is activated upstream of p38 MAP kinase in pancreatic islet beta cell signaling. Group VIA phospholipase A₂ (iPLA₂β) in pancreatic islet β-cells participates in glucose-stimulated insulin secretion and sarco(endo)plasmic reticulum ATPase (SERCA) inhibitor-induced apoptosis, and both are attenuated by pharmacologic or genetic reductions in iPLA2 activity and amplified by iPLA₂β overexpression. While exploring signaling events that occur downstream of iPLA₂ activation, we found that p38 MAPK is activated by phosphorylation in INS-1 insulinoma cells and mouse pancreatic islets, that this increases with iPLA₂β expression level, and that it is stimulated by the iPLA₂β reaction product arachidonic acid (14). The insulin secretagogue D-glucose also stimulates β-cell p38 MAPK phosphorylation, and this is prevented by the iPLA₂β inhibitor bromoenol lactone. Insulin secretion induced by D-glucose and forskolin is amplified by overexpressing iPLA₂β in INS-1 cells and in mouse islets, and the p38 MAPK inhibitor PD169316 prevents both responses. The SERCA inhibitor thapsigargin also stimulates phosphorylation of both β-cell MAPK kinase isoforms and p38 MAPK, and bromoenol lactone prevents both events. Others have reported that iPLA₂β products activate Rho family G-proteins that promote MAPK kinase activation via a mechanism inhibited by Clostridium difficile toxin B, which we find to inhibit thapsigargin-induced β-cell p38 MAPK phosphorylation. Thapsigargin-induced cell apoptosis and ceramide generation are also prevented by the p38 MAPK inhibitor PD169316. These observations indicate that p38 MAPK is activated downstream of iPLA₂β in β-cells incubated with insulin secretagogues or...
thapsigargin, that this requires prior iPLA$_2^β$ activation, and that p38 MAPK is involved in the β-cell functional responses of insulin secretion and apoptosis in which iPLA$_2^β$ participates (14). (J. Biol. Chem. 2012; 287: 5528-5541.)
Figure 60. Schematic diagram of signaling pathways in β-cells in which iPLA₂β participates.
Signals that activate iPLA₂β in insulin-secreting β-cells include 1) incubation with D-glucose concentrations sufficient to stimulate insulin secretion and 2) incubation with SERCA inhibitors (e.g. thapsigargin) that deplete ER Ca²⁺ content, induce ER stress, and trigger β-cell apoptosis. iPLA₂β activation occurs in signaling pathways involved both in D-glucose-stimulated insulin secretion and in SERCA inhibitor-induced apoptosis because both responses are attenuated by pharmacologic or genetic reductions in iPLA₂β activity, and both are amplified by iPLA₂β overexpression. Involvement of iPLA₂β in insulin secretion may reflect effects of one of its products, arachidonic acid, to inhibit membrane Kv2.1 channel activity and to prolong the D-glucose-induced action potential in β-cells. Amplification of ceramide generation by iPLA₂β may be a component of its participation in apoptosis. Data presented here indicate that p38 MAPK is activated during both glucose-stimulated insulin secretion and SERCA inhibitor-induced apoptosis in β-cells and that this occurs downstream of and requires prior iPLA₂β activation. Intermediate events between iPLA₂β activation and p38 MAPK activation include arachidonic acid release, its enzymatic oxygenation to bioactive eicosanoids (e.g. 12/15-lipoxygenase products), activation of Rho family small G-proteins (e.g. Rac1 and Cdc42), and activation of MAPK kinases (e.g. MEK3).
B.6) **Group VIA Phospholipase A	extsubscript{2} mitigates palmitate-induced \( \beta \)-cell mitochondrial injury and apoptosis.** Palmitate (C16:0) induces apoptosis of insulin-secreting \( \beta \)-cells by processes that involve generation of reactive oxygen species, and chronically elevated blood long chain free fatty acid levels may contribute to \( \beta \)-cell lipotoxicity and the development of diabetes mellitus. Group VIA phospholipase A\( _2 \) (iPLA\( _2 \beta \)) affects \( \beta \)-cell sensitivity to apoptosis, and here we examined iPLA\( _2 \beta \) effects on events that occur in \( \beta \)-cells incubated with C16:0 (15). In INS-1 insulinoma cells these events included caspase-3 activation, stress response genes (C/EBP homologous protein and activating transcription factor 4) expression, ceramide accumulation, mitochondrial membrane potential loss, and apoptosis. All those responses were blunted in INS-1 cells that overexpress iPLA\( _2 \beta \), which is proposed to facilitate repair of oxidized mitochondrial phospholipids, e.g. cardiolipin (CL), by excising oxidized polyunsaturated fatty acid residues, e.g. linoleate (C18:2), to yield lysophospholipids, e.g. monolysocardiolipin (MLCL), that can be reacylated to regenerate native phospholipid structures. Here mouse pancreatic islet MLCL was found to rise with increasing iPLA\( _2 \beta \) expression, and recombinant iPLA\( _2 \beta \) hydrolyzed CL to MLCL and released oxygenated C18:2 residues from oxidized CL in preference to native C18:2. C16:0 induced accumulation of oxidized CL species and the oxidized phospholipid (C18:0/hydroxyeicosatetraenoic acid)-glycerophosphoethanolamine, and these effects were blunted in INS-1 cells that overexpress iPLA\( _2 \beta \), consistent with iPLA\( _2 \beta \)-mediated removal of oxidized phospholipids. C16:0 also induced iPLA\( _2 \beta \) association with INS-1 cell mitochondria, consistent with a role in mitochondrial repair (15). Thus, iPLA\( _2 \beta \) confers protection of \( \beta \)-cells against C16:0-induced injury. (J. Biol. Chem. 2014; 289:14194-210.)
Fig. 64. \textit{iPLA}_{2\beta} catalyzes hydrolysis of oxidized (C18:2)$_{4}$-cardiolipin species to release oxygenated free fatty acids (FFA). A is the negative ion ESI/MS spectrum of standard (C18:2)$_{4}$-CL and shows the [M-H]$^-$ ion and $^{13}$C isotopomers and little signal from m/z 1460 to 1600. B is a spectrum after oxidation with cytochrome c/H$_{2}$O$_{2}$ that shows formation of oxidation products reflected by peaks with m/z 1448$+$(nx16) where n = 1–8. C is the ESI/MS spectrum from m/z 250 to 320 of the lipid extract of an incubation mixture of partially oxidized CL and His-\textit{iPLA}_{2\beta} and shows [M-H]$^-$ ions of internal standard [H$_{4}$]palmitate (d4-C16:0, m/z 259), linoleate (C18:2, m/z 279), oxylinoleate (O-C18:2, m/z 295), and dioxylinoleate (O$_{2}$-C18:2, m/z 311). D is the time course of \textit{iPLA}_{2\beta}-catalyzed FFA release from oxidized CL.

