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(54) Title: LIPIDS THAT INCREASE INSULIN SENSITIVITY AND METHODS OF USING THE SAME

(57) Abstract: The invention provides, *inter alia*, fatty acyl hydroxy fatty acid (FAHFA; a novel class of estolide-related molecules) and diagnostic and treatment methods for a variety of disorders— including diabetes-related disorders, Metabolic Syndrome, polycystic ovarian syndrome, cancer, and inflammatory disorders— using them; as well as methods of screening for additional compounds that are useful in treating these disorders and/or that modulate FAHFA levels, FAHFA-mediated signaling, and FAHFA-mediated biological effects.

LIPIDS THAT INCREASE INSULIN SENSITIVITY AND METHODS OF USING THE SAME

RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Application No. 61/642,176, filed on May 3, 2012 and U.S. Provisional Application No. 61/792,505, filed March 15, 2013. The entire teachings of the above applications are incorporated herein by reference.

GOVERNMENT SUPPORT

[0002] This invention was made with government support under P30DK57521, P30DK046200 and R37DK43051 from the National Institutes of Health. The government has certain rights in the invention.

BACKGROUND

[0003] The prevalence of obesity and type 2-diabetes is increasing worldwide and threatens to shorten lifespan. Impaired insulin action in peripheral tissues is a major pathogenic factor. Insulin stimulates glucose uptake in adipose tissue through the Glut4-glucose transporter and alterations in adipose-Glut4 expression or function regulate systemic insulin sensitivity. Downregulation of adipose tissue-Glut4 occurs early in diabetes development. Complications from obesity and type-2 diabetes include vascular disease, which detracts from quality of life further and increases mortality. Other disorders, such as polycystic ovarian syndrome and some inflammatory disorders have higher prevalence in individuals with diabetes related disorders—and the signaling pathways driving certain diabetes related disorders cross-talk with pathways that regulate inflammation. In addition, cancer, a prevalent and devastating disorder can be characterized by changes in metabolic flux, *e.g.*, via the so-called Warburg effect, by which cancer cells substantially upregulate the level of glycolysis. Many cancer cells also have increased de novo lipogenesis. In view of the prevalence of these disorders and their relation to changes in metabolism, a

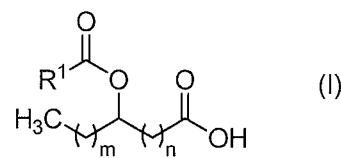
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need exists for methods of detecting and monitoring disease states and/or treatment programs in subjects with diabetes-related disorders, obesity, polycystic ovarian syndrome (PCOS), gestational diabetes, inflammatory disorders, vascular disease, or cancer, as well as tools and method of identifying agents that modulate key metabolic pathways.

SUMMARY OF THE INVENTION

[0004] The invention provides, *inter alia*, methods of monitoring disease states and/or treatment responses for a variety of metabolic disorders, such as obesity and type 2 diabetes (and diabetes-related disorders) as well as common complications such as vascular disease, polycystic ovarian syndrome, gestational diabetes, inflammatory disorders and other disorders associated with changes in metabolic flux, such as cancer. The invention is based, at least in part, on Applicants' unexpected discovery of a novel class of lipids upregulated in AG40X mice—termed fatty acyl fatty hydroxy acids, referred to herein as "FAHFAs"—as well as 1) human clinical data that indicates a strong correlation between levels of these lipids and insulin sensitivity and/or obesity and 2) the ability of these lipids to inhibit dendritic cell activation.

[0005] Accordingly, in one aspect, the invention provides an isolated fatty acyl hydroxy fatty acid (FAHFA) of formula (I):



Wherein: m is an integer from 0 to 21;
 n is an integer from 0 to 21;
 the sum of m and n is an integer from 11 to 21;
 R¹ is an alkyl group;
 or a salt thereof.

[0006] In particular embodiments, R¹ of formula (I) is a (C₁₅-C₁₇) alkyl group. In other particular embodiments, n is 7; and R¹ is C₁₅H₃₁ or C₁₇H₃₃ in of formula (I). In other particular embodiments of formula (I) m is 12; n is 3; and R¹ is a C₁₅H₃₁. h₁

certain embodiments, any of the forgoing particular embodiments are detectably labeled. For example, the FAHFA can be isotopically labeled and/or ester- or amide-bound to a detectable moiety, such as biotin, streptavidin, GST, a fluorous affinity tag, an alkyne suitable for click chemistry, an epitope tag such as FLAG, 6x His, or another affinity tag. In certain embodiments, the invention also provides a FAHFA incorporated into structures such as phospholipids, glycerophospholipids, carbohydrates, polypeptides and proteins, di- and triglycerides, and conjugates to metabolic cofactors such as CoA or acyl carnitine. In further aspects, the invention provides compositions and formulations comprising any of the foregoing.

[0007] In another aspect, the invention provides methods of assessing the disease state and/or treatment response of a mammalian subject for a disease or disorder selected from obesity, type 2 diabetes (T2D), impaired glucose tolerance, maturity onset diabetes of the young (MODY), impaired fasting glucose, metabolic syndrome, insulin resistance, polycystic ovarian syndrome, gestational diabetes, cardiovascular disease, inflammatory disorders, and cancer and the like by determining the level of one or more FAHFAs, or a precursor or derivative thereof, in a biological sample from the subject (such as an isolated sample of, *e.g.*, serum or plasma or a biopsy), where the level of the one or more FAHFAs indicates the subject's disease state and/or treatment response for the disease or disorder.

Suitable biological samples include a blood fraction, bile salt, pancreas secretions, or a tissue biopsy. In more particular embodiments, the blood fraction is plasma or serum. In other particular embodiments, the tissue biopsy comprises adipose tissue. In still other particular embodiments, the tissue biopsy comprises pancreas, liver, kidney, or tumor tissue. In any of these methods, the FAHFA, or precursor or derivative thereof, is detected by any suitable means, including methods comprising MS/MS or an immunoassay.

[0008] Exemplary inflammatory disorders include sepsis, rheumatoid arthritis (RA), ulcerative colitis, inflammatory bowel disease, Crohn's disease, systemic lupus erythematosus, celiac disease, uveitis, pancreatitis, adult respiratory distress syndrome, asthma, multiple sclerosis, graft-versus host disease, atopic dermatitis, ankylosing spondylitis, and the like. MODY can be one or more of MODY 1, 2, 3,

4, 5, 6, 7, 8, 9, 10, or 11 and, in some embodiments, the subject is determined to be heterozygous or homozygous for a germline mutation in one or more genes selected from HNF4A, GCK, HNFIA, PDX1, TCF2, NEUROD1, KLF11, CEL, PAX4, INS, or BLK.

[0009] In another aspect, the invention provides methods of decreasing pro-inflammatory signaling or increasing: glucose uptake, glucose tolerance, insulin secretion, or insulin sensitivity in a cell, by contacting the cell with an agent that increases the level of one or more FAHFAs. In a related aspect, the invention also provides methods of decreasing pro-inflammatory signaling or increasing: glucose uptake, glucose tolerance, insulin secretion, or insulin sensitivity in a mammalian subject in need thereof, by administering to the subject a therapeutically effective amount of an agent that increases the level of one or more FAHFAs. In either of these related aspects, the agent that increases the level of one or more FAHFAs can be an exogenous FAHFA, or a substrate of carboxyl ester lipase (CEL), an inhibitor of CEL, a CHREBP expression product, or a PPAR agonist, such as an agonist of one or more of PPAR α , PPAR β , or PPAR γ . In particular *in vivo* embodiments, the subject has type 2 diabetes (T2D). In some embodiments, the biologic effect of the FAHFA is to block dendritic cell maturation, activation, or proliferation; macrophage maturation, activation, or proliferation; T lymphocyte maturation, activation, or proliferation; and pro-inflammatory signaling, as well as combinations thereof. In more particular embodiments, the dendritic cell maturation, activation, or proliferation comprises an increase in CD40+, CD80+, CD86+, MHCII+ cells, or combinations thereof. In still more particular embodiments, the increased number of CD40+, CD80+, CD86+, or MHCII+ cells are also CD11c+. In other particular embodiments, the pro-inflammatory signaling is release of a proinflammatory cytokine selected from TNF- α , IL-1 β , IL-12p70, or combinations thereof.

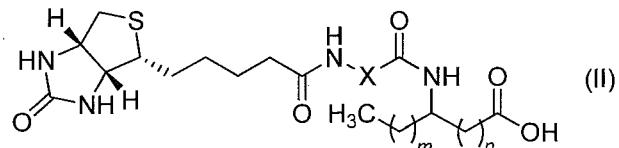
[0010] In another aspect, the invention provides methods of identifying an agent that modulates the level of one or more FAHFAs. These methods include the steps of contacting a candidate agent with a cell and measuring the level of one or more FAHFAs, where a change in the level of one or more FAHFAs in the cell, relative a control cell not contacted with the agent, indicates that the agent modulates the level

of one or more FAHFAs. In particular embodiments, the agent increases the levels of one or more FAHFAs in the cell. In other particular embodiments, the agent decreases the levels of one or more FAHFAs in the cell. In other particular embodiments, the agent modulates the level of a FAHFA synthase. In still other particular embodiments, the agent modulates the level of a FAHFA esterase.

[0011] In particular embodiments, the cell used in these methods is an isolated, cultured animal cell, such as a macrophage (such as a RAW cell), an islet cell (such as Ins₁ cell line), or a hepatic cell (such as a HepG2 cell). In more particular embodiments, the cell is cultured in the presence of low glucose. In other particular embodiments, the cell is cultured in the presence of a modulator of PPAR α , γ , or δ .

[0012] In other particular embodiments, the cell is located in a non-human animal—*i.e.* the method is performed, at least in part, *in vivo*. In more particular embodiments, the non-human animal is a mammal and in still more particular embodiments, is murine, *e.g.* an AG40X mouse, an AG4KO mouse, a ChREBPKO mouse, or a ChREBPOX mouse, and combinations thereof, *et cetera*.

[0013] In another aspect, the invention provides a detectable biotinylated amino-FAHFA of formula (II):



Wherein: m is an integer from 0 to 21;

n is an integer from 0 to 21;

the sum of m and n is an integer from 11 to 21;

X is selected from (OCH₂CH₂)_p or (CH₂)_p, wherein p is an integer from 2 to 20;

or a salt thereof.

[0014] In another aspect, the invention provides methods of identifying FAHFA-binding molecules, such as FAHFA-binding proteins. In some embodiments, detectably labeled FAHFAs are used. In particular embodiments, one or more detectably labeled FAHFAs are contacted with whole cells, cell extracts, or synthetic biomixtures, including, for example isolated proteins. After a suitable

amount of time to allow binding of the one or more FAHFAs to other molecules in the mixture, a complex of the detectably labeled one or more FAHFAs and any binding partners is detected—optionally including isolation of the complex.

Molecules bound to one or more FAHFAs are then identified by any suitable means, including MS/MS (*e.g.*, proteolytic digestion of FAHFA-bound proteins, and detection of peptides by MS/MS). In other embodiments, FAHFAs are detected in co-IP or other pull-down assay; *e.g.*, either the binding partner is isolated by suitable means and one or more FAHFAs are detected by any suitable means or vice versa.

One exemplary methodology of identifying FAHFA binding partners, using SILAC (stable isotope labeling by/with amino acids in cell culture), is shown in FIG. 44.

[0015] In another aspect, the invention provides methods of identifying FAHFA-binding molecules, such as FAHFA-binding proteins. In some embodiments, detectably labeled FAHFAs are used. In particular embodiments, one or more detectably labeled FAHFAs are contacted with whole cells, cell extracts, tissues, serum or plasma, or synthetic biomixtures, including, for example isolated proteins. After a suitable amount of time to allow binding of the one or more FAHFAs to other molecules in the mixture, a complex of the detectably labeled one or more FAHFAs and any binding partners is detected—optionally including isolation of the complex. Molecules bound to one or more FAHFAs are then identified by any suitable means, including MS/MS (*e.g.*, proteolytic digestion of FAHFA-bound proteins, and detection of peptides by MS/MS). In other embodiments, FAHFAs are detected in co-IP or other pull-down assay; *e.g.*, either the binding partner is isolated by suitable means and one or more FAHFAs are detected by any suitable means or vice versa. One exemplary methodology of identifying FAHFA binding partners, using SILAC (stable isotope labeling by/with amino acids in cell culture), is shown in FIG. 44.

[0016] In yet another aspect, the invention provides methods of identifying a modulator of FAHFA-mediated signaling and/or FAHFA-mediated biological effects. The methods include forming a mixture with an isolated mammalian cell that expresses a G-protein coupled receptor (GPCR), one or more FAHFAs, and a test compound and monitoring the FAHFA-mediated signaling. A change in the

FAHFA-mediated signaling indicates that the test compounds is a modulator of FAHFA-mediated signaling. In certain embodiments, the GPCR is GPR120 (human GenelD No. 338557, *see also* OMIM 609044) or GPR40 (human GenelD No. 2864; OMIM 603820). In some embodiments, the one or more FAHFAs comprise 9-PAHSA, 5-PAHSA, 9-OAHSA, or a combination thereof. In other embodiments, the FAHFA-mediated biologic effect is selected from decreasing pro-inflammatory signaling, stimulating insulin secretion, stimulating glucagon like peptide- 1 (GLP1; see human GenelD No. 2641 and OMIM 138030) or other incretin secretion, stimulating calcium flux into the cell or from intracellular organelles into the cytoplasm, G-protein activation, and combinations thereof. In more particular embodiments, the FAHFA-mediated biological effect is stimulating insulin secretion and, in more particular embodiments, the isolated mammalian cell is a pancreatic islet cell. In other particular embodiments the FAHFA-mediated biological effect is stimulating GLP1 secretion and, in more particular embodiments, the cell is an enteroendocrine cell from the intestine of a mammal (such as STC-1 cells). In any of these related embodiments, the mammalian cell may be a human cell.

BRIEF DESCRIPTION OF THE DRAWINGS

[0017] The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the Office upon request and payment of the necessary fee.

[0018] FIG. 1 shows a novel FAHFA pathway.

[0019] FIG. 2 shows comparative metabolite profiling of adipose GLUT4-overexpressing (AG40X) and wild-type mice (WT) by LC-MS-based metabolomics.

[0020] FIG. 3 shows lipid metabolite profiling of AG40X and WT mice, which revealed a highly upregulated lipid family.

[0021] FIGs. 4a and 4b show (a) an LC-MS trace of m/z 537 ion, upregulated in transgenic mice (AG40X) and (b) a fragmentation pattern of novel mammalian lipid.

[0022] FIG. 5 shows a general FAHFA structure and the specific isomer, 9-PAHSA.

[0023] FIGs. 6a and 6b show (a) FAHFA-containing fractions of HPLC-purified WAT sample exhibiting a diagnostic fragment pattern at 50V by MS/MS, and (b) a coextraction with [¹³C]9-PAHSA shows coelution with the natural isomer.

[0024] FIGs. 7A-7B show tissue distribution of total PAHSA (7A) and 5-PAHSA (7B) levels in WT mice on CHOW diet (Female on chow (5008), fed, n=4-5).

[0025] FIGs. 8a and 8b show (a) FAHFA hydrolysis activity enriched in liver membrane, and (b) the hydrolytic activity is a serine hydrolase.

[0026] FIG. 9 shows tissue distribution of FAHFA hydrolysis activity (n = 3).

[0027] FIG. 10 shows hydrolysis of FAHFA by transfected HEK293T cell lysates (top) and expression levels by ABP gel (bottom).

[0028] FIGs. 11a, 11b, and 11c show that (a) WWL92 inhibits FAHFA hydrolysis partially in liver, but (b) almost completely in the pancreas; (c) shows CEL expression in the pancreas and liver.

[0029] FIGs. 12a and 12b show supplementation of FAHFA hydrolase activity assays in a) CEL-transfected cell lysates, liver and pancreas membrane lysates and (b) pancreas membrane lysates treated with WWL92 (20 uM).

[0030] FIG. 13 shows the reaction of 9-PAHSA CEL in the presence of micellar NaTC concentrations.

[0031] FIG. 14a and 14b show the production of 9-OASHA (*m/z* 563.5) by tissue membrane lysates.

[0032] FIG. 15 shows the tissue distribution of FA-CoA:HFA acyl transferase activity.

[0033] FIGs. 16a and 16b show that (a) FAHFA biosynthesis is unaffected by DGAT1 or ACAT inhibitors, and (b) that increased FA-CoA:HFA acyltransferase activity is not observed in HEK293T cells transfected with acyltransferase clones (n = 3).

[0034] FIG. 17 shows FA-CoA:HFA acyl transferase activity in 3T3-L1 cell lysates.

[0035] FIG. 18 shows an LC-MS trace of isomers of hydroxystearic acid in AG40X fat with comparison to commercially purchased 12- and 9-HSA standards. Samples were analyzed on a QQQ-MS in selected ion monitoring (SIM) mode targeting the *m/z* 299.3 ion.

[0036] FIG. 19 shows two exemplary biotinylated FAHFAs, Biotin-FAHFA-2 (BF-2) and Biotin-FAHFA-3 (BF-3).

[0037] FIG. 20 shows that adipose tissue ChREBP gene expression in obese humans correlates highly with systemic insulin sensitivity.

[0038] FIG. 21 shows FAHFAs present in human serum and fat.

[0039] FIG. 22 shows that total FAHFA levels are markedly increased in adipose tissue of Adipose Glut4 overexpressing mice and that FAHFAs are regulated by ChREBP in adipose tissue of normal mice and adipose Glut4 overexpressing mice (*p<0.05 vs wild type; #p<0.05 vs adipose Glut4 OX).

[0040] FIG. 23 shows that the levels of FAHFAs are lower in ChREBP KO serum while total fatty acids are unchanged (*p<0.05 vs wild type). Isomer 2 is 5-PAHSA.

[0041] FIG. 24 shows that the novel lipid levels in serum are regulated by fasting and refeeding (*p<0.05 vs all other groups). Isomer 2 is 5-PAHSA.

[0042] FIG. 25 is a table that shows baseline characteristics of human subjects undergoing a euglycemic-hyperinsulinemic clamp study, a test of insulin sensitivity. Serum samples were from the Lundberg Laboratory for Diabetes Research in Gothenburg, Sweden. Serum samples used in this analysis were obtained from individuals prior to the study. Percent glycosylated hemoglobin (HbA1c) was measured to assess the subject's average recent blood glucose levels (ref. range = 4.0-5.9%) and to confirm the absence of frank diabetes ($\geq 6.0\%$). Body mass index is calculated by dividing the subject's total mass in kilograms by the square of the height in meters. The glucose infusion rate reflects the steady-state rate of glucose infusion, normalized to lean body mass or to body weight, that is required to maintain euglycemia over a period between 60-180 minutes during which insulin is infused at a fixed rate (240 pmol/m²/min). See the methodology of Norseen *et al.*

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Mol Cell Biol. 32(10):2010-9 (Epub 2012), which is incorporated by reference in its entirety, for additional description of these subjects.

[0043] FIG. 26 shows the resolution of positional isomers of PAHSA *via* a high-resolution chromatographic method. Coelution studies with 4-, 5- or 6-PAHSA synthetic standards showed that the later eluting peak in AG40X brown adipose tissue was 5-PAHSA, and not 4- or 6-PAHSA.

[0044] FIG. 27 shows a comparison of serum 12-PAHSA, 10-PAHSA and 5-PAHSA levels in lean insulin-sensitive (lean, IS), lean insulin-resistant (lean, IR), and obese insulin-resistant human subjects. Serum samples were collected after an overnight fast, prior to a hyperinsulinemic-euglycemic clamp study that measured insulin-sensitivity in these individuals. Samples were extracted in the presence of 10 pmol [$^{13}\text{C}_{16}$]-9-PAHSA and analyzed by LC-MS using an isocratic chromatographic method (ANOVA: *, $p < 0.05$ versus lean insulin sensitive; $n = 8$ per group).

[0045] FIG. 28 shows a representative LC-MS trace of 5-PAHSA in serum from fasted human subjects. The lean insulin-sensitive subject had a body mass index (BMI) of 24.0 kg/m² and a glucose infusion rate normalized to lean body mass (GIR/LBM) of 23.6 mg/min/kg. The obese insulin-resistant subjected had a BMI of 31.2 kg/m² and a GIR/LBM of 8.6 mg/min/kg. Extracted serum samples were analyzed on an isocratic LC-MS method in MRM mode.

[0046] FIGs. 29 and 30 are scatter plots of glucose infusion rate (GIR) versus 5-PAHSA levels in human subjects showing strongly positive correlations between insulin sensitivity measured by euglycemic clamp study and the serum levels of 5-PAHSA.

[0047] FIGs. 31A, 31B, 32A and 32B are bar graphs that illustrate the effects of FAHFAs on insulin release from islet cells. FIG. 31A is in a cultured islet cell line, Insl cells, with 2.5mM glucose in the media. FIG. 31B shows that 5- and 9-PAHSA potentiate insulin secretion in Insl cells with 25 mM glucose in the media. FIG. 32A is in primary islet cells from normal rats with 2.5 mM glucose in the media. FIG. 32B shows that 5-PAHSA potentiates glucose-stimulated insulin secretion in isolated rat islets with 25 mM glucose in the media. While FAHFAs increase insulin secretion from Insl cells at both low and high glucose under this particular

set of cell culture conditions, in human islets FAHFAs stimulate insulin secretion only at high glucose levels (see FIGs. 47 and 48). Thus, the effect of FAHFAs on insulin secretion in human islets is physiologic.

[0048] FIGs. 33 and 34 are bar graphs that summarize the effect of different PPAR agonists on FAHFA levels in a cultured liver cell line.

[0049] FIG. 35 is a graphical representation of the experimental design used to examine the effect of FAHFAs on the inflammatory cytokine cascade.

[0050] FIGs. 36A and 36B are graphs of secreted IL-12 levels from LPS-stimulated macrophages treated with different levels of FAHFAs.

[0051] FIG. 37 shows that upregulation of FAHFA levels in subcutaneous white adipose tissue (SQ fat) upon GLUT4 overexpression (AG40X) requires ChREBP. To determine whether adipose ChREBP is required for the increased PAHSA levels in adipose tissue resulting from GLUT4 overexpression, we crossbred AG40X mice with whole-body ChREBP KO mice. Total PAHSA levels normalized in adipose tissue after deletion of ChREBP in AG40X mice. Statistical significance was assessed by a Student's *t* test (**, p < 0.01; ***, p < 0.001; n = 4).

[0052] FIG. 38 shows a FAHFA gavage experiment workflow. Mice were fasted overnight then injected with WWL92. After 30 minutes, the were gavaged with the double-labeled FAHFA, [¹³C]-9-PAHHA, dissolved in olive oil. After three hours, mice were sacrificed and plasma is collected and extracted with chloroform-methanol. Lipid extracts were analyzed by LC-MS in targeted MRM mode to look for either intact [¹³C]-9-PAHHA (*m/z* 539.5) or reesterified lipids containing HHA. Analysis of 9-HHA itself can be performed in selected ion monitoring (SIM) mode (*m/z* 285.3).

[0053] FIG. 39 shows complete inhibition of CEL by WWL92 *in vivo*. Labeling of CEL was by an activity-based probe was inhibited in mice treated with WWL92. Mice were fasted overnight and an intraperitoneal injection of WWL92 was administered at the indicated doses. After either 1 or 2 hours, the mice were sacrificed and the pancreas was harvested. Membrane lysates of the pancreas tissue were prepared, and they were first reacted with either WWL92 (20 μ M) or DMSO to ensure that WWL92 could inhibit CEL *ex vivo*. Reaction with an activity-based

probe (FP-Rh) followed by separation of proteins by SDS-PAGE permitted fluorescent detection of FP-Rh-labeled proteins. The lower intensity of bands in WWL92-treated mice indicated that a greater proportion of the CEL was pre-labeled with WWL92 and not available for labeling by FP-Rh.

[0054] FIG. 40 shows bar graphs illustrating that plasma levels of [¹³C]-9-PAHHA and its hydrolyzed product 9-HHA in mice after treatment with 40 mg/kg WWL92. Mice were injected intraperitoneally with an aqueous solution of the inhibitor 0.5 hour prior to gavage with 0.1 mL olive oil containing 0.15 mg [¹³C]-9-PAHHA. Mouse plasma was collected after 3 hours and the levels of the metabolites were measured by QQQ-MS using [¹³C]-9-PAHSA as an internal standard. Values for 9-HHA are presented as average signal intensity. Statistical significance was determined by an unpaired Student's *t* test (*, n < 0.05; n = 3-4).

[0055] FIG. 41 shows a bar graph illustrating that plasma levels of transesterified products of [¹³C]-9-PAHHA. The levels of PAHHA and OAHHA were measured by QQQ-MS using [¹³C]-9-PAHSA as an internal standard, all of which possessed unique MRM transitions. Statistical significance was determined by an unpaired Student's *t* test (**, n < 0.01; n = 3-4).

[0056] FIG. 42 shows CEL degradation of FAHFAAs may follow a model similar to the canonical lipid absorption pathway. Double-labeled FAHFA (*PA*HHA) dissolved in extra light olive oil is ingested and proceeds from the stomach to the duodenum *via* gastric emptying. Lipid content in the chyme triggers release of bile and pancreatic enzymes, which include CEL. Labeled FAHFA is hydrolyzed in intestinal lumen to *PA and *HHA, permitting uptake by the enterocytes lining the lumen wall. Once inside, *HHA can be re-esterified with a natural fatty acyl group (FA) to give FA*HHA or exported directly to bloodstream as *HHA.

[0057] FIG. 43 illustrates an experimental design for *in vivo* experiments to show that CEL is an endogenous FAHFA-hydrolase.

[0058] FIG. 44 illustrates an experimental design for enrichment of FAHFA-binding proteins using SILAC.

[0059] FIG. 45 shows bar graphs that illustrate that 9-PAHSA prevents LPS-induced dendritic cell activation by reducing expression of MHCII and co-

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stimulatory molecules (CD40, CD80, CD86) by dendritic cells as a percentage of activated dendritic cell (top row) and in fluorescence units (bottom row). *p<0.05 vs all other groups.

[0060] FIG. 46 shows line graphs that illustrate 9-PAHSA prevents LPS-induced dendritic cell activation in a dose dependent manner.

[0061] FIG. 47 is a bar graph evidencing that 5-PAHSA (Lipid A-5) induces Glucose Stimulated Insulin Secretion in Human islets from Donor 1. * p < 0.05 vs. 2.5 mM glucose alone; # p < 0.05 vs. respective 2.5 mM glucose controls; \$ p < 0.05 vs. 20 mM glucose alone.

[0062] FIG. 48 is a bar graph evidencing that 5-PAHSA (Lipid A-5) induces Glucose Stimulated Insulin Secretion in Human islets from Donor 2. * p < 0.05 vs. 2.5 mM glucose alone; # p < 0.05 vs. respective 2.5 mM glucose controls; \$ p < 0.05 vs. 20 mM glucose alone.

[0063] FIGs. 49A-C are line graphs showing that lipids A9 (9-PAHSA) and A5 (5-PAHSA) induce Ca^{2+} flux from intracellular stores in STC-1 cells. Lipids were applied at t = 3 min, Results = average of 10 cells. 49A is STC-1 Linoleic Acid 100uM (positive control for Ca^{2+} flux) No Ca^{2+} ; 49B is STC-1 Lipid A9 100uM No Ca^{2+} ; 49C is STC-1 Lipid A5 100uM No Ca^{2+} .

[0064] FIG. 50 is a Western Blot illustrating that lipids A9 (9-PAHSA), B9 (9-OAHSAs), and A5 (5-PAHSA) induce ERK phosphorylation in STC-1 cells.

[0065] FIG. 51 is a bar graph illustrating that lipids (9-PAHSA), B9 (9-OAHSAs), and A5 (5-PAHSA) induce GLP-1 secretion in Stc-1 enteroendocrine cells.

[0066] FIG. 52 is a bar graph time series illustrating that the time course for GLP-1 secretion by 9-PAHSA in Stc-1 cells is very rapid and sustained.

[0067] FIG. 53 is a bar graph evidencing that lipid A9 (9-PAHSA) is a ligand for the GPCR, GPR120.

[0068] FIG. 54 is a titration curve, further illustrating that lipid A9 (9-PAHSA) is a ligand for the GPCR, GPR120.

[0069] FIG. 55 is a series of bar graphs illustrating that 9-PAHSA and 5-PAHSA, but not 90AHSA, activate GPR120.

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[0070] FIGs. 56A and 56B illustrate that glucose-insulin infusion (clamp) lowers 10 - PAHSA levels in the plasma of insulin sensitive (FIG. 56A) and insulin resistant (FIG. 56B) human subjects.

[0071] FIG. 57 illustrates that the levels of 5-PAHSA in plasma of insulin sensitive and insulin resistant subjects were lower after clamp than at baseline.

[0072] FIGs. 58A and 58B illustrate that glucose-insulin infusion (clamp) lowers 5-PAHSA levels in plasma of insulin sensitive (FIG. 58A) and insulin resistant (FIG. 58B) human subjects.

[0073] FIG. 59 is a scatterplot illustrating the correlation between the levels of 5-PAHSA in plasma from humans in the fasting state with insulin sensitivity.

[0074] FIG. 60 is a scatterplot illustrating the inverse correlation between the levels of 5-PAHSA in plasma from humans with plasma triglycerides in the fasting state.

DETAILED DESCRIPTION OF THE INVENTION

[0075] The present invention provides methods for assessing the disease state and/or treatment response of a mammalian subject for a disease or disorder selected from obesity, type 2 diabetes (T2D), impaired glucose tolerance, maturity onset diabetes of the young (MODY), impaired fasting glucose, metabolic syndrome, insulin resistance, polycystic ovarian syndrome (PCOS), gestational diabetes, cardiovascular disease, inflammatory disorders, cancer, and other neoplastic disorders. To assess the disease state, a biological sample is obtained from the mammalian subject, and the level of one or more fatty acyl hydroxy fatty acids (FAHFAs) is determined. The invention also provides methods of treating the foregoing disorders, which, in particular embodiments, comprise administering an agent that increases the levels of one or more FAHFAs. The invention also provides isolated or purified preparations comprising FAHFAs.

I. *Certain Definitions*

[0076] As used herein, a "fatty acyl hydroxy fatty acid" or "FAHFA" means an estolide having an estolide number of 1, in which a hydroxy fatty acid is esterified at

the hydroxyl group by another fatty acid. In the present invention, the hydroxyl group of the fatty acid is not on the terminal carbon of the fatty acid. A FAHFA may exist as a salt or may be incorporated into other structures, including, but not limited to, phospholipids, glycerophospholipids, carbohydrates, polypeptides, proteins (*e.g.* analogues to cysteine palmitoylation and myristoylation), di- and triglycerides, and may be conjugates to CoA or acyl carnitine.

[0077] "Estolide number" or "EN" is the number of fatty acid units added to the primary fatty acid.

[0078] "Alkyl" means a saturated or unsaturated aliphatic branched or straight-chain hydrocarbon radical having the specified number of carbon atoms that can be substituted or unsubstituted. Thus, "(C_i-C_6) alkyl" means a radical having from 1- 6 carbon atoms in a linear or branched arrangement. "(C_i-C_6)alkyl" includes methyl, ethyl, propyl, butyl, pentyl and hexyl.

[0079] A "subject" is a mammal, including primates (*e.g.*, humans or monkeys), cows, sheep, goats, horses, dogs, cats, rabbits, guinea pigs, rats, mice or other bovine, ovine, equine, canine, feline, rodent or murine species. Examples of suitable subjects include, but are not limited to, human patients (*e.g.*, obese, diabetic, non-diabetic, having a diabetes-related disorder, cancer or vascular disease) and in more particular embodiments human patients (*e.g.*, obese, non-obese) who have, or are at risk for developing, a diabetes-related disorder, PCOS, an inflammatory disorder, vascular disease or cancer. Examples of high-risk groups for the development of PCOS, metabolic syndrome, insulin resistance or type 2 diabetes include medically overweight and obese individuals. In preferred embodiments, the subject is human. In particular embodiments, the subjects to be tested or treated by the methods provided by the invention have, or are at increased risk for developing obesity or a diabetes-related disorder, PCOS, an inflammatory disorder, cancer or vascular disease. In more particular embodiments, the vascular disease may be secondary to either obesity and/or a diabetes-related disorder. Similarly, the diabetes-related disorder may be secondary to obesity, or vice-versa. While subjects may be of any stage of life and any age, *e.g.*, neonate, infant, toddler, child, young adult, adult, or geriatric; in particular embodiments the subject is an adult, *e.g.* a human adult, *i.e.*

18 years old, or older, *e.g.*, 18-70, 20-60, 25-55, 25-50, 30-50, 25-65 years old, as well as greater than 30, 40, 50, 60, 70, 80 or 90 years old.

[0080] "Determining" a level of a FAHFA, requires contacting a sample (*e.g.* an isolated biological sample) with isolated analytic tools, such as laboratory equipment (*e.g.* a mass spectrometer) for measuring the level, and, in certain embodiments, additional isolated reagents, such as chemical solutions, isolated oligonucleotides (*e.g.* aptamers), cloned enzymes, *et cetera*), antibodies (including antigen-binding fragments thereof; optionally where the antibody or antigen-binding fragment thereof is detectably labeled) to measure the level of a FAHFA by an analytical laboratory method. Determining a level of a FAHFA may be done directly in the course of the analytical laboratory methods or, in some embodiments, by evaluating the quantitative output of the analytical laboratory methods.

[0081] As used herein, the terms "treat," "treating," or "treatment," mean to counteract a medical condition (*e.g.*, obesity, a diabetes-related disorder, PCOS, an inflammatory disorder, cancer, vascular disease) to the extent that the medical condition is improved according to a clinically-acceptable standard. For example, an improvement in a medical condition related to obesity can be determined according to one or more of the following: 1) reduction of body weight, 2) reduction of body mass index (BMI), 3) reduction of waist-to-hip ratio (WHR); improvement relative to diabetes can include 1) improved glucose tolerance, 2) reduced glycated hemoglobin, 3) improved insulin sensitivity, 4) improved glycemia; improvement in PCOS can include: 1) increased fertility, 2) reduced ovary volume 3) resolution of hirsutism, 4) resolution of amenorrhea, 5) reduced levels of PSA; improvement in an inflammatory disorder can include: 1) reduced levels of pro-inflammatory cytokines, 2) increased levels of anti-inflammatory cytokines, 3) reduced pain, 4) reduced macrophage or dendritic cell counts at sites of inflammation; improvement in cancer can include: 1) reduced tumor growth, 2) tumor shrinking, 3) remission, 4) reduction in metastases, 5) reduced glucose uptake or utilization; improvement in vascular disease can include 1) reduced blood pressure, 2) lowered LDL cholesterol, 3) increased HDL cholesterol, 4) lowered triglycerides, 5) reduced atherosclerotic

burden, 6) improved cardiac output. "Treatment response" is the change in a clinically-acceptable standard in response to a treatment, as defined above.

[0082] The terms "prevent," "preventing," or "prevention," as used herein, mean reducing the probability/likelihood, progression, onset, risk or severity of a disorder—including, for example, obesity or a diabetes-related disorder, PCOS, an inflammatory disorder, cancer or vascular disease—in a subject. In general, a subject undergoing a preventative regimen most likely will be categorized as being "at-risk" for a given disorder, *e.g.*, the risk for the subject developing obesity, a diabetes-related condition, PCOS, an inflammatory disorder, vascular disease or cancer is higher than the risk for an individual represented by the relevant baseline population.

[0083] As used herein, a "therapeutically effective amount" is an amount sufficient to achieve the desired therapeutic or prophylactic effect under the conditions of administration, such as an amount sufficient to inhibit (*e.g.*, reduce, prevent), *e.g.*, obesity, diabetes-related disorder, vascular disease or cancer. The effectiveness of a therapy can be determined by one skilled in the art using standard measures and routine methods.

[0084] The term "obese" or "obesity" refers to the condition of a subject having a body mass index (BMI) of about 30 kg/m² or higher, *e.g.*, a BMI of 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37 kg/m², or more. In particular embodiments, an obese subject has a BMI within the ranges defined as "obese" by the Center for Disease Control. *See*, //www.cdc.gov/obesity/defining.html. For example, in some embodiments, an adult who has a BMI >=30.0 kg/m² is obese.

[0085] "Type 2 diabetes" or "T2D" (OMIM 125853), in some embodiments, is defined as provided by the World Health Organization and the International Diabetes Federation in "Definition and diagnosis of diabetes mellitus and intermediate hyperglycaemia," published in 2006, which is incorporated by reference in its entirety. In more particular embodiments, a diabetic subject exhibits a fasting plasma glucose of >=126mg/dL or a 2-hour plasma glucose (2 hours after oral administration of 75 grams of glucose) >=200mg/dL. In some embodiments a diabetic or pre-diabetic subject exhibits elevated levels of glycated hemoglobin, *e.g.*,

greater than 4.5, 4.6, 4.7, 4.8, 4.9, 5.0, 5.1, 5.2, 5.3, 5.4, 5.5, 5.6, 5.8, 6.0, 6.2, 6.4, 6.6., 6.8, 7.0, 7.2, 7.4, 7.6%, or more of total hemoglobin.

[0086] "Gestational diabetes" is a condition in which women without previously diagnosed diabetes exhibit high blood glucose levels during pregnancy, with a higher prevalence during the third trimester of pregnancy. Gestational diabetes typically resolves itself after pregnancy. In certain embodiments, gestational diabetes is classified as MODY 2.

[0087] "Insulin resistance," which may be identified by any means known in the art, and is characterized by a reduced ability of insulin to lower blood glucose levels.