Fig. 65. Palmitate induces accumulation of an oxy-cardiolipin species in \textit{\beta}-cells, and this is attenuated by overexpression of \textit{iPLA}_{2\beta}. 
C.) Group VIB Phospholipase A₂ (iPLA₂γ): Mice deficient in Group VIB Phospholipase A₂ exhibit relative resistance to obesity and metabolic abnormalities induced by a Western Diet. Phospholipases A₂ (PLA₂) play important roles in metabolic processes, and the Group VI PLA₂ family is comprised of intracellular enzymes.
that do not require Ca\(^{2+}\) for catalysis. Mice deficient in Group VIA PLA\(_2\) (iPLA\(_2\)\(\beta\)) develop more severe glucose intolerance than wild-type (WT) mice in response to dietary stress. Group VIB PLA\(_2\) (iPLA\(_2\)\(\gamma\)) is a related enzyme distributed in membranous organelles, including mitochondria, and iPLA\(_2\)\(\gamma\) knockout (KO) mice exhibit altered mitochondrial morphology and function. We have compared metabolic responses of iPLA\(_2\)\(\gamma\)-KO and
WT mice fed a Western diet (WD) with a high fat content (16). We find that KO mice are resistant to WD-induced increases in body weight and adiposity and in blood levels of cholesterol, glucose, and insulin, even though WT and KO mice exhibit similar food consumption and dietary fat digestion and absorption. KO mice are also relatively resistant to WD-induced insulin resistance, glucose intolerance, and altered patterns of fat vs. carbohydrate fuel utilization. KO skeletal muscle exhibits impaired mitochondrial β-oxidation of fatty acids, as reflected by accumulation of larger amounts of long-chain acylcarnitine (LCAC) species in KO muscle and liver compared with WT in response to WD feeding. This is associated with increased urinary excretion of LCAC and much reduced deposition of triacylglycerols in liver by WD-fed KO compared with WT mice (16). The iPLAγ-deficient genotype thus results in a phenotype characterized by impaired mitochondrial oxidation of fatty acids and relative resistance to the metabolic abnormalities induced by WD.

D.) Literature Cited

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