[0088] The term "metabolic syndrome" refers to a group of symptoms that occur together and increase the risk for coronary artery disease, stroke and type 2 diabetes. In some embodiments the subject has central obesity (waist circumference ≥ 80 cm for women; ≥ 90 cm for Asian men, including ethnic South and Central Americans, and ≥ 94 cm for all other males), $\text{BMI} > 30 \text{ kg/m}^2$, raised triglycerides ($\geq 150 \text{ mg/dL}$, or specific treatment for this lipid abnormality), reduced HDL cholesterol ($< 40 \text{ mg/dL}$ in males, $< 50 \text{ mg/dL}$ in females or specific treatment for this lipid abnormality), raised blood pressure ($\text{sBP} \geq 130 \text{ mm HG}$ or $\text{dBP} \geq 85 \text{ mm HG}$ or treatment of previously diagnosed hypertension) or raised fasting plasma glucose ($\text{FPG} \geq 100 \text{ mg/dL}$ or previous type 2 diabetes diagnosis), including combinations thereof. In more particular embodiments, the subject to be treated by the methods provided by the invention has or is at increased risk for metabolic syndrome, as defined by the International Diabetes Federation in "The IDF consensus worldwide definition of the metabolic syndrome," published in 2006, which is incorporated by reference in its entirety, *i.e.*, the subject has central obesity (as described above, and/or $\text{BMI} > 30 \text{ kg/m}^2$) AND any two of raised triglycerides, reduced HDL cholesterol, raised blood pressure, or raised fasting plasma glucose.

[0089] "Diabetes-related disorders" include T2D, gestational diabetes, MODY, impaired fasting glucose, impaired glucose tolerance, insulin resistance and metabolic syndrome.

[0090] "Cancer" refers to mammalian cancers, in some embodiments, human cancers, and carcinomas, sarcomas, adenocarcinomas, lymphomas, leukemias, *et*

cetera, including solid and lymphoid cancers, kidney, breast, lung, kidney, bladder, colon, ovarian, prostate, pancreas, stomach, brain, head and neck, skin, uterine, testicular, esophagus, and liver cancer, including hepatocarcinoma, lymphoma, including non-Hodgkin's lymphomas (e.g., Burkitt's, Small Cell, and Large Cell lymphomas) and Hodgkin's lymphoma, leukemia, and multiple myeloma. Cancers embraced in the current application include both metastatic and non-metastatic cancers. In certain embodiments, a cancer cell may exhibit one or more of loss of contact inhibition when cultured, abnormal karyotype, abnormal cellular morphology or altered metabolic state consonant with what is termed the Warburg effect. Additional states that may be related to cancer and that can be diagnosed, monitored and/or treated by the methods provided by the invention include precancerous lesions and neoplasias.

[0091] "Vascular disease" is a pathological state of large, medium, or small sized arteries and may be triggered by endothelial cell dysfunction (e.g. including aneurisms, blockage, collapse) in central, peripheral or cerebral vasculature and can include angina, as well as severe complications such as stroke (ischemia), myocardial infarct (heart attack), arrhythmia, congestive heart failure, or ischemia resulting in gangrene or amputation.

[0092] "Polycystic ovarian syndrome" or "polycystic ovary syndrome" or "PCOS" is characterized by one or more of bilateral enlarged ovaries, abnormal 24-hour urinary ketosteroids, and evidence of virilization and, in particular embodiments, all three indications. In certain particular embodiments, PCOS is correlated with one or more of obesity, hirsutism, and amenorrhea, e.g., 1, 2, or all 3 indications. In other embodiments, a subject with PCOS exhibits elevated urinary levels of PSA (human GenelD No. 354) and/or kallikrein-2 (human GenelD No. 3817). In certain particular embodiments, PCOS is characterized by the presence of a mutation in any one of the loci identified in OMIM accession number 184700, which is incorporated by reference, including follistatin, CYP11A, CAPN10, or INSR (human GenelD No. 3643).

[0093] "Inflammatory disorders" are characterized by abnormally high levels of pro-inflammatory cytokines (e.g. IL-2, IL-3, GM-CSF, IL-6, IL-8, IL-18, HMGB1,

TNF- α , and IFN- γ) and/or abnormally low levels of anti-inflammatory cytokines (*e.g.* IL-10). Exemplary inflammatory disorders include sepsis, rheumatoid arthritis (RA), ulcerative colitis, inflammatory bowel disease, Crohn's disease, systemic lupus erythematosus, celiac disease, uveitis, pancreatitis, adult respiratory distress syndrome, asthma, multiple sclerosis, graft-versus host disease, atopic dermatitis, ankylosing spondylitis, and the like.

[0094] "Maturity onset diabetes of the young," "MODY," and the like are a group of disorders (*see* OMIM 606391, incorporated by reference)— autosomal dominants form of diabetes typically occurring before 25 years of age and caused by primary insulin secretion defects.

[0095] "Antibody" and the like refers to both whole immunoglobulins as well as antigen-binding fragments of immunoglobulins that contain an antigen-binding domain comprising at least 3, 4, 5, or 6 complementary determining regions (CDRs). Antibodies can be from any source including human, orangutan, mouse, rat, goat, sheep, rabbit and chicken antibodies, as well as synthetic, engineered antibodies. Antibodies may be polyclonal, monoclonal, monospecific, polyspecific, non-specific, humanized, camelized, single-chain, chimeric, synthetic, recombinant, hybrid, mutated, or CDR-grafted antibodies.

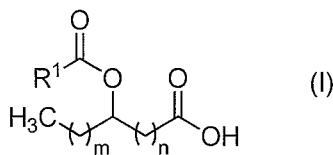
[0096] "Highly stringent hybridization" conditions refers to at least about 6X SSC and 1% SDS at 65°C, with a first wash for 10 minutes at about 42°C with about 20% (v/v) formamide in 0.1 X SSC, and with a subsequent wash with 0.2 X SSC and 0.1% SDS at 65°C.

II. FAHFAS

[0097] As noted above, in the present application a "fatty acyl hydroxy fatty acid" or "FAHFA" means an estolide having an estolide number of 1, in which a hydroxy fatty acid is esterified at the hydroxyl group by another fatty acid. In the present invention, the hydroxyl group of the fatty acid is not on the terminal carbon of the fatty acid. A FAHFA may exist as a salt or may be incorporated into other structures, including, but not limited to, phospholipids, glycerophospholipids,

carbohydrates, polypeptides, proteins (*e.g.* analogous to cysteine palmitoylation and myristoylation), di- and triglycerides, and may be conjugated to other molecules involved in metabolism, particularly lipid metabolism, such as CoA or acyl carnitine.

[0098] In certain embodiments, FAHFAs and the like are estolides comprising a hydroxy fatty acid that is esterified at the hydroxyl group by a fatty acid. The FAHFAs provided by the invention are, in certain embodiments, structures of formula (I).



wherein: m is an integer from 0 to 21;
 n is an integer from 0 to 21;
 the sum of m and n is an integer from 11 to 21; and
 R^1 is an alkyl group.

[0099] In some embodiments of the invention, the FAHFA may exist as a salt or may be incorporated into other structures, including, but not limited to, phospholipids, glycerophospholipids, carbohydrates, polypeptides and proteins, di- and triglycerides, and other metabolic co-factors such as CoA or acyl carnitine.

[00100] In certain embodiments of the invention, the base unit of the FAHFA, the hydroxy fatty acid, is hydroxytetradecanoic acid (14 carbon atoms), hydroxypentadecanoic acid (15 carbon atoms), hydroxypalmitic acid (16 carbon atoms), hydroxyheptadecanoic acid (17 carbon atoms), hydroxystearic acid (18 carbon atoms), hydroxynonadecylic acid (19 carbon atoms), hydroxyicosanoic acid (20 carbon atoms), hydroxyhenicosanoic acid (21 carbon atoms), hydroxydocosanoic acid (22 carbon atoms), hydroxytricosanoic acid (23 carbon atoms), or hydroxytetracosanoic acid (24 carbon atoms), where, for each of the above, the hydroxyl group may substitute any of positions 2 through p-1, where p is the total number of carbons in the fatty acid. The fatty acid ester can be a saturated or unsaturated, linear or branched (C_i-C_{23}) alkyl group.

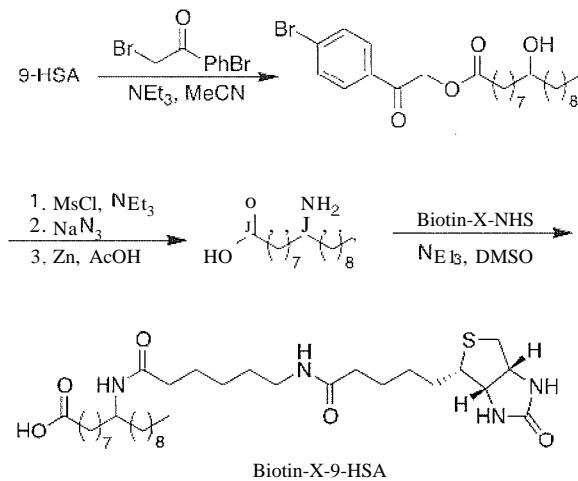
[00101] In particular embodiments, R^1 is a C15 residue derived from palmitic acid, a C₁₆ residue derived from margaric acid, a C₁₇ residue derived from stearic acid, or an unsaturated C₁₇ residue derived from oleic acid.

[00102] In a more particular embodiment of the invention, the FAHFA is 9-PAHSA, as shown in FIG. 5, and is 9-hydroxystearic acid esterified with palmitic acid. In the case of 9-PAHSA, m = 8, n = 7, and R¹ is C15H31 in formula (I). In another particular embodiment of the invention, the FAHFA is 5-hydroxystearic acid esterified with palmitic acid (5-PAHSA). In the case of 5-PAHSA, m = 12, n = 3, and R¹ is C15H31 in formula (I).

[00103] The present invention also discloses a method to identify specific protein-metabolite interactions through the synthesis of FAHFA derivatives, such as detectably labeled FAHFAs. For example, in some embodiments, FAHFAs are isotopically labeled and/or ester- or amide-bound to a detectable moiety, such as streptavidin, GST, an epitope tag such as FLAG, 6x His, or another affinity tag. In certain embodiments of the invention, the FAHFA is fluorinated for use in fluorous affinity chromatography, or bound to an alkyne for use with click chemistry. In a particular embodiment, FAHFAs are biotinylated FAHFAs. Because biotin binds tightly to streptavidin protein (*e.g.* immobilized streptavidin), the biotinylated FAHFA may be used in "pull down" assays that enable the identification of FAHFA binding partners, such a FAHFA-binding proteins. The FAHFA is biotinylated through an amide or ester linkage. In certain embodiments of the invention, the carboxylic acid of the FAHFA is amidated with a biotinylated alkyl amine (FIG. 19).

[00104] In a particular embodiment, hydroxyl group of the FAHFA is amidated and acylated with a biotin derivative. 9-hydroxystearic acid is protected as the ester with 2,4'-dibromoacetophenone. The product is mesylated with mesyl chloride, then the mesyl group is displaced by sodium azide. The azide is reduced with zinc dust in acetic acid to afford the aminated fatty acid. The amine is then acylated with a biotin derivative to afford a biotinylated FAHFA, as in Scheme 1.

[00105] Scheme 1.

**JJJ. Diagnostic methods, antibodies and kits**

[00106] The invention provides a variety of methods for the diagnosis, prognosis, and monitoring (*e.g.* disease progression and/or treatment efficacy) for a variety of disorders by determining the level of one or more FAHFAs in a biological sample from a subject and, *e.g.*, comparing the level of the one or more levels to suitable controls, such as annotated reference values for a particular disease or disorder, as well as monitoring them over time in a subject. Accordingly, the invention also provides kits and antibodies for performing these methods.

[00107] For the diagnostic, prognostic, or monitoring methods provided by the invention, the one or more FAHFAs can be detected by measuring the level of precursors (such as a hydroxy! fatty acid), metabolites, or derivatives of the one or more FAHFAs. The one or more FAHFAs—or precursor, metabolite, or derivative thereof—need only be detected at resolution at which they are distinguishable from other molecules in the given biological sample (or derived fraction thereof), such as plasma, serum, total lipids, *et cetera*.

[00108] As defined above, any mammalian subject can be evaluated by the methods of the invention, while human subjects are one particular exemplification. Also, the subject may be of any age, with adult human subjects serving as particular exemplifications. However veterinary applications, particularly in a research

context to develop treatments for human subjects are clearly encompassed by the invention as well.

[00109] Samples for use in the methods provided by the invention include any suitable biological sample or fraction thereof (*e.g.*, extracted total lipids or further subfractions thereof). In particular embodiments, the biological sample may be isolated from blood (*e.g.* serum or plasma), liver, adipose tissue, brown adipose tissue, muscle, pancreas, islet cells, kidney, breast, small intestine, bone marrow, nervous tissue (central, including brain or spine, and/or peripheral), ovary, or prostate. In some embodiments, the biological sample includes cancerous, precancerous, or neoplastic tissue that may include tissue obtained from any of the foregoing tissues. In more particular embodiments, the biological sample comprises serum, plasma, liver or adipose tissue. Biological samples may be assayed from fresh or fixed (or otherwise preserved) samples.

[00110] The classification of a sample as normal (or well-controlled) or associated with a disease state (and/or requiring a modified treatment protocol) depends on the particular indication being assayed for (*e.g.*, cancer versus obesity and diabetes-related disorders, including, in some embodiments, associated vascular disease). For example, in adipose tissue, levels of FAHFAs are negatively correlated with disease state—*i.e.* reduced levels FAHFAs in adipose tissue are associated with disease states such as diabetes-related disorders.

[00111] Levels of FAHFAs can be determined by any means known in the art, including tandem mass spectrometry (MS/MS) and in particular embodiments, MS/MS with multiple reaction monitoring (MRM). Additional detection methods include HPLC (high precision liquid chromatography; optionally coupled to MS/MS, with or without MRM), TLC (thin layer chromatography), NMR (Nuclear Magnetic Resonance) spectroscopy, IR (Infrared) spectroscopy, UV-VIS spectroscopy, GC (gas chromatography, optionally coupling to MS/MS), and capillary electrophoresis. Further detection methods include immune-methods employing antibodies that specifically bind to FAHFAs, including polyacrylimide electrophoresis, RIA, ELISA *et cetera*; nucleic acid-based or protein-based aptamer techniques), SPR (surface plasmon resonance), and SAT (suspension array

technology—including both immune-based, aptamer-based, or combination methods).

[00112] Levels of one or more FAHFAs can be evaluated and classified by a variety of means such as general linear model (GLM), ANOVA, regression (including logistic regression), support vector machines (SVM), linear discriminant analysis (LDA), principal component analysis (PCA), k-nearest neighbor (kNN), neural network (NN), nearest mean/centroid (NM), and bayesian covariate predictor (BCP). Suitable cutoffs for evaluating levels of one or more FAHFAs (*e.g.*, for classification as abnormal (obese; positive or at risks for a diabetes-related disorder, cancer, PCOS, an inflammatory disorder, or vascular disease; requiring modification of a treatment regime) or normal (or low risk, or positive response to treatment) can be determined using routine methods, such as ROC (receiver operating characteristic) analysis, and may be adjusted to achieve the desired sensitivity (*e.g.*, at least about 50, 52, 55, 57, 60, 62, 65, 67, 70, 72, 75, 77, 80, 82, 85, 87, 90, 92, 95, 97, or 99% sensitivity) and specificity (*e.g.*, at least about 50, 52, 55, 57, 60, 62, 65, 67, 70, 72, 75, 77, 80, 82, 85, 87, 90, 92, 95, 97, or 99% specificity).

[00113] For example, in particular embodiments, levels of one or more FAHFAs are converted to a disease index. In particular embodiments, a disease index can use raw or transformed (*e.g.* normalized to any suitable metabolite, log-normalized, percentile ranked, ranked as quartiles, *et cetera*) levels of FAHFAs. A disease index for a particular individual can be compared to reference values as, for example, a percentile rank. Using percentile ranks the skilled artisan can then diagnose, prognose, or otherwise clinically stratify a subject by comparing the subject's disease index to these reference values. For example, in certain embodiments, a subject with a disease index percentile rank of at least 40, 45, 50, 60, 65, 70, 75, 80, 85, 90, or 95 may be classified as having, being at an increased risk for developing, or needing additional/ alternative treatment for obesity, a diabetes-related disorder, cancer, PCOS, an inflammatory disorder, or vascular disease. In more particular embodiments, a subject exhibiting a disease index in at least the 60th, *e.g.*, at least 70th or at least 75th percentile is classified as having, being at an increased risk for developing, or needing additional/ alternative treatment for obesity, a diabetes-

related disorder, PCOS, an inflammatory disorder, cancer or vascular disease. A selected threshold for a disease index can be set to achieve a desired sensitivity or specificity, as described above, and/or to stratify subjects based on a relative hazard ratio between stratification groups. For example, in some embodiments, a disease index threshold is set to achieve a "hazard ratio" (ratio of frequency of a disorder between two stratification groups, *e.g.*, high and low risk of disease or complication) of about 1.1, 1.2, 1.4, 1.6, 1.8, 2.0, 2.2, 2.4, 2.5, 2.6, 2.8, 3.0, 3.5, 4.0, or more. In more particular embodiments, the index threshold is set to achieve a hazard ratio of at least about 3.8, 3.9, 4.0, 4.1, 4.2, 4.3, 4.4, or more, *e.g.*, 5, 6, 7, 8, 9, or 10.

"Stratification groups" are the groups of a data set satisfying one or more stratification criteria—for example, a percentile rank of disease index, such as all group members with a disease index greater than or equal to the 60th percentile. Stratification groups may be compared by any means by any statistic, such as, mean, median, mode, and/or standard deviation of any clinical parameter, such as age, duration of disease, frequency of death, *et cetera*.

[00114] Kits provided by the invention contain reagents to perform any of the methods provided by the invention, *e.g.* antibodies or other reagents to detect one or more FAHFAs, as described above. In particular embodiments, the kits include instructions for use. Optionally, the kits may include "suitable positive controls," which are compositions comprising (consisting essentially of, or consisting of) lipids that contain known concentrations of one or more FAHFAs. For example, suitable controls may be from a clinical source known to have obesity, a diabetes-related disorder, PCOS, an inflammatory disorder, cancer or vascular disease and may include either fixed or preserved but otherwise unprocessed biological sample, or, alternatively, isolated fractions from such samples, including fractions comprising (consisting of or consisting essentially of) lipids (*e.g.*, at least 20, 40, 50, 60, 70, 80, 90, 95, 97, 99% by dry weight, or more, lipids). Alternatively, in certain embodiments, the suitable positive controls may comprise artificial mixtures of lipids, *e.g.*, combined in proportions characteristic of an abnormal levels of one or more FAHFAs for a particular disorder and/or particular ranges of concentrations.

IV. Treatment methods

[00115] In another aspect, the invention provides methods of treatment comprising administering a suitable prophylaxis or treatment to a subject in need thereof, as determined by the methods provided by the invention—*e.g.*, according to any of the methods described under the previous subheading. Specifically, in certain embodiments, the subject is administered any clinically acceptable prophylaxis or treatment (including new regimens or modifications of existing regimens) for their given indication, based on the determination that the subject exhibits an abnormal level of one or more FAHFAs according to the methods provided by the invention. In more particular embodiments, any of the methods described in the claims or under the previous subheading further comprise the steps of follow-on diagnosis, prognosis or treatment. In other embodiments, the follow-on diagnosis, prognosis or treatment is performed by a provider advised of the presence of an abnormal level of one or more FAHFAs, but who did not necessarily make the determination. For example, where the subject has been identified as having, being at an increased risk for, or needing further treatment for obesity, a diabetes-related disorder, PCOS, an inflammatory disorder, cancer or vascular disease, at least in part, on the basis of abnormal levels of one or more FAHFAs, a provider administers or directs the subject to undergo a prophylactic and/or treatment regime (or a change in an existing treatment) suitable for the indication.

[00116] Suitable prophylaxes or treatments for diabetes-related disorders include, for example, weight loss programs, increased exercise, modified diet (*e.g.*, reduced glycemic index), GLP-1R (human GenelD 2740) agonists (such as exenatide and liraglutide); DPP-4 antagonists (*e.g.*, saxagliptin, vildagliptin); pramlintide; insulins (*e.g.*, glulisine, detemir, glargine, lispro, aspart); SGLT2 (human GenelD No. 6524) inhibitors; inhibitors of glucose synthesis or release (FR-225654, CS-917 and MB07803; including starch blockers, such as acarbose); inhibitors of pyruvate kinase M2 (human GenelD No. 5315) (including agents described in U.S. Patent Application Publication No. 20100099726 A1, incorporated by reference in its entirety); insulin sensitizers (such as biguanidines, including metformin); adiponectin receptor 1 (human GenelD No. 51094) and adiponectin receptor 2

(human GenelD No. 79602) agonists; leptin receptor (human GenelD No. 3953) agonists; anoretics (*e.g.*, sibutramine, rimonabant, bupropion); and the like, including combinations of the foregoing.

[00117] Suitable prophylaxes or treatments for obesity include weight loss programs, increased exercise, modified diet (*e.g.* reduced caloric, carbohydrate, or fat diets), gastric bypass or laproscopic banding, SGLT2 (human GenelD No. 6524) inhibitors; inhibitors of glucose synthesis or release (SB-204990, 2-deoxy-D-glucose (2DG), 3-bromopyruvate (3-BrPA, Bromopyruvic acid, or bromopyruvate), 3-BrOP, 5-thioglucose and dichloroacetic acid (DCA), FR-225654, CS-917 and MB07803); inhibitors of pyruvate kinase M2 (human GenelD No. 5315) (including agents described in U.S. Patent Application Publication No. 20100099726 A1, incorporated by reference in its entirety); leptin receptor (human GenelD No. 3953) agonists; anoretics (*e.g.*, sibutramine, rimonabant, bupropion); fat absorption inhibitors (such as orlistat); and the like, including combinations of the foregoing.

[00118] Suitable prophylaxes or treatments for cancer include chemotherapy, hormonal therapy, immunotherapy, radiotherapy, surgery, targeted gene therapies (*e.g.*, epidermal growth factor receptor-tyrosine kinase inhibitors, such as gefitinib; and agents targeting ALK mutations and rearrangements, such as crizotinib, *et cetera*), glycolytic inhibitors (*e.g.*, SB-204990, 2DG, 3-BrOP, 5-thioglucose, DCA, as well as those agents described in U.S. Patent Application Publication No. 20100099726 A1), inhibitors of ATP-citrate lyase, inhibitors acetyl-CoA carboxylase, inhibitors fatty acid synthase, and combinations of the foregoing.

[00119] Suitable prophylaxes or treatments for vascular disease include weight loss programs (such as the pharmaceutical obesity treatments, above), increased exercise, modified diet (*e.g.* reduced salt), statin treatment (*e.g.* atorvastatin, fluvastatin, lovastatin, pitavastatin, pravastatin, rosuvastatin and simvastatin), bypass surgery and stenting; as well as combinations of the foregoing.

[00120] Suitable prophylaxes or treatments for PCOS include weight loss, hormonal therapy (*e.g.* birth control pills, progesterone), meformin, LH-releasing hormone analogs, thiazolidinediones, and cyproterone or spironolactone (or other

antiandrogen), and clomifene (or other selective estrogen receptor modulator), including combinations of the foregoing.

[00121] Suitable prophylaxes or treatments for inflammatory disorders include immunomodulators, including immunosuppressants, such as methotrexate or steroids; NSAIDs; COX-2 inhibitors (coxibs); neutralizing antibodies directed to a pro-inflammatory cytokine; a soluble receptor for a pro-inflammatory cytokine; or exogenous anti-inflammatory cytokines, as well as agents that increase the levels of anti-inflammatory cytokines, *e.g.*, at either the nucleic acid or protein level; including combinations of the above.

[00122] It is, of course, encompassed by the methods provided by the invention that a subject determined to have obesity, a diabetes-related disorder, PCOS, an inflammatory disorder, cancer or vascular disease may have one or more of these disorders, *e.g.* 2, 3 or 4 or more of these disorders, and may therefore provide an indication for treatment with combinations of prophylaxes or treatments for the different disorders, *i.e.* combinations of prophylaxes or treatments for obesity, a diabetes-related disorder, PCOS, an inflammatory disorder, cancer or vascular disease as described above.

[00123] In another aspect, the invention provides methods of treating a subject with any one or more of obesity, a diabetes-related disorder, PCOS, an inflammatory disorder, cancer or vascular disease, comprising administering to the subject a therapeutically effective amount of one or more agents that increase the level of one or more FAHFAs. In particular embodiments, the agent that increases the level of one or more FAHFAs is one or more exogenous FAHFAs—*i.e.*, one or more of any of the FAHFAs described herein, *e.g.*, a composition comprising 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more FAHFA species. For example, in particular embodiments, the composition comprises 5-PAHSA and/or 9-PAHSA. In certain embodiments the one or more FAHFAs administered to the subject are detectably labeled, as described above.

[00124] In other embodiments, the agent that increases the level of one or more FAHFAs is an inhibitor (antagonist or negative agonist) of carboxyl ester lipase (CEL)(human GenelD No. 1056). Inhibitors of CEL include siRNAs *{e.g.,* which

target a mRNA at least 60%, *e.g.*, 60, 65, 70, 75, 80, 85, 90, 95, 96, 97, 98, 99, or 100% identical to NM_001807, or a fragment thereof, *e.g.*, a contiguous fragment of at least 20, 25, 30, 35, 40, 45, 50, 100, 200, 300, 400, 500, 1000, 1500, 2000, or 2384 bp of any of the foregoing; or a nucleic acid that hybridizes under highly stringent hybridization conditions to any of the foregoing); neutralizing antibodies {*e.g.*, which specifically bind to polypeptide comprising an amino acid sequence at least 60%, *e.g.*, 60, 65, 70, 75, 80, 85, 90, 95, 96, 97, 98, 99, or 100% identical to NP_001798, or a fragment thereof, *e.g.*, a contiguous fragment of at least 5, 10, 20, 25, 30, 35, 40, 45, 50, 100, 200, 300, 400, 500, 600, 700, or 756 amino acids of any of the foregoing; in particular embodiments the antibody specifically binds a portion of CEL comprising one of the amino acid residues of the catalytic triad (*see* conserved domain cd00312; *e.g.* S217, D343, and H458 of NP_001798) or substrate binding pocket (*see* conserved domain cd00312); an aptamer (*e.g.* polypeptide or nucleic acid, including analogs of either) directed to the catalytic triad or substrate binding pocket of CEL, as exemplified above; or any of the compounds described in U.S. Patent Application Publication No. 20100324075, or U.S. Patent Nos. 5,391,571; 5,512,565; 5,942,631; 6,034,255; 6,114,5455,017,565; and 5,063,210, which are incorporated by reference; as well as natural, non-FAHFA substrates of CEL, which act as competitive inhibitors; and combinations of any of the foregoing.

[00125] In different embodiments, the agent that increases FAHFA levels is a ChREBP expression product, such as a ChREBP α expression product, a ChREBP β expression product, or a combination of ChREBP α and β expression products, (collectively "ChREBP expression products") as further described in U.S. Provisional Application No. 61/590,012, filed January 24, 2012, which is incorporated by reference in its entirety. "ChREBP β expression product" or simply "ChREBP β " is a nucleic acid encoding an N-terminally truncated isoform of ChREBP that exhibits enhanced transcriptional activation relative to ChREBP protein that is not N-terminally truncated—termed "ChREBP α ," here; "ChREBP β " and "ChREBP β expression product" also encompasses a protein expression product encoded by a nucleic acid that encodes an N-terminally truncated isoform of ChREBP that exhibits enhanced transcriptional activation relative to ChREBP α .

The ChREBP expression products for use in the inventions can be either recombinant or non-recombinant. In a particular embodiment, ChREBP α is exemplified by the reference sequence NP_1 16569, and encompasses proteins comprising an amino acid sequence at least 60, 65, 70, 75, 80, 85, 90, 95, 96, 97, 98, 99, or 100% identical to this reference sequence. In some embodiments a ChREBP β expression product has (or, in the case of a nucleic acid, encodes) an N-terminal truncation, relative to ChREBP α , of at least about 10, 20, 30, 40, 50, 75, 100, 125, 150, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, or 180 amino acids. In certain embodiments, the ChREBP β expression product is encoded by a transcript lacking the first exon of a ChREBP transcript encoding a ChREBP α protein (such as the reference sequence NM_032951.2, as well as nucleic acids at least 60, 65, 70, 75, 80, 85, 90, 95, 96, 97, 98, 99, or 100% identical to this reference sequence or that hybridize to its complement under highly stringent hybridization conditions). In more particular embodiments, a ChREBP β expression product is encoded by a transcript comprising an alternative first exon, termed "exon 1b" herein, preferably wherein the exon 1b does not encode protein sequence. ChREBP expression products will typically retain the function of structurally conserved regions, including those depicted in FIG. 18 of Application No. 61/590,012 and, in more preferred embodiments, which preserve the function of the "P2" domain depicted in Li *et al.* 2006 (see FIG. 2 in Li, which is incorporated herein by reference, and is contained within the GRACE domain), which is required for ChREBP function.

[00126] In other embodiments, an agent that increases the level of one or more FAHFA is a PPAR agonist, such as an agonist of PPAR α (human GenelD No. 5465), PPAR γ (human GenelD No. 5468), or PPAR δ (human GenelD No. 5467) activity, and in particular embodiments, PPAR the PPAR agonist is WY14643, CAY10592, or Pioglitazone or other thiazolediendiones including rosiglitazone, troglitazone or other PPAR gamma agonists, or any of the agents described in Kahn and McGraw *N. Engl. J. Med.* 363(27):2667-9 (2010) or paragraph 301 of U.S. Patent Application Publication No. 20120052040, which are incorporated by reference in their entirety. In the examples of the present application, Applicants show that levels of the 5 PAHFA are highest in liver and brown fat and increase in

serum and liver with fasting. PPAR α is a master metabolic regulator of the fasting state and in the liver upregulates FA oxidation, enhancing ketogenesis. PPAR α is also critical for FA oxidation required for BAT (brown adipose tissue) thermogenesis. The data in this application indicates that circulating 5 FAHFA levels are regulated in a PPAR α dependent manner. These data suggest that the 5 FAHFA might be important for mediating biological actions of PPAR α activation. In the liver, this may include activating FA oxidation which may be therapeutically useful for treating fatty liver disease. Increasing FA oxidation in BAT may enhance thermogenesis and whole-body energy expenditure which may help treat obesity and obesity associated metabolic diseases. 5 FAHFA may be one means by which BAT affects systemic metabolic controls.

[00127] The one or more agents that increase the level of one or more FAHFA is administered in a therapeutically effective amount to the subject, in any suitable formulation and by any suitably mode of administration. Suitable modes of administration include *e.g.*, intra venous, or intra peritoneal, rectal, oral or nasal administration, with oral being a particular mode of administration. Appropriate formulations include tablets, gelcaps, syrups, oil mixtures, emulsions, *et cetera*. For example, oil mixtures include preparing the FAHFA in a formulation with a pharmaceutical or dietary oil, for example, in a gelcap or as a dietary supplement in cooking oil, salad oil, or in a dropper formulation for direct oral administration or mixing in, *e.g.*, yogurt, milk, *et cetera*. In certain embodiments, the agent that increases FAHFA levels is *e.g.*, supported by a solid carrier, such as starch, dextrose, yeast extract, wheat germ, *et cetera*, for sprinkling onto food. In other embodiments, one or more FAHFAs are delivered by slow release subcutaneous pellets or infrequent subcutaneous depot injection with release over months.

[00128] In other aspects, the invention provides methods of decreasing pro-inflammatory signaling or increasing: glucose uptake, glucose tolerance, insulin secretion, or insulin sensitivity in either an isolated cell or in a subject. These methods comprise contacting the cell, or administering to the subject, an agent that increases the level of one or more FAHFAs, as described, above. In particular embodiments, these methods comprise contacting the cell with one or more

FAHFAAs alone or in combination with any of the foregoing agents that increase the levels of one or more FAHFAAs, *e.g.*, a CEL antagonist, a ChREBP expression product, or a PPAR agonist.

V. Screening methods

[00129] In another aspect, the invention provides screening methods for identifying an agent that modulates FAHFA levels. The methods comprise determining the level of one or more FAHFAAs in a cell contacted with a candidate agent, where a change in the level of one or more FAHFAAs in the cell relative to a control cell not contacted with the agent indicates that the agent modulates the level of one or more FAHFAAs.

[00130] Any cell type may be useful in such methods including, yeast or insect cells, or a mammalian cell, *e.g.* a primate, murine, bovine, ovine, leporine, or porcine cell. The cell may be isolated, *e.g.*, the method is performed *in vitro*, in a suitable culture, *e.g.*, with either primary or established cell lines. Suitable cells include macrophages (such as a RAW cell), islet cells (such as the Insl cell line), or a hepatic cell (such as HepG2 cells). In some embodiments, the cells are recombinant while in other embodiments the cells are non-recombinant. In other embodiments, the cell is *in situ*, *i.e.*, the method is performed *in vivo* in a non-human animal, and in more particular embodiments in a non-human mammal, such as a non-human primate, a leporine or murine. The non-human animal may be transgenic or non-transgenic and such animals may also serve as the source of cells for the *in vitro* screening methods provided by the invention. In particular embodiments, the non-human mammal is a mouse and in more particular embodiments is an AG40X mouse, an AG4KO mouse, a ChREBPKO mouse, or a ChREBPOX mouse.

[00131] In some embodiments, the cell expresses ChREBP and, in particular embodiments, overexpress ChREBP α or β , and in still more particular embodiments, overexpress ChREBP α or β in adipose tissue. In other embodiments, the cell may express reduced levels of ChREBP α or β or express a hypomorphic or dominant negative form of ChREBP or a dominant negative form of its dimerization

partner Mix (human GenelD No. 6945), and in still more particular embodiments, the cell may be a ChREBP or Mix knockout (ChREBPKO or MlxKO). In other embodiments, the cell expresses GLUT4 (SLC2A4, human GenelD No. 6517), and in more particular embodiments overexpresses GLUT4 and in still more particular embodiments, overexpresses GLUT4 in adipose tissue (AG40X). In other embodiments, the cell may express reduced levels of GLUT4 or express a hypomorphic or dominant negative form of GLUT4 and in still more particular embodiments, the cell may be a GLUT4 knockout (GLUT4KO) or more particularly, a knockout in adipose tissue only, AG4KO).

[00132] The cell, in certain embodiments, further expresses Mix. In other embodiments, the cell further expresses GLUT4 and in more particular embodiments, the cell further expresses both Mix and GLUT4. Although not essential to all aspects of these screening methods, in some embodiments, the methods include incubating the cell in the presence of glucose or fructose or their metabolites or analogs such as glucose-6-phosphate, xyulose-5-phosphate, fructose-2,6-bisphosphate, mannoheptulose, 2-deoxyglucose (2DG), or fluorodeoxyglucose. In other embodiments, the cell is incubated in low glucose conditions (*e.g.* the cell is cultured in low glucose conditions or the non-human mammal is maintained on a low carbohydrate diet). For example, in particular embodiments, the cell is cultured in <25mM glucose, *e.g.* less than 10 or 5 mM glucose, *e.g.* about 1-5 mM, 2-3 mM, or 2.5 mM glucose.

[00133] The screening methods provided by the invention can, in some embodiments, include screening the candidate agent in the presence of additional agents, *e.g.*, a second (or more) candidate therapeutic. For example, second therapeutic agents can include one or more of any of the therapeutics described under the previous subheading in order to identify combinations of agents with synergistic interactions. In other embodiments, the cell is contacted with a modulator of PPAR α (human GenelD No. 5465), PPAR γ (human GenelD No. 5468), or PPAR δ (human GenelD No. 5467) activity, and in particular embodiments, PPAR agonists, such as WY14643, CAY10592, or Pioglitazone.

[00134] Agents identified by these screening methods may either increase or decrease the level of one or more FAHFAs. Methods of treating obesity, a diabetes-related disorder, PCOS, an inflammatory disorder, cancer or vascular disease by administering an effective amount of one or more of the agents identified by these screening methods are contemplated and encompassed by the present invention.

EXAMPLES

[00135] The following examples serve to illustrate, and in no way to limit, the present invention.

Example 1.

Identification of a novel class of fatty acid conjugates by metabolomics.

[00136] As described herein, a novel class of lipid metabolites has been identified through the application of metabolomics to the analysis of adipose tissue from transgenic mice that overexpress the insulin-responsive glucose transporter, GLUT4, in adipose tissue but not in muscle [1-3]. Glucose disposal was enhanced in isolated adipocytes from transgenic mice versus wild-type controls. This led to altered gene expression, including genes involved in lipid metabolism, and whole-body insulin sensitivity was improved despite the fact that GLUT4 overexpression was localized to adipose tissue. Furthermore, adipose GLUT4 overexpression can reverse the insulin resistance and diabetes in mice lacking GLUT4 in muscle [3]. These data suggested that a circulating factor, perhaps a lipid, may exert anti-diabetic effects on peripheral tissues in these mice.

[00137] Given the elevated expression of genes involved in lipid metabolism, it was decided to measure changes in adipose tissue from these mice to identify any specific changes in their lipid metabolism. Subcutaneous adipose tissue from GLUT4-overexpressing or wild-type mice was homogenized and extracted with chloroform-methanol-water[4-6] to yield lipid extracts that were analyzed by LC-MS (FIG. 2). The analysis was performed on a time-of-flight (TOF) mass spectrometer whose high mass accuracy permits the direct calculation of candidate molecular formulae [6]. The LC-MS chromatograms were aligned and compared by XCMS [7], a data analysis software for metabolomics applications. Metabolite ions

were ranked by statistical significance and fold change to highlight changing ions of interest; these data can be plotted on a volcano plot (FIG. 3). One group of highly regulated metabolite ions in our negative mode data set was identified that seemed to belong to the same lipid family but whose identity was unknown. These ions were observed in both transgenic and wild type samples, though at 40-fold higher levels in the transgenic samples.

Structural characterization of novel metabolite.

[00138] The most abundant ion in this family exhibited a mass-to-charge ratio of m/z 537.487 that was consistent with the molecular formula $C_{34}H_{66}O_4$ (FIG. 4a). Analysis of the fragmentation pattern of each member of the ion family on a quadrupole TOF mass spectrometer revealed that these metabolites were likely fatty acyl-hydroxy fatty acid conjugates (FIG. 4b), herein abbreviated as FAHFA. The m/z 537 species, in particular, was thus comprised of palmitic acid (PA) and hydroxystearic acid (HSA) components, abbreviated as PAHSA (FIG. 5).

[00139] The precise isomer of hydroxystearic acid that was elevated in the transgenic mice was then determined. At high collisional dissociation energies (50 V), 12-hydroxystearic acid produced diagnostic fragment ions, m/z 113 and 169, resulting from bond cleavage at predictable locations near the hydroxyl group [8]. 12-PAHSA was synthesized from commercially available 12-HSA and, in both species, we confirmed that the same diagnostic fragment ions were observed. This indicated that the high energy HFA fragmentation method could extend to the identification of the position of the hydroxyl group of FAHFAs. From the fragmentation pattern of FAHFAs seen *in vivo*, it was determined that the major isomer of the upregulated lipid was 9-PAHSA (FIG. 6a). To confirm the structure [^{13}C]-labeled 9-PAHSA was synthesized from commercially available 9-HSA and all-[^{13}C]-palmitic acid, which was then coextracted with transgenic adipose tissue and analyzed without further purification. It was observed by LC-MS that the authentic standard coeluted at the same retention time with the major natural isomer (FIG. 6b).

Tissue distribution of PAHSA levels in wild type mice.

[00140] In order to assess the relevance of these novel lipids in normal mammalian biology, PAHSA levels were measured in several wild-type tissues by isotope-dilution mass spectrometry (IDMS) using our [¹³C]-9-PAHSA standard and a highly sensitive multiple reaction monitoring (MRM) method. Tissues were extracted in the presence of a known amount of internal standard, and the ion intensities were ratioed to obtain absolute levels of PAHSA in the tissues, expressed as nmol/g tissue. The presence of PAHSA was observed in all tissues measured, and PAHSA levels were highest in pancreas and kidney (~10 nmol/g tissue), indicating that their presence was not merely an artifact of the transgenic mouse model (FIG. 7). These lipid levels are below those of unesterified saturated fatty acids such as stearate or palmitate but are comparable to the levels of some signaling molecules such as 2-arachidonoylglycerol or oleoylethanolamine in brain tissue [9-10]. Nevertheless, it is believed that these specific lipid structures have never been previously described, and they constitute a novel class of fatty acid conjugates.

Development of an assay to measure FAHFA degradation activity.

[00141] To establish whether or not FAHFA can be enzymatically degraded, an LC-MS-based *in vitro* activity assay was developed to measure the hydrolysis of 9-PAHSA to 9-HSA. Liver tissue was initially profiled because it contains a large number and variety of hydrolytic enzymes [11-12]. Liver lysate (1 mg/ml protein) was incubated with 100 μ M 9-PAHSA at 37 °C for two hours. The reaction mixture was then extracted with one volume ethyl acetate, concentrated under nitrogen gas stream, and reconstituted in 1:1 chloroform: methanol for MS analysis. Substantial 9-HSA production was observed that was enriched in the membrane fraction (isolated by ultracentrifugation) and depleted in the soluble fraction (FIG. 8a). To determine the class of enzyme responsible for FAHFA hydrolysis, several classes of enzyme inhibitors were screened. Only the serine hydrolase inhibitors demonstrated nearly complete abrogation of FAHFA hydrolysis (FIG. 8b), and inhibition by the fluorophosphonate inhibitor DFP was dose-dependent (not shown).

Identification of a FAHFA-degrading enzyme.

[00142] A global database of serine hydrolases, complete with tissue distribution data, was recently assembled by Cravatt and colleagues [11]. The candidates were narrowed down further by comparing the tissue distribution of FAHFA hydrolysis activity to the tissue distribution of activity for every membrane-localized serine hydrolase. Membrane fractions were prepared from pancreas, liver, fat, muscle, brain and kidney, then diluted to 1 mg/ml protein concentration and incubated with 100 μ M 9-PAHSA for 30 min at room temperature. Pancreas membrane exhibited the highest FAHFA hydrolysis activity and was ~10-fold higher than in liver membrane (FIG. 9).

[00143] Only three membrane-bound serine hydrolases were reported to possess significant activity in pancreas: carboxyl ester lipase (CEL), arylacetamide deacetylase-like 1 (AADACLI), and carboxylesterase 3 (CES3). Plasmid expression clones for these enzymes were obtained, which were under the control of the cytomegalovirus promoter (from the Cravatt lab), with which we transfected HEK293T cells to assess the ability of these enzymes to hydrolyze 9-PAHSA. For the activity assays, cell lysates were diluted according to the relative expression of the three clones according to ABP intensities. Remarkably, only CEL-transfected cell lysate was able to hydrolyze FAHFA above the level of background hydrolysis activity present in the untransfected HEK cell lysate (FIG. 10). The increased activity in CEL-transfected cell lysates was reversed by addition of the CEL-selective inhibitor, WWL92 [13] (not shown).

[00144] Inhibition of FAHFA hydrolysis by WWL92 treatment was observed in liver and pancreas membrane lysates in a dose-dependent manner. Liver activity was only partially inhibited, while the higher pancreas activity was almost completely extinguished by WWL92 treatment (FIGs. 11a, b). High levels of WWL92-sensitive hydrolysis activity in the pancreas were consistent with the relative tissue abundance of CEL expression as measured by immunoblotting (FIG. 11c).

Bile-salt activation of FAHFA hydrolysis.

[00145] Originally characterized as "bile salt-activated lipase", CEL is secreted by acinar pancreatic cells into the duodenum where it plays a significant role in dietary lipid digestion [14]. Its activity is enhanced by primary bile salts, such as sodium taurocholate (NaTC), which promote substrate access to the lipase active site [15]. We first treated CEL-transfected cell lysates with increasing concentrations of NaTC in the presence of 9-PAHSA and verified that FAHFA hydrolysis was enhanced (FIG. 12a). The experiment was repeated with either liver or pancreas membrane lysate. Notably, FAHFA hydrolysis in liver lysate was not enhanced by NaTC, while FAHFA hydrolysis in pancreas lysate was markedly increased (FIG. 12a). Pancreas membrane lysate was then pretreated with WWL92 and verified that the increase in FAHFA activity upon treatment with NaTC was due to increased CEL activity (FIG. 12b).

Preliminary experiments with purified CEL.

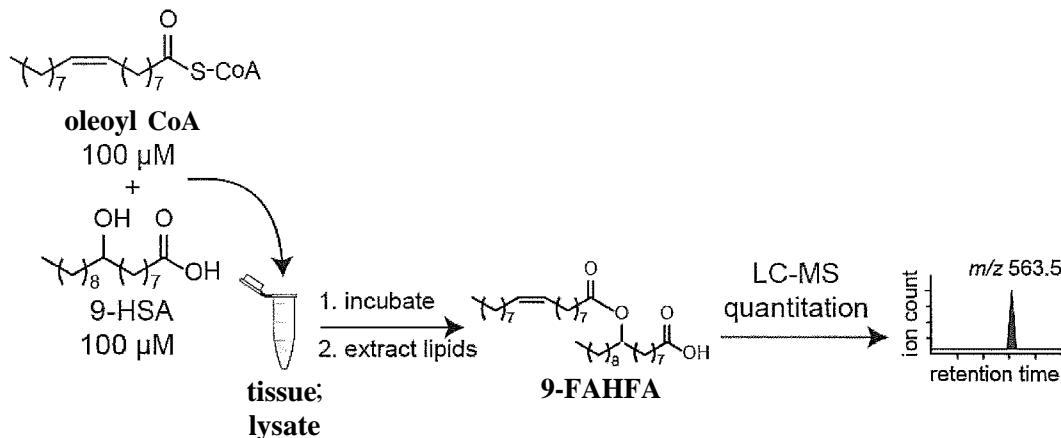
[00146] To confirm the identification of CEL as a FAHFA hydrolase, a small amount of purified enzyme (available commercially from R&D Systems) was used. As expected, substantial hydrolysis of 9-PAHSA was observed, as measured by 9-HSA production, when incubated with purified CEL in the presence of micellar concentrations of NaTC (FIG. 13). The results were comparable to CEL hydrolysis activity of a known substrate, dioleylglycerol (DOG) [14].

Development of an assay to measure FAHFA acyltransferase activity.

[00147] In order to understand the biology of the new FAHFA lipid class more completely, experiments were performed to identify an enzyme capable of synthesizing FAHFAs as well. By structural analogy to other lipid metabolites such as diacylglycerols or wax esters, it was hypothesized that hydroxy fatty acids were the immediate precursors to FAHFAs. Candidate acyltransferases were identified by reacting the common biochemical acyl carrier, oleoyl-CoA, with 9-hydroxystearic acid (9-HSA) in the presence of cell lysates or tissue membrane lysates to form 9-OAHSA (Scheme 2).

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[00148] Scheme 2.



[00149] After first establishing that FA-CoA:HFA acyltransferase activity was membrane-localized in liver (not shown), we isolated membrane fractions from liver or WAT tissue lysate by ultracentrifugation, then resuspended the pellets in PBS buffer and diluted to 1 mg/ml protein concentration. Oleoyl-CoA and 9-HAS were incubated, both at 100 μ M concentration, with the membrane lysates for 2 hours at 37 °C. The reaction mixtures were extracted with ethyl acetate, concentrated, and reconstituted in 1:1 chloroform:methanol for MS analysis. Formation of 9-OAHSA was measured by MS/MS, with which the appearance of the parent ion m/z 563 was monitored. Substantial 9-OAHSA production was observed in both liver and WAT samples, but not in heat-treated samples (FIG. 14).

[00150] To assess whether the observed activity was limited to liver and WAT, the tissue distribution of HFA acylation activity was explored by performing the FAHFA biosynthesis assay with membrane lysates from pancreas, liver, fat, muscle, brain and kidney (FIG. 15). Production of 9-OAHSA was observed at comparable levels in all tissues, well in excess of background activity. Highest levels were detected in pancreas and kidney, which parallels the tissue distribution of FAHFA levels *in vivo*.

Efforts to identify candidate **acyltransferases** with **FAHFA** biosynthesis activity.

[00151] In light of the above results acyltransferase families that have been reported to have a membrane-bound localization and to have the ability to conjugate FA-CoA to a hydrophobic alcohol were targeted [16-19]. Two of the enzymes, DGAT1 and ACAT, had selective inhibitors that were commercially available, A922500 [20] and CI976 [21], which were used to test their ability to reduce FAHFA production in the biosynthesis assay (inhibitors purchased from Tocris Bioscience). Tissue lysates were incubated with a reduced concentration of 9-HSA and oleoyl-CoA (10 μ M) and with increasing doses of A922500 or CI976, but no change in 9-OAHSA levels was observed (FIG. 16a). This suggested that neither DGAT1 nor ACAT, two FA-CoA:HFA acyltransferase candidates, were responsible for the observed biosynthesis activity in the tissues studied.

[00152] Next it was attempted to increase FAHFA production by transfecting HEK293T cells with expression-ready clones of DGAT1/2, MGAT1/2, and ACAT2 enzymes (from Dr. Robert Farese, UCSF) [22]. Overexpression of these enzymes did not lead to elevated FA-CoA:HFA acyltransferase activity compared to the background activity in HEK293T cell lysate (FIG. 16b). This experiment indicates that these five enzymes are likely not FA-CoA:HFA acyltransferases.

FAHFA biosynthesis activity in 3T3-L1 adipocytes.

[00153] Since FAHFAs were initially discovered in adipose tissue, and CEL is not known to be significantly expressed in fat, it was next investigated whether adipose FAHFA levels could be controlled by an acyl-CoA:HFA acyltransferase. As a first step, whole cell lysates from mature 3T3-L1 cells were incubated with oleoyl-CoA and 9-HAS, then extracted and analyzed by LC-MS (FIG. 17). Meaningful production of 9-OAHSA was observed in these cells, implying that 3T3-L1 cells possess the ability to produce, not merely store, the conjugated FAHFAs.

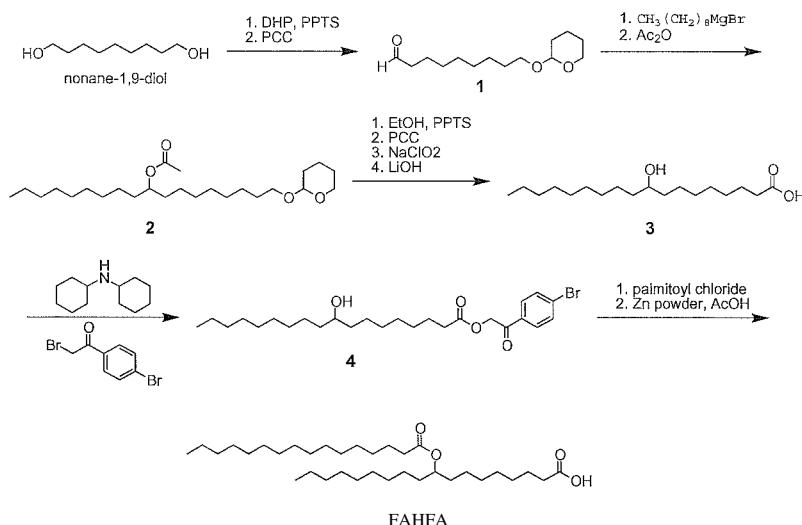
Correlation of changes in HFA levels with changes in FAHFA levels.

[00154] HFA levels in the lipid extracts from AG40X and WT mice were measured to analyze their hydroxyl fatty acids (HFAs) (FIG. 18). Representative lipid extracts from WT and AG40X adipose tissue were compared to 9- and 12-HSA standards (Indofme Chemical Company and Sigma-Aldrich). Interestingly, AG40X fat exhibited a three-fold enrichment in 9-HSA abundance compared to WT fat, which was more modest than the robust fold change observed in 9-PAHSA levels in these tissues. It is reasonable to believe that 9-PAHSA, acting as a storage form of 9-HSA, may preferentially accumulate in tissues rather than 9-HSA in response to increased 9-HSA levels.

Chemical synthesis of the FAHFAs.

[00155] The identification of the FAHFAs required that these compounds be synthesized to confirm that the structural assignments were correct. To enable the synthesis a simple approach was developed that would enable one to make a variety of FAHFA derivatives (Scheme 3). In the initial synthesis, large amounts of the 9-hydroxy FAHFA were prepared. The strategy began with the simple desymmetrization of a 1,9-nonanediol, available in large quantities using substoichiometric equivalents of a dihydropyran protecting group, which afforded the mono-THP protected alcohol (30% yield). This alcohol was then oxidized to the aldehyde (1) using PCC^{58,59} (53% yield). Addition of the Grignard reagent *n*-nonylmagnesium bromide to 1 resulted in a secondary alcohol at position 9 of the alkyl chain (63% yield). This material was acylated with acetic anhydride to afford the THP-protected acetate (2) (100% yield). In a series of steps then carried out without purification, the THP group was removed with PPTS, the resulting alcohol oxidized to an aldehyde with PCC and the aldehyde oxidized to the carboxylate with sodium chlorite. Finally, the acetate was removed with lithium hydroxide to afford 9-HSA (3, 52% overall yield for the four steps).

[00156] Scheme 3



[00157] Next, using method developed by Zhang and colleagues the 9-HAS was reacted with 2-bromo-1-(4-bromophenyl)ethanone to give the protected ester (4, 20% yield) [23]. Our yield was much lower than the reactions reported in the original paper so further optimization will be necessary. Nevertheless, sufficient material was isolated to complete the synthesis by acylating the hydroxyl group of 4 with palmitoyl chloride in the presence of 4-dimethylaminopyridine (DMAP) followed by deprotection with zinc dust in acetic acid to provide the FAHFA (53% overall yield for the two steps). Importantly, this synthetic route is highly modular and the key pieces are commercially available, which will enable the rapid synthesis of additional derivatives. Different FAHFA derivatives can be synthesized using this route to enable the isolation and identification of FAHFA-binding proteins.

Example 2.

FAHFAs in White Adipose Tissue (WAT).

[00158] From a previous study, it was found that there is a strong correlation between adipose-ChREBP expression and insulin sensitivity in humans (FIG. 20). Because enhanced lipogenesis in WAT of mice with adipose-selective overexpression of Glut4 was found to be critical for the enhanced insulin sensitivity in this model [24-25], and because Glut4 is markedly down-regulated in WAT in

obese and insulin resistant humans [1], the determinates of which lipids were being synthesized in response to increased adipose-Glut4 expression was carried out by an untargeted lipidomics analysis. Many classes of lipids were altered in WAT of adipose-Glut4 overexpressing mice but one class stood out as very highly upregulated (FIG. 3, cluster in upper right). Using Mass Spectrometry, it was determined that this is a novel class of lipids that has not been described before in mammalian tissues. Structural studies revealed that they were fatty acyl hydroxy fatty acids (FAHFA). When the fatty acyl group is palmitate and the ester bond with the hydroxy fatty acid (stearic acid) is at position 9, this yields palmitoyl-9-hydroxystearic acid (9-PAHSA) whereas when the fatty acyl group is oleate and the ester bond is at position 8, this yields oleoyl-8-hydroxystearic acid (8-OAHSAs). There were at least 8 FAHFA family members in tissues including WAT, BAT, pancreas, liver, muscle, kidney and brain (FIG. 7) and several of these forms were present in serum (FIGs 21, 23, 24). Levels were particularly high in pancreas and kidney (FIG. 7).

[00159] To determine the relevance of FAHFAs to humans, FAHFA levels were measured in human serum and adipose biopsies. FAHFAs were present in serum of normal people at a concentration of 45-100 nanomolar and in adipose tissue at ~1 pmol/mg of lipid (FIG. 21). The serum concentrations were somewhat higher than hormones including testosterone, estradiol, vitamin D and triiodothyronine and were lower than most other fatty acids. The tissue levels were lower than nonesterified saturated fatty acids such as stearate or palmitate but were comparable to levels of signaling lipids such as 2-arachidonoylglycerol (a physiological ligand for cannabinoid receptors) and oleoylethanolamine (a ligand for PPARalpha) in brain tissue [9-10].

[00160] Since ChREBP expression and ChREBP-driven lipogenesis in WAT is highly associated with insulin sensitivity in rodents and humans [24], studies were carried out to determine whether the production of FAHFAs is regulated by ChREBP. FIG. 22 shows that FAHFA levels were increased ~6-fold in WAT of adipose-Glut4 overexpressing mice compared to wildtype (WT). FAHFA levels were reduced in WAT of ChREBP knockout (KO) mice and when adipose-Glut4

overexpressing mice were bred with ChREBP KO mice, the elevated FAHFA levels were markedly reduced (FIG. 22). In serum of ChREBP KO mice, levels of the 2 major isomers of FAHFAs were reduced by 50-60% (FIG. 23). This indicated that FAHFA synthesis and/or degradation is linked to ChREBP and that the beneficial metabolic effects of ChREBP-induced lipogenesis in WAT could be mediated, at least in part, by FAHFAs. We next investigated the physiologic regulation of FAHFAs. The two major FAHFA isomers in serum are reduced by fasting and rapidly restored or increased by refeeding (FIG. 24).

Example 3.

PAHSA levels in serum from insulin-resistant human subjects.

[00161] Since the FAHFAs were first found to be elevated in diabetes-resistant mouse model (AG40X mice), it was important to determine whether FAHFA levels correlated with insulin sensitivity in a well-defined cohort of human subjects. Samples of human serum were obtained from a cohort of twenty-four Swedish volunteers at the Lundberg Laboratory for Diabetes Research. Eight of these volunteers were lean and insulin-sensitive, eight were lean but insulin-resistant, and eight were obese and insulin-resistant (FIG. 25). Subjects with a body mass index (BMI), calculated as their total mass in kilograms divided by the square of their height in meters, below 25 kg/m² were considered to be lean, and subjects with a BMI above 30 kg/m² were considered to be obese. The volunteers had fasted for 12 hours, after which their blood serum was sampled. In order to divide the cohort into insulin-sensitive and insulin-resistant groups, subjects underwent a hyperinsulinemic-euglycemic clamp study, which measures the rate of intravenous glucose infusion that is required to avoid a drop in blood sugar in response to intravenous infusion of insulin. Individuals that required a glucose-infusion rate above 15 mg/kg/min to maintain euglycemia were considered to be insulin-sensitive and subjects that could not tolerate a glucose infusion rate above 12 mg/kg/min were considered to be insulin-resistant.

[00162] Serum from these subjects was diluted with PBS buffer and extracted with chloroform-methanol using the protocol for serum extractions we developed.

PAHSA levels in these samples were analyzed by the isocratic LC-MS method used previously (FIG. 26) and described above. This method permitted higher throughput analysis while still resolving the PAHSA isomers. The results of this analysis showed that there was no significant difference between the groups in the levels of the major isomer, 10-PAHSA, nor did we observe a significant difference in the levels of a minor isomer, 12-PAHSA (FIG. 27). Nevertheless, it was notable that this novel lipid class, identified in mouse adipose tissue, was found to be present in the serum of human subjects at levels similar to those found in mouse serum.

[00163] Strikingly, 5-PAHSA was detectable in serum from all of the insulin-sensitive subjects, was present in the serum of only some of lean insulin-resistant subjects, and was nearly entirely undetectable in serum from obese insulin-resistant subjects (FIG. 27). Comparison of two representative LC-MS traces from a lean insulin-sensitive subject and an obese insulin-resistant subject illustrates the qualitative difference between the two samples (FIG. 28). These data suggested that 5-PAHSA may be an important serum marker of increased insulin sensitivity in humans.

Example 4.

Chemical synthesis of biotin-X-9-HAS (Scheme 1).

[00164] 9-Hydroxystearic was protected with 2,4'-dibromoacetophenone by the method developed by Zhang and colleagues [23] to give the esterified FAHFA. Mesylation of this compound set up the displacement of mesylate by sodium azide and was followed by reduction with zinc dust in glacial acetic acid. The zinc reduction step also removed the bromoacetophenone protecting group to give the fatty amino acid, which was filtered and used without further purification (crude yield: 0.27 g, 85% over three steps). To a solution of the fatty amino acid (25 mg, 0.083 mmol) in DMSO (4 mL) was added commercially available biotinamidohexanoyl-6-aminohexanoic acid N-hydroxysuccinimide ester (Biotin-X-NHS) (34 mg, 0.074 mmol) in the presence of triethylamine (40 uL) afforded Biotin-X-9-HSA, which was purified by reversed phase HPLC (3.5 mg, 6.6%). Calculated: *m/z* 637.4368 [M-Hf, found: *m/z* 637.4363.

Example 5.**FAHFA effects on insulin secretion**

[00165] Ins1 cells (FIG. 31A and 31B) or primary rat islet cells (FIG. 32A and FIG. 32B) were seeded in 24 well-plates [~ 106 cells/well]. At confluence, cells were incubated with Low Serum [2.5%] and Low Glucose [2.5 mM] media for 12 hours. Next, cells were incubated with KRB buffer for 1 h and stimulated with glucose in the presence of 5-PAHSA (FIG. 31a and 31B) or 9-PAHSA (FIG. 31B) for 45 min in KRB. At the end of the stimulation, 100 μ L of media was collected and 10 μ L was used to quantitate the amount of insulin released by ELISA.

Treatment Conditions for figures 31A, 31B and 32B were:

5-PAHSA or 9-PAHSA [Stock] = 20.0 mM [10.76 mg/mL Methanol]

A] 1 μ M = 0.3 μ L/6 mL [Amount of methanol/mL = 0.05 μ L]

B] 5 μ M = 1.5 μ L/6 mL [Amount of methanol/mL = 0.25 μ L]

C] 10 μ M = 3.0 μ L/6 mL [Amount of methanol/mL = 0.50 μ L]

D] 20 μ M = 6.0 μ L/6 mL [Amount of methanol/mL = 1.00 μ L]

[00166] For FIG. 32A, the vehicle for desolving the lipids is DMSO instead of methanol.

Glucose [Stock] = 500 mM in KRB

A] 2.5 mM Glucose = 150 μ L/30 mL

B] 6.0 mM Glucose = 360 μ L/30 mL

C] 25.0 mM Glucose = 1.5 mL/30 mL

[00167] Results are summarized in FIGs. 31A, 31B, 32A and 32B. For the results in FIG. 32A, 5-PAHSA was 100 μ M, 45 min; DMSO = 5 μ L/mL, Palmitate = 200 μ M.

Example 6.**Agonists for PPARs increase 5PAHSA levels in HepG2 liver cells**

[00168] In this example, HepG2 cells were seeded in 6-well plates. At 80 % confluence, cells were treated with two different concentrations [0.5 μ M and 1.0 μ M] of an agonist of PPAR α (WY14643), δ (CAY10592), or γ (Pioglitazone) for 48 hr in HepG2 media with 2.5% FBS. At the end of incubation, 500 μ L of media was collected and the cells were collected in PBS for lipid extraction. Lipids were

extracted from both media and cells to quantitate the FAHFA levels by Mass Spec. The results are summarized in FIGs. 33 and 34 for the cells and media, respectively. These results indicate that PPAR agonists stimulate FAHFA production in liver cells, and the FAHFAs are released into the media.

Example 7.

FAHFA effects on LPS stimulated cytokine secretion and maturation status in dendritic cells

[00169] Macrophage-related inflammation in adipose tissue contributes to obesity-induced insulin resistance. Wellen KE and Hotamisligil GS, *J Clin. Invest.* 2003. The Experimental design used in this example is shown in FIG. 35 and is described further in the materials and methods. Results are shown in FIGs. 36A and 36B. These results demonstrate that 9-PAHSA blocks IL-12 production by LPS-activated dendritic cells and thus can likely be used to treat insulin resistance as well as other disorders mediated by the inflammatory cytokine cascade. Additional data is shown in FIGs. 45 and 46.

[00170] Generation and treatment of bone marrow-derived dendritic cells (BMDCs):

[00171] Mouse bone marrow cells were flushed from the femurs and tibiae. The red blood cells were eliminated and the cells plated at a density of 1.0×10^6 cells/mL in RBMI complete medium containing 10% FCS plus 20 ng/ml GM-CSF. The medium was replaced on day 4, and the cells were harvested on day 6 to obtain immature DCs. To obtain mature DCs, LPS was added to the cultures at a final concentration of 100 ng/mL on day 6, and the cells were cultured for an additional 24 h, as previously described by Macia, L., *et al.* Impairment of dendritic cell functionality and steady-state number in obese mice. *J Immunol* **177**, 5997-6006 (2006), which is incorporated by reference in its entirety. To test the anti-inflammatory effects of 9-PAHSA, immature BMDC were treated with 9-PAHSA in a dose response dependent manner in the presence of 100ng/mL of LPS. The activation status of the BMDCs was evaluated by measuring the expression of MHCII and co-stimulatory molecules using Flow Cytometry and by the production

of pro-inflammatory cytokines (IL-6, TNF, IL-12 and IL-1 β) which was assessed by ELISA.

Example 8.

Probing the hydrolysis of FAHFAs *in vivo*.

[00172] Using a FAHFA probe that was labeled on both fatty acyl chains, the significance of CEL hydrolysis of the FAHFAs *in vivo* was studied. On its own, [^{13}C]-9-PAHHA had a unique molecular mass that could be targeted by mass spectrometer without concern for interfering signal from naturally occurring FAHFAs (*i.e.*, "bio-orthogonal"), permitting us to assess only the effects on the FAHFAs that we introduced into the system. Upon hydrolysis [$^{13}\text{C}_{16}$]-9-PAHHA liberated two labeled fatty acids: [$^{13}\text{C}_{16}$]-PA and the odd-chain 9-HHA. Each had a bio-orthogonal molecular mass, permitting specific targeting by mass spectrometry. Since the 17-carbon hydroxy fatty acid was not naturally occurring, re-acylation of this compound—that is, exchange of [$^{13}\text{C}_{16}$]-PA for a naturally occurring fatty acyl chain on the 9-HHA backbone—was evidence that [$^{13}\text{C}_{16}$]-9-PAHHA was hydrolyzed and re-esterified.

[00173] The experimental design used to confirm that CEL was relevant to FAHFA hydrolysis *in vivo* used [$^{13}\text{C}_{16}$]-9-PAHHA as a molecular probe (FIG. 38). It was reasoned that feeding mice labeled FAHFA in vegetable oil would most closely mirror the natural situation in which CEL is released from the pancreas, where it is most abundant, in response to ingestion of lipids. This way CEL would likely encounter the labeled FAHFA in the duodenal lumen. By analogy with existing pharmacologic therapies aimed at preventing triglyceride absorption, inhibition of CEL was expected to lead to lower absorption of FAHFAs. Since the initial destination of lipids taken up by enterocytes is the bloodstream, it was expected to observe not only lower levels of [^{13}C]-9-PAHHA itself, but also lower levels of its downstream metabolites such as 9-HHA and products re-esterified with palmitoyl or oleoyl acyl chains such as 9-PAHHA or 9-OAHHA.

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Visiialization of *in vivo* inhibition of CEL by WWL92.

[00174] To establish that WWL92 was capable of inhibiting CEL *in vivo*, mice were fasted overnight and an intraperitoneal injection of WWL92 was administered at doses of 0, 3, or 30 mg/kg. After either 1 or 2 hours, the mice were sacrificed and the pancreas was harvested. Membrane lysates of the pancreas tissue were prepared, and they were first reacted with either WWL92 (20 μ M) or DMSO to verify that WWL92 inhibited CEL *ex vivo*. Reaction with an activity-based probe (FP-Rh), followed by separation of proteins by SDS-PAGE, permitted fluorescent detection of FP-Rh-labeled proteins. A decreased band intensity at 75 kD indicates that a greater proportion of CEL was pre-labeled by the CEL inhibitor during incubation in the body and was not available for labeling by FP-Rh.

[00175] The results show that WWL92 was indeed capable of inhibiting CEL *in vivo* (FIG. 39). There was a dose-dependent decrease in CEL band intensity that was also time-dependent. The WWL92-treated bands decreased in intensity when comparing mice that were sacrificed after two hours rather than one hour. Pancreas membrane lysates from vehicle-treated mice were reacted with WWL92 *ex vivo* and CEL activity was nearly extinguished in these samples, verifying that WWL92 was active against CEL. Importantly, after two hours mice treated with 30 mg/kg WWL92 were devoid of CEL activity in the pancreas. This dose and timepoint served as an appropriate guidepost for the subsequent gavage experiment with WWL92-treated mice.

Gavage of WWL92-treated mice with double-labeled FAHFA.

[00176] Fasted wild type mice were injected intraperitoneally with 40 mg/kg WWL92 or vehicle. After 30 minutes all of the mice (n = 3-4) were oral-gavaged with 0.1 mL refined olive oil containing 0.15 mg [^{13}C]-9-PAHFA. Three hours later, the mice were sacrificed and plasma was harvested and flash frozen on liquid nitrogen. Extraction of serum lipids with chloroform-methanol-PBS buffer was performed using [^{13}C]-9-PAHSA as an internal standard to control for any differences in extraction efficiency.

[00177] The results of the experiment support the hypothesis that CEL is important in FAHFA hydrolysis and absorption *in vivo*. Significantly both lower plasma levels of [¹³C]-9-PAHHA in the WWL92-treated mice and markedly lower levels of the 9-HHA hydrolysis product were observed (FIG. 40). This indicated that WWL92 had successfully inhibited CEL hydrolysis of FAHFAs. Importantly, it was also observed that lower plasma levels of the re-esterified 9-PAHHA and 9-OAHHA products in the WWL92-treated mice (FIG. 41), which confirmed the observation of lower 9-HHA levels and underscored the parallels between the enteric absorption of FAHFAs and other natural lipids such as triglycerides (FIG. 42).

Example 9.

Insulin release from primary human islet cells

[00178] Two human donors provided cells used in these studies. Donor1 was a 48 year old female, 50", 157 lbs, with a BMI of 30.5, HbA1C was 5.8, who died from a stroke, but it was witnessed and she had no downtime. Islets were 80% pure and 95% viable. After arrival, islets were incubated overnight in human islet media before stimulation with glucose in presence and absence of 5-PAHSA (lipid A-5).

Note 1 IU of Human Insulin = 6 nmol = 34.8 µg Insulin.

[00179] Human islets were divided into ten groups [100 islets per condition]

[00180] A] Low glucose [2.5 mM]; B] High glucose [20.0 mM]; C] Low glucose [2.5 mM] + 2.5 µL/mL Methanol; D] High glucose [20.0 mM] + 2.5 µL/mL Methanol; E] Low glucose + 20 µM Lipid A - 5; F] High glucose + 20 µM Lipid A - 5; G] Low glucose + 50 µM Lipid A - 5; H] High glucose + 50 µM Lipid A - 5.

[00181] Islets were cultured overnight in Human Islet media and next day incubated with KRB buffer for 4 h prior to glucose stimulation.

[00182] Following KRB incubation, islets were stimulated with glucose [2.5 mM & 20.0 mM] in the presence and absence of Lipid A - 5 [20 & 50 µM] for 45 min

[00183] At the end of glucose stimulation the amount of insulin released [µ IU/mL] into the media was measured using Human Insulin ELISA kit. And the

insulin content from the islets were extracted to express insulin released into the media as μ IU/mL/islet content.

[00184] Results for Donor 1 are shown in FIG. 47.

[00185] Donor 2 was a 44 year old male, 183 cm, 176 lbs, with a BMI of 23.9, HbA1C was 5.0, died from a stroke, but it was witnessed and he had no downtime. Islets were 85% pure and 95% viable. After arrival, islets were incubated overnight in human islet media before stimulation with glucose in presence and absence of 5-PAHSA (Lipid A-5).

[00186] Human islets were divided into ten groups [75 islets per condition]:

A] Low glucose [2.5 mM]; B] High glucose [20.0 mM]; C] Low glucose [2.5 mM] + 2.5 μ VmL Methanol; D] High glucose [20.0 mM] + 2.5 μ LmL Methanol; E] Low glucose + 20 μ M Lipid A - 5; F] High glucose + 20 μ M Lipid A - 5.

[00187] Islets were cultured overnight in Human Islet media and next day incubated with KRB buffer for 4 h prior to glucose stimulation.

[00188] Following KRB incubation, islets were stimulated with glucose [2.5 mM & 20.0 mM] in the presence and absence of Lipid A - 5 [20 μ M] for 45 min.

[00189] At the end of glucose stimulation the amount of insulin released [μ IU/mL] into the media was measured using Human Insulin ELISA kit. And the insulin content from the islets were extracted to express insulin released into the media as μ IU/mL/islet content.

[00190] Results for Donor 2 are shown in FIG. 48.

Example 10.

Additional FAHFA functional studies

[00191] For the experiments summarized in FIG. 49 the method is: Cells grown on collagen coated glass slides were preloaded with the calcium indicator, FURA2, for 60 minutes. Buffer containing either FAHFAs or vehicle was incubated with cells for a period of 3 minutes followed by washout with buffer alone. FURA2 fluorescence, an indicator of Calcium mobilization, was monitored continuously before and during the addition of compounds and throughout the washout period. Results were analyzed with metamorph software. Calcium Flux in STC-1 (intestinal

enteroendocrine) cells in the absence of extracellular calcium (calcium free buffer). Ligands were injected after 3 minutes and washed out after an additional 3 minutes. The data presented is the average fold change in Fura2 fluorescence for 10 individual STC-1 cells in the same field.

[00192] DMSO control had no effect. Top panel is linoleic acid, a positive control. Middle panel 9-PAHSA (100 uM). Bottom panel 5-PAHSA (100 μ M). These experiments were done in the absence of extracellular calcium. Calcium flux was also stimulated by FAHFAs in the presence of extracellular calcium indicating that FAHFAs stimulate influx of extracellular calcium into the cell as well as release from the endoplasmic reticulum.

[00193] For the experiments summarized in FIG. 50, STC-1 cells were incubated with 9-PAHSA, 5-PAHSA or 9-OAHSA at the indicated concentrations. Cells were harvested and lysed and proteins were separated by SDS PAGE and transferred onto nitrocellulose filters. The phosphorylation of ERK and the amount of total ERK was determined by immunoblotting with antibodies specific for total or phosphor-ERK. DMSO was the control. For ERK phosphorylation, cells were serum deprived overnight and stimulated with FAHFAs or vehicle as indicated for 15 minutes followed by lysis in RIPA buffer and analysis by SDS-PAGE

[00194] For the experiments summarized in FIG. 51 STC-1 cells were incubated with vehicle (DMSO), 9-PAHSA, 5-PAHSA or 9-OAHSA at the indicated concentrations or linoleic acid (100 uM) as a positive control. Media was harvested and GLP-1 was measured by ELISA.

[00195] For the experiments summarized in FIG. 52, the same protocol as described for FIG. 51 was used, except in this experiment a time course was performed. For both FIG. 51 and FIG. 52, for GLP-1 secretion STC1 cells were washed 3X with serum free culture media followed by stimulation with FAHFAs or vehicle at the indicated concentrations and timepoints. Levels of active GLP-1 were measured by ELISA.

[00196] For the experiments summarized in FIGs. 53- 55, the fragment based GPCR assay from DiscoveRx (PATHHUNTER \circledR β -Arrestin assay) was used to as per manufacturer's instructions to determine activation of specific GPCRs as

indicated in FIGs. 53-55.

Example 10.

FAHFA Analysis in plasma of insulin-sensitive (**n=12**) and insulin-resistant (**n=9**) female subjects before and during hyperinsulinemic euglycemic clamp [00197] A total of 21 non-diabetic apparently healthy Caucasian women were recruited on the basis of the following inclusion criteria: (1) age 18-60 years, and (2) no known acute or chronic disease other than obesity. Physical and biochemical characteristics of the study subjects is shown in the Table 1. These subjects were from a different study from that summarized in Example 3.

[00198] Table 1.

	Insulin-sensitive	Insulin-resistant	p value
Number	11	10	—
Age (years)	32±3	40±3	NS
Body weight (kg)	69±4	90±4	<0.01
BMT (kg/nr)	24.7±1.1	32.7±1.8	<0.001
Whole-body fat (%)	28±2	36±1	<0.001
Fat mass (kg)	20±2	35±4	<0.01
Waist-to-hip-ratio	0.86±0.01	0.91±0.01	<0.01
Fasting plasma glucose (mmol/l)	5.1±0.1	5.6±0.2	<0.01
Fasting serum insulin (mU/l)	3±1	10±1	<0.001
Fasting serum C-peptide (nmol/l)	0.4±0.1	0.8±0.1	<0.001
Fasting serum LDL cholesterol (mmol/l)	2.2±0.1	3.1±0.1	<0.01
Fasting serum triglycerides (mmol/l)	0.8±0.1	1.4±0.2	<0.01
Fasting serum HDL cholesterol (mmol/l)	1.4±0.1	1.3±0.1	<0.001
Fasting serum adiponectin (mg/l)	18±2	12±1	<0.01

[00199] Whole body insulin sensitivity was measured using the euglycaemic insulin clamp technique (insulin infusion rate 1 mU kg⁻¹ min⁻¹ for 6 h) after an overnight fast. The women were divided into insulin sensitive and insulin-resistant groups on the basis of their rate of whole-body insulin sensitivity. Normoglycemia was maintained by adjusting the rate of a 20% glucose infusion based on plasma glucose measurements. Whole body insulin sensitivity was determined from the glucose infusion rate required to maintain normoglycaemia between 30 and 360 min. Hyperinsulinemic euglycemic clamp measurements were made at 0, 180, and 360 minutes. Markers of insulin resistance, including serum fasting insulin, C-peptide and triglyceride concentrations were higher and HDL cholesterol and adiponectin concentrations were lower in the insulin-resistant than in the insulin-sensitive group.

Lipid extraction was performed by Folch's method (chloroform : methanol = 2:1), and lipids were extracted from ~300 μ L serum and plasma, and concentrated in 100 μ L chloroform. Lipids were measured by MS with a gradient of Methanol : Water of (94:6) and an injection volume of 2 μ L.

[00200] FIGs. 56A and 56B show that glucose-insulin infusion (clamp) lowered 10 - PAHSA levels in plasma of insulin sensitive (FIG. 56A) & insulin resistant (FIG. 56B) human subjects. The levels of 5-PAHSA in plasma of insulin sensitive & insulin resistant were lower after clamp than at baseline (FIGs. 57 and 58A and 58B).

[00201] Experimentation also demonstrated that 5-PAHSA levels in human plasma in the fasting state correlated with insulin sensitivity measured by euglycemic hyperinsulinemic clamp studies (FIG. 59), while 5-PAHSA levels correlated inversely with plasma triglycerides in the fasting state (FIG. 60).

Materials and methods

[00202] For all cell culture experiments, FAHFAs were solubilized in DMSO at a concentration of 20mM and further diluted in assay buffer or cell culture media to the working concentrations indicated for each assay. In all instances an equivalent volume of vehicle (DMSO) was added to control treatment groups.

Animal studies

[00203] AG40X and AG40X-ChREBP-KO mice were described previously [1, 24]. Briefly, the fat-specific human GLUT4 transgene was constructed using the fat-specific promoter/enhancer from the fatty acid-binding protein gene, aP2. The AG40X mice used were raised at Beth Israel Deaconess Medical Center, Boston, MA and were on a FVB background. ChREBP-KO mice were on a C57BL/6J background. Age- and sex-matched wild type littermates were used as controls. Animals were kept on a 12-hour light, 12-h dark schedule and fed *ad libitum* unless specified otherwise. For tissue collection, animals were euthanized with carbon dioxide and their tissues were dissected, flash frozen with liquid nitrogen, and stored at -80 °C. All animal care and use procedures were in strict accordance with the

standing committee on the use of animals in research and teaching at Harvard University and at the Beth Israel Deaconess Medical Center Animal Research Facility.

Lipid extraction from serum or plasma

[00204] To a labeled 8 mL glass vial was added 0.9 mL phosphate-buffered saline, 1 mL methanol, and 2 mL chloroform with internal standards 5 nM [¹³C₁₆]-9-PAHSA and 1 uM [¹³C₁₆]-palmitic acid. After thawing serum or plasma samples at 4 °C, 100 uL serum/plasma was transferred to the glass vial. The vial was capped with a PTFE-lined cap, shaken vigorously for 10 seconds, and inserted in a 50 mL conical tube. The samples were centrifuged at 3220 x g for 5 min at 4 °C to separate the immiscible layers. The bottom organic layer was transferred to a fresh 4 mL glass vial and the volatiles were removed under a nitrogen gas stream, leaving a lipid residue that was stored at -80 °C. For analysis by LC-MS, samples were reconstituted in 50 uL chloroform and 2 uL injection volumes were typically used.

Lipid extraction from animal tissues

[00205] A frozen section of animal tissue (50-100 mg) was quickly sectioned off with a clean razor blade, weighed on an analytical balance, placed in a 1.7 mL plastic tube, and kept on dry ice. To a cold Dounce tissue grinder was added 1 mL ice-cold PBS, 1 mL methanol, and 2 mL chloroform that contained 10 nM [¹³C]-9-PAHSA internal standard. The tissue sample was added to the tissue grinder, which was kept on ice, and the tissue was homogenized with the high clearance "A" pestle (30-40 strokes). Adipose tissue was homogenized with the low clearance "B" pestle owing to its low connective tissue content. The mixture was carefully poured into a labeled 8 mL glass vial. The vial was loaded into a 50 mL plastic conical tube and centrifuged at 3200 x g for 10 minutes at 4 °C. The bottom organic layer was transferred to a clean 4 mL glass vial using a Pasteur pipet and the volatiles were evaporated under a nitrogen gas stream. The lipid residue was stored at -80 °C and was reconstituted in 200-500 uL chloroform for MS analysis, with injection volumes

of 10-30 μ L for injection on 4.6 mm diameter columns and 1-3 μ L for injection on 2.1 mm diameter columns.

Analysis by QQQ mass spectrometry

[00206] Analysis of FAHFAs was performed in negative mode on an Agilent 6410 triple quadrupole mass spectrometer (QQQ-MS). For targeted profiling experiments, analysis was performed in multiple reaction monitoring mode, and the precursor ion and product ion targeted for each FAHFA species is detailed in Table 2. For FAHFAs, the fragmentor voltage was set to 180 V and the collision energy was 30 V. Dwell times for quantitated species ranged from 150-250 ms, and the cycle time did not exceed 1000 ms.

[00207] Table 2.

FAHFA name	Acy carbons: double bonds	Fatty Acyl Group	Hydroxy Fatty Acid	Parent ion	MRM product ion
POHPA	C32:1	palmitoleic acid	hydroxypalmitic acid	507.4	253.2
PAHPA	C32:0	palmitic acid	hydroxypalmitic acid	509.5	255.2
OAHPA	C34:1	oleic acid	hydroxypalmitic acid	535.5	281.2
POHSA	C34:1	palmitoleic acid	hydroxystearic acid	535.5	253.2
PAHSA	C34:0	palmitic acid	hydroxystearic acid	537.5	255.2
OAHSA	C36:1	oleic acid	hydroxystearic acid	563.5	281.2
[¹³ C]-PAHSA	C34:0	[¹³ C ₁₆]-palmitic acid	hydroxystearic acid	553.5	271.3

[00208] Analysis of free fatty acids by QQQ-MS was performed in selected ion monitoring (SIM) mode with the fragmentor voltage set to 150 V in negative mode and dwell times of 25 ms for each ion. Selected ions were *m/z* 227.2, 253.2, 255.2, 271.3, 277.2, 279.2, 281.2, 283.3, 301.2, 303.2, 309.3, and 327.2 for free fatty acid ions C14:0, C16:1, C16:0, [¹³C₁₆]-C16:0, C18:3, C18:2, C18:1, C18:0, C20:5, C20:4, C20:1, and C22:6, respectively.

Chromatographic conditions

[00209] The QQQ-MS system was connected to an Agilent 1200 Binary Pump. For analyses of total PAHSA content without separation of PAHSA isomers (FIG.

39), a Gemini C18 reversed phase column (5 um, 4.6 x 50 mm, Phenomenex) and a C18 reversed phase guard column (3.5 um, 2 mm x 20 mm, Western Analytical) was used for LC-MS analysis in negative mode. Mobile phase A consisted of a 95:5 water:methanol mixture and mobile phase B consisted of 60:35:5, 2-propanol:methanol:water, both containing 0.1% ammonium hydroxide. An Agilent 1200 series binary pump was set to a flow rate of 0.1 mL/min for the first 5 min followed by 0.4 mL/min for the remainder of the gradient. At 5 min, concomitant with the increase in flow rate, the gradient was increased from 0% B to 20% B. The gradient increased linearly to 100% B at 45 min, followed by an 8 minute wash at 0.5 mL/min with 100% B before re-equilibrating the column with 0% B for 7 min.

[00210] For separation of PAHS A isomers in WAT, BAT, and liver extracts, we used an analytical C18 column purchased from Phenomenex (Torrance, CA). The Kinetex C18 column had dimensions of 2.1 x 150 mm, pore size of 100 Å, and particle size of 2.6 µm (core shell). Buffer A was 100% water and buffer B was 100% methanol, both containing 10 mM ammonium acetate. Samples were loaded with injection volumes of 2 µL onto a column preequilibrated with a solvent composition of 80% B. After 2 minutes at 80% B, the composition was linearly increased to 100% B over 60 minutes. The column was washed with 100% B for 10 minutes before returning the solvent composition to 80% B for reequilibration for 13 minutes. The flow rate was 0.3 mL/min throughout the LC method.

[00211] Serum samples from HFD-fed mice were analyzed by a method similar to the above gradient method, but buffer A was 90:10 methanol: water and buffer B was 100% methanol as before, both buffers containing 10 mM ammonium acetate. The solvent composition was held at 0% B for 2 minutes, after which it increased linearly over 40 minutes to 100% B and held at 100% B for 8 minutes. The flow rate was 0.3 mL/min and the Kinetex C18 column dimensions were 2.1 x 100 mm.

[00212] For analysis of human serum samples, the Kinetex C18 2.1 x 150 mm column was used with an isocratic elution method that consisted of 94:6 methanol: water containing 10 mM ammonium acetate and a flow rate of 0.3 mL/min. The PAHSAs eluted typically eluted with a retention time of approximately 9 minutes, with little loss of chromatographic resolution compared to

the 80→100% gradient method. Analysis of WAT or liver from HFD-fed mice was performed by a similar method using a Kinetex 0 8 2.1 x 100 mm column using a 0.3 mL/min flow rate and a solvent buffer that was composed of 93:7 methanol: water containing 10 mM ammonium acetate.

Biotin-FAHFA pulldown

[00213] The specific Ins1 cell line used was Ins1 832/13, a subclone of the original Ins1 cell line that stably expresses the human insulin gene [26]. The culture medium was RPMI-1640 with 11.1 mM D-glucose supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 ug/ml streptomycin, 10 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, 2 g/L sodium bicarbonate and 50 uM β -mercaptoethanol. Ins1 cells were cultured to 80% confluence in 150-mm dishes. Cells were scraped into PBS, washed with PBS, and resuspended in optimized lysis buffer (20 mM Tris-HCl, 2 mM EDTA, 500 mM NaCl, 0.1% Triton-X100). Cell suspension was sonicated with probe sonicator then centrifuged at 13,000 x g for 15 minutes. Protein concentration was determined by BCA assay.

[00214] Paramagnetic avidin-linked polystyrene Dynabeads (Invitrogen) were washed twice and incubated in 0.1% fatty acid-free bovine serum albumin to while preparing cell lysates. Blocking buffer was removed by magnetic separation. Lysates were pre-cleared by diluting 2 mg protein sample to a final volume 1 mL with lysis buffer and incubating with 10 uL beads, after which the lysate was separated from the beads by magnetic separation. To the pre-cleared lysate was then added 400 uM 9-PAHSA or DMSO (2%) and the sample was incubated 2 hours at 4 °C.

[00215] Following the blocking procedure, the beads were washed and resuspended in 1 mL of buffer containing 20 mM Tris-Cl, 2 mM EDTA and 150 mM NaCl. To this was added 5 uL biotin-X-9-HSA ("Bt-FAHFA") (100 uM in DMSO) or 5 ul of DMSO for control beads. The suspension was incubated for 2 hours at 4 °C. The beads were washed three times with lysis buffer to remove any unbound Bt-FAHFA. Pre-cleared cell lysate (1 mL) was then added tubes containing the Bt-FAHFA-conjugated beads and suspension was incubated overnight at 4 °C.

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[00216] The next morning, the lysate supernatant containing unbound proteins was aspirated and the beads were washed four times with lysis buffer. Beads were resuspended in 30 uL 4x SDS-PAGE loading buffer and heated for 10 minutes at 95 °C to remove any bound target proteins from the beads. Each sample (25 uL) was then analyzed by SDS-PAGE on a 4-15% gradient gel, which was stained with Coomassie Blue.

Racl activation assay

[00217] Ins1 cells were incubated overnight in a low serum (2.5% FBS) - low glucose (2.5 mM) containing medium, followed by an additional 1 h incubation in Krebs-Ringer bicarbonate buffer (KRB). At the end of the incubation cells were further stimulated with low glucose (5.0 mM) and high glucose (25 mM) for 30 min in the presence or absence of 9-PAHSA (50 µM; dissolved in DMSO) as indicated in the figure legend. An equal amount of DMSO was added to the vehicle. Lysates [~200 µg protein] were clarified by centrifugation for 5 min at 4800xg, and PAK-PBD [p21-activated kinase-binding domain] beads [20 µl] were added to the supernatant. The mixture was then rotated for 1 h at 4 °C and pelleted by centrifugation at 4,000xg for 3 min. The resulting pellet was washed once with lysis buffer followed by a rinse [3x] in wash buffer [25 mM Tris, pH 7.5, 30 mM MgCl₂, 40 mM NaCl, and 150 mM EDTA]. Proteins in the pellet were resolved by SDS-PAGE and transferred onto a nitrocellulose membrane, and Western blotting method determined the relative abundance of activated Racl. [Racl Activation Assay Biochem Kit (bead pull-down method); Catalog # BK035; Cytoskeleton Inc.]

Insulin release studies in Ins1 cells

[00218] Ins1 cells were seeded in 24 well-plates [~ 10⁶ cells/well] and at 80% confluence, were cultured overnight in RPMI 1640 medium containing 2.5 mM glucose and 2.5% fetal bovine serum supplemented with 100 IU/ml penicillin and 100 IU/ml streptomycin, 1 mM sodium pyruvate, 2-mercaptoethanol (50 µM) and 10 mM HEPES (pH 7.4). The cells were further incubated with Krebs-Ringer bicarbonate buffer, pH 7.4 for 1 h prior to stimulation with low (2.5 mM) or high

glucose (25 mM) in the continuous presence or absence of 9-PAHSA or 5-PAHSA (dissolved in Methanol) at different concentrations mentioned in the figure legend for 45 min at 37°C. An equal amount of Methanol was added to the respective control. At the end of the stimulation supernatant was collected and insulin released into the medium was quantified by ELISA as per manufacturer's protocol. [Insulin (Rat) High Range Elisa Kit; Catalog # 80-INSRTH-E01; Alpco Diagnostics].

Isolation of pancreatic islets for Insulin release studies

[00219] Intact pancreatic islets were isolated from adult Sprague-Dawley rats using collagenase digestion method and separated from acinar tissue and debris on Ficoll gradient. Islets were hand-picked under a stereo-microscope twice to avoid contamination by acinar cells. All experiments, including isolation of pancreatic islets from normal Sprague-Dawley rats, were reviewed and approved by the Beth Israel Deaconess Medical Center Animal Care and Use Committee.

Insulin release studies in rat islets

[00220] Isolated rat islets were cultured overnight in RPMI 1640 medium containing 2.5 mM glucose and 2.5% fetal bovine serum supplemented with 100 IU/ml penicillin and 100 IU/ml streptomycin, 1 mM sodium pyruvate, and 10 mM HEPES (pH 7.4). The islets were further incubated with Krebs-Ringer bicarbonate buffer for 1 h prior to stimulation with low (2.5 mM), high glucose (25 mM) or Palmitate (200 μ M) in the continuous presence or absence of 9-PAHSA (100 μ M; dissolved in DMSO) as mentioned in the figure legend for 45 min at 37°C. An equal amount of DMSO was added to the vehicle. At the end of the stimulation supernatant was collected and insulin released into the medium was quantified by ELISA as per manufacturer's protocol. The islets were lysed and protein content was measured and the amount of insulin released into the media was quantitated as ng/mL/ μ g of protein.

PPAR agonist studies

[00221] HepG2 cells were seeded in 6-well plate. At 80% confluence, cells were treated with two different concentrations [0.5 μ M and 1.0 μ M] of PPAR $\alpha/\delta/\gamma$ agonists for 48 h in HepG2 media containing 2.5% fetal bovine serum (PPAR α agonist - WY14643; PPAR δ agonist – CAY10592; PPAR γ agonist - Pioglitazone). At the end of incubation, 500 μ L of media and the cells were collected in PBS. Lipids were extracted as per Folch Method from both media and cells to quantitate the FAHFA levels by Mass Spec. Briefly, chloroform and methanol were added to the media or cells in 2:1 ratio and sonicated for 2-3 min and centrifuged at 3000 rpm for 10 min at 4°C. The bottom chloroform layer containing lipids was taken and dried under Nitrogen. Calculated volume of chloroform is added to the dissolve the lipids and 2 μ L of injection volume was used to analyze FAHFAs through Mass spec. [HepG2 Cells - ATCC # HB-8065].

Generation of bone marrow-derived dendritic cells (BMDCs)

[00222] Mouse bone marrow cells were flushed from the femurs and tibiae, The red blood cells were lysed, and the cells were plated at a density of 0.5 \times 10⁶ cells/mL in RPMI 1640 Low medium (Gibco, USA) containing 10% FCS (Gibco, USA) and 20 ng/mL GM-CSF. The medium was replaced on day 5, and the cells were harvested on day 6 to obtain immature DCs. To obtain mature DCs, LPS was added to the cultures at a final concentration of 100 ng/mL on day 6, and the cells were cultured for an additional 24 h.

9-PAHSA affects on LPS induced dendritic cells maturation

[00223] The immature dendritic cells obtained in the day 6 were matured with LPS in the presence of different concentrations of 9-PAHSA varying between 100 ng/mL to 20 ug/mL. The cells were matured for 24h and the maturation status analyzed by cytokine production and co-stimulatory molecules expression.

Flow Cytometry

[00224] The cells were analyzed by Multicolor Flow cytometry. The mature dendritic cells were harvested on day 7 and re-suspended in PBS supplemented with 2% FCS and stained with saturating amounts of the following mAbs: CD11c PE, MHCII APC, CD40 PE-Cy7, CD80 FITC and CD86 Percp. The cells were analyzed with an LSR II flow cytometer (BD) and FlowJo software.

Intracellular cytokine analysis and Foxp3 staining

[00225] Matured dendrite cells were analyzed for cytokine production by flow cytometry. For intracellular cytokine staining, 1×10^6 cells were stimulated *in vitro* for 4 h at 37 °C in 5% CO₂ with phorbol-12-myristate-13-acetate (PMA; 100 ng/ml) and ionomycin (1 µg/ml) and brefeldin A (1 µg/ml). The cells were then washed and stained with PE anti-CD11c and permeabilized using the BD Cytofix/Cytoperm Fixation/Permeabilization solution kit (BD Biosciences, USA). Intracellular staining was performed with APC-conjugated anti-IL-12p40 (Biolegend, USA).

ELISA Assay for IL-12p70

[00226] An ELISA assay (Biolegend) was used to measure the concentration of IL-12p70 protein in conditioned media from immature and mature BMDCs. This assay allowed for the detection of total IL-12p70 concentrations in the range of 15.6-1,000 pg/ml, and the results are expressed as pg/mL IL-12p70.

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[00227] It should be understood that for all numerical bounds describing some parameter in this application, such as "about," "at least," "less than," and "more than," the description also necessarily encompasses any range bounded by the recited values. Accordingly, for example, the description at least 1, 2, 3, 4, or 5 also describes, *inter alia*, the ranges 1-2, 1-3, 1-4, 1-5, 2-3, 2-4, 2-5, 3-4, 3-5, and 4-5, *et cetera*.

[00228] It should be understood that for all numerical bounds describing some parameter in this application, such as "about," "at least," "less than," and "more than," the description also necessarily encompasses any range bounded by the recited values. Accordingly, for example, the description at least 1, 2, 3, 4, or 5 also describes, *inter alia*, the ranges 1-2, 1-3, 1-4, 1-5, 2-3, 2-4, 2-5, 3-4, 3-5, and 4-5, *et cetera*.

[00229] For all patents, applications, or other reference cited herein, such as non-patent literature and reference sequence information, it should be understood that it is incorporated by reference in its entirety for all purposes as well as for the proposition that is recited. Where any conflict exists between a document incorporated by reference and the present application, this application will control. All information associated with reference gene sequences disclosed in this application, such as GenelDs or accession numbers (typically referencing NCBI accession numbers), including, for example, genomic loci, genomic sequences, functional annotations, allelic variants, and reference mRNA (including, *e.g.*, exon boundaries or response elements) and protein sequences (such as conserved domain structures) are hereby incorporated by reference in their entirety.

[00230] Headings used in this application are for convenience only and do not affect the interpretation of this application.

[00231] Preferred features of each of the aspects provided by the invention are applicable to all of the other aspects of the invention *mutatis mutandis* and, without limitation, are exemplified by the dependent claims and also encompass combinations and permutations of individual features (*e.g.* elements, including numerical ranges and exemplary embodiments) of particular embodiments and

aspects of the invention including the working examples. For example, particular experimental parameters exemplified in the working examples can be adapted for use in the methods provided by the invention piecemeal without departing from the invention. For example, for materials that are disclosed or used in the methods provided by the invention, while specific reference of each various individual and collective combinations and permutation of these compounds may not be explicitly disclosed, each is specifically contemplated and described herein. Thus, if a class of elements A, B, and C are disclosed as well as a class of elements D, E, and F and an example of a combination of elements, A-D is disclosed, then even if each is not individually recited, each is individually and collectively contemplated. Thus, in this example, each of the combinations A-E, A-F, B-D, B-E, B-F, C-D, C-E, and C-F are specifically contemplated and should be considered disclosed from disclosure of A, B, and C; D, E, and F; and the example combination A-D. Likewise, any subset or combination of these is also specifically contemplated and disclosed. Thus, for example, the sub-group of A-E, B-F, and C-E are specifically contemplated and should be considered disclosed from disclosure of A, B, and C; D, E, and F; and the example combination A-D. This concept applies to all aspects of this application including, elements of a composition of matter and steps of method of making or using the compositions.

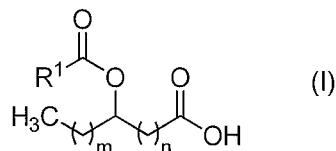
[00232] The forgoing aspects of the invention, as recognized by the person having ordinary skill in the art following the teachings of the specification, can be claimed in any combination or permutation to the extent that they are novel and non-obvious over the prior art—thus to the extent an element is described in one or more references known to the person having ordinary skill in the art, they may be excluded from the claimed invention by, *inter alia*, a negative proviso or disclaimer of the feature or combination of features.

While this invention has been particularly shown and described with references to example embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the scope of the invention encompassed by the appended claims.

CLAIMS

What is claimed is:

1. An isolated fatty acyl hydroxy fatty acid (FAHFA) of formula (I):



Wherein: m is an integer from 0 to 21;
n is an integer from 0 to 21;
the sum of m and n is an integer from 11 to 21;
R¹ is an alkyl group;
or a salt thereof.

2. The FAHFA of claim 1, wherein R¹ is a (C₁₅-C₁₇)alkyl group.

3. The FAHFA of claim 1, wherein:

m is 8;
n is 7; and
R¹ is C₁₅H₃₁ or C₁₇H₃₃.

4. The FAHFA of claim 1, wherein:

m is 12;
n is 3; and
R¹ is a C₁₅H₃₁.

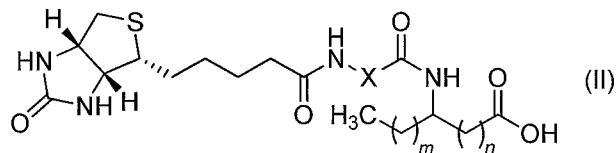
5. A FAHFA of any one of claims 1 through 4, wherein the FAHFA is detectably labeled.

6. A FAHFA of claim 5, wherein the FAHFA is isotopically labeled and/or ester- or amide-bound to a detectable moiety, selected from biotin, streptavidin, GST, a fluorous affinity tag, an alkyne suitable for click chemistry, an epitope tag such as FLAG, 6x His, or another affinity tag.
7. A method of assessing the disease state and/or treatment response of a mammalian subject for a disease or disorder selected from obesity, type 2 diabetes (T2D), impaired glucose tolerance, maturity onset diabetes of the young (MODY), impaired fasting glucose, metabolic syndrome, insulin resistance, polycystic ovarian syndrome, gestational diabetes, cardiovascular disease, inflammatory disorders, and cancer comprising determining the level of one or more fatty acyl-hydroxy fatty acids (FAHFAs), or a precursor or derivative thereof, in an isolated biological sample obtained from the subject, wherein the level of the one or more FAHFAs is indicative of the subject's disease state and/or treatment response for the disease or disorder.
8. The method of Claim 7, wherein the inflammatory disorder is selected from sepsis, rheumatoid arthritis (RA), ulcerative colitis, inflammatory bowel disease, Crohn's disease, systemic lupus erythematosus, celiac disease, uveitis, pancreatitis, adult respiratory distress syndrome, asthma, multiple sclerosis, graft-versus host disease, atopic dermatitis, and ankylosing spondylitis.
9. The method of Claim 7, wherein the isolated biological sample is a blood fraction, bile salt, pancreas secretions, or a tissue biopsy.
10. The method of Claim 9, wherein the blood fraction is plasma or serum.
11. The method of Claim 9, wherein the tissue biopsy comprises adipose tissue.
12. The method of Claim 9, wherein the tissue biopsy comprises pancreas, liver, kidney, or tumor tissue.

13. The method of any one of Claims 7-12, wherem the FAHFA, or precursor or derivative thereof, is detected by a method comprising MS/MS or an immunoassay.
14. The method of any one of Claims 7-13, wherein the disorder is one or more of MODY 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or 11 and, in some embodiments, is determined to be heterozygous or homozygous for a germline mutation in one or more genes selected from HNF4A, GCK, HNF1A, PDX1, TCF2, NEUROD1, KLF1 1, CEL, PAX4, FNS, or BLK.
15. A method of decreasing pro-inflammatory signaling or increasing glucose uptake, glucose tolerance, insulin secretion, or insulin sensitivity in a cell, comprising contacting the cell with an agent that increases the level of one or more FAHFAs.
16. A method of decreasing pro-inflammatory signaling or increasing glucose uptake, glucose tolerance, insulin secretion, or insulin sensitivity in a mammalian subject in need thereof, comprising administering to the subject a therapeutically effective amount of an agent that increases the level of one or more FAHFAs.
17. The method of Claim 15 or 16, wherem the agent that increases the level of one or more FAHFAs is an exogenous FAHFA, or a substrate of carboxyl ester lipase (CEL) or an inhibitor of CEL.
18. The method of Claim 15 or 16, wherein the agent is a CHREBP expression product.
19. The method of Claim 16, wherein the subject has Type-2 Diabetes.
20. The method of any one of Claims 16-19, wherein the pro-inflammatory signaling is dendritic cell maturation, activation, or proliferation; or macrophage cell maturation, activation, or proliferation.

21. The method of Claim 20, wherein the dendritic cell maturation, activation, or proliferation comprises an increase in CD40+, CD80+, CD86+, MHCII+ cells, or combinations thereof. And/or cytokine production.
22. The method of Claim 21, wherein the increased number of CD40+, CD80+, CD86+, or MHCII+ cells are also CD1 lc+.
23. The method of any one of Claims 16-22, wherein the pro-inflammatory signaling is release of a proinflammatory cytokine selected from TNF-a, IL-1 β , IL-12p70, IL-6, or combinations thereof. .
24. A method of identifying an agent that modulates the level of one or more FAHFAs, comprising contacting a cell with a candidate agent and measuring the level of one or more FAHFAs in the cell, wherem a change in the level of one or more FAHFAs in the cell, relative to a control cell not contacted with the agent, indicates that the agent modulates the level of one or more FAHFAs.
25. The methods of Claim 24, wherein the modulation results in an increase in the level of one or more FAHFAs.
26. The method of Claim 24, wherein the modulation results in a decrease in the level of one or more FAHFAs.
27. The method of Claim 24, wherem the agent modulates the level of a FAHFA synthase.
28. The method of Claim 24, wherein the agent modulates the level of a FAHFA esterase.
29. The method of Claim 24, wherein the cell is an isolated, cultured animal cell.
30. The method of Claim 29, wherein the cell is a macrophage (such as a RAW cell), an islet cell (such as Insl cell line), or a hepatic cell (such as HepG2).

31. The method of Claim 29, wherein the cell is cultured in the presence of low glucose.
32. The method of Claim 29, wherein the cell is cultured in the presence of a modulator of PPAR α , γ , or δ .
33. The method of Claim 29, wherein the cell is located in a non-human animal.
34. The method of Claim 33, wherein the non-human animal is a mammal.
35. The method of Claim 34, wherein the mammal is murine.
36. The method of Claim 35, wherein the murine is an AG40X mouse, an AG4KO mouse, a ChREBPKO mouse, or a ChREBPOX mouse.
37. A detectable biotinylated amino-FAHFA of formula (II):



Wherein: m is an integer from 0 to 21;
 n is an integer from 0 to 21;
 the sum of m and n is an integer from 11 to 21;
 X is selected from $(OCH_2CH_2)_p$ or $(CH_2)_p$, wherein p is an integer from 2 to 20;
 or a salt thereof.

38. A method of identifying a modulator of FAHFA-mediated signaling, and/or FAHFA-mediated biological effects, comprising forming a mixture comprising an isolated mammalian cell that expresses a G-protein coupled receptor (GPCR), one or more FAHFAs, and a test compound and monitoring the FAHFA-mediated signaling, wherein a change in the FAHFA-mediated signaling indicates that the test compound is a modulator of FAHFA-mediated signaling.

39. The method of Claim 38, wherein the GCPR is GPR120 or GPR40.
40. The method of any one of Claims 38-39, wherein the one or more FAHFAs comprise 9-PAHSA, 5-PAHSA, 9-OAHSA, or a combination thereof.
41. The method of any one of Claims 38-40, wherein FAHFA-mediated signaling and/or FAHFA-mediated biological effect is selected from decreasing pro-inflammatory signaling, stimulating insulin secretion, stimulating GLP1 secretion, stimulating calcium flux including calcium entry into cells or calcium exit from intracellular organelles into the cytoplasm, G-protein activation, and combinations thereof.
42. The method of Claim 41, wherein the FAHFA-mediated signaling is stimulating insulin secretion and the isolated mammalian cell is a pancreatic islet cell, an intestinal enteroendocrine cell, a macrophage, a T lymphocyte, or dendritic cell.
43. The method of any one of Claims 38-42, wherein the mammalian cell is a human cell.

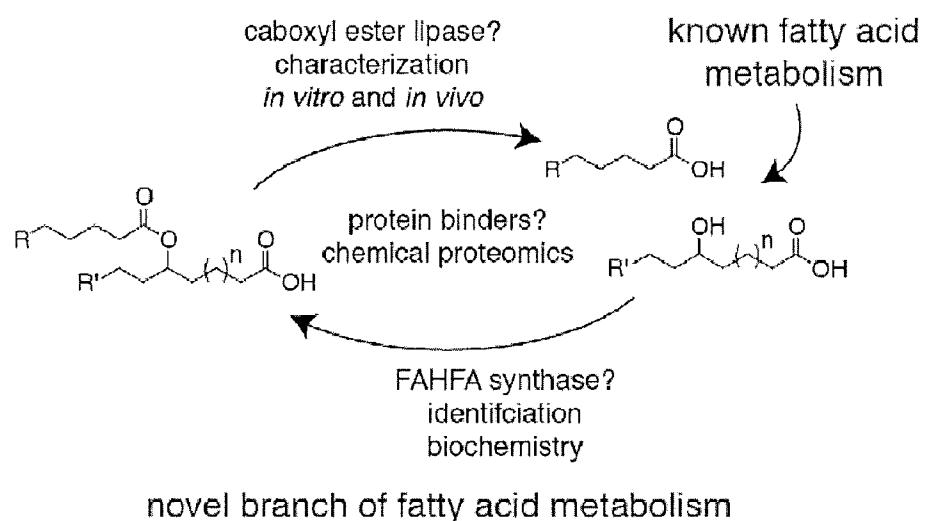


FIG. 1

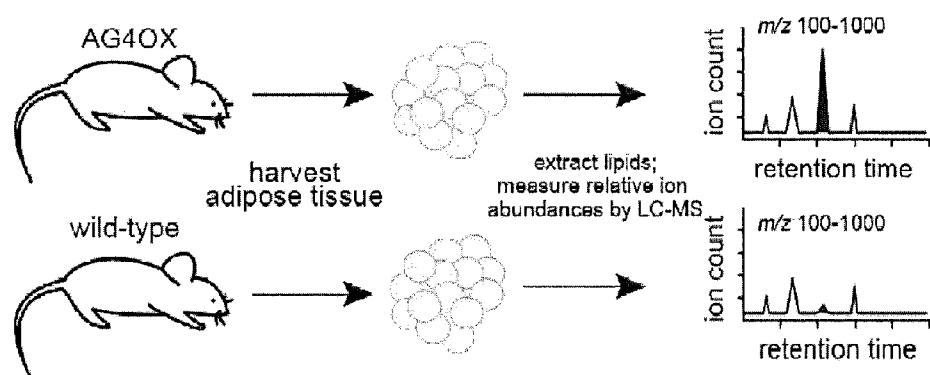


FIG. 2

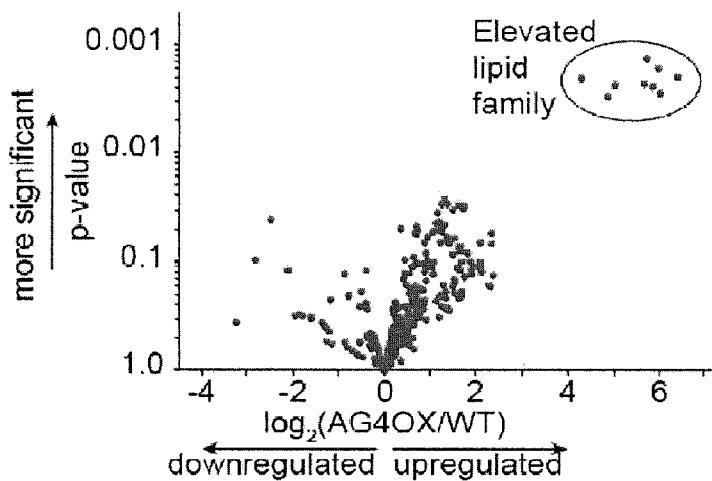


FIG. 3

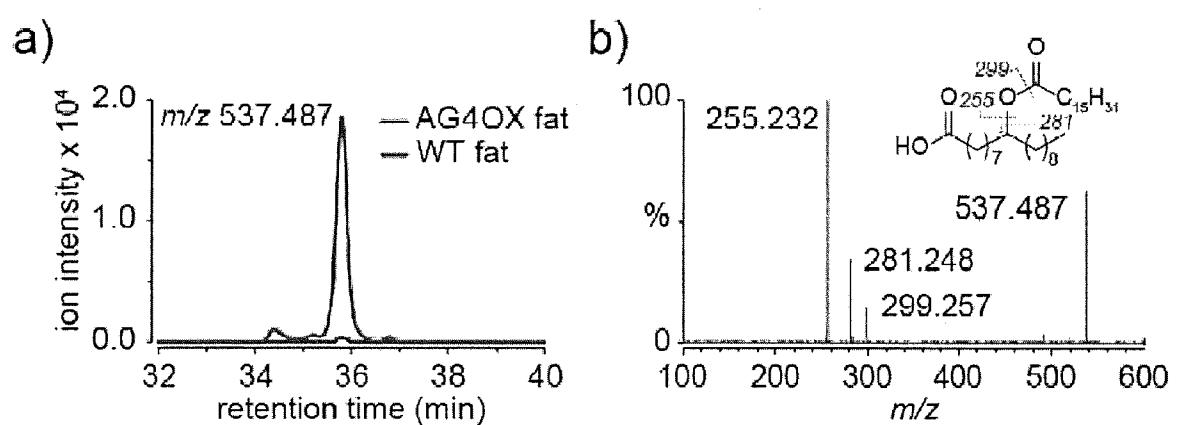


FIG. 4

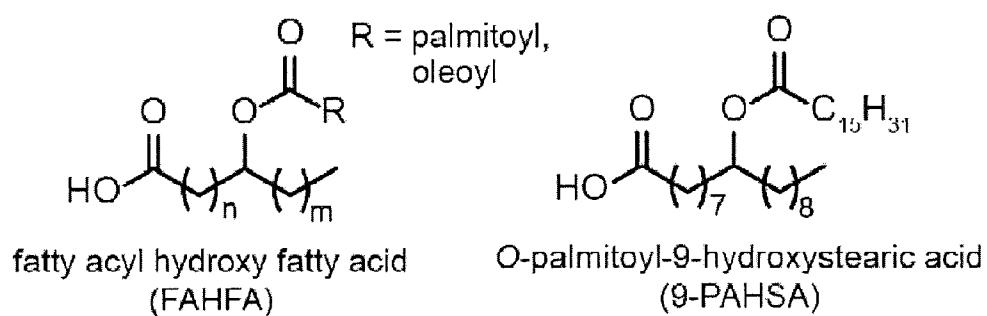


FIG. 5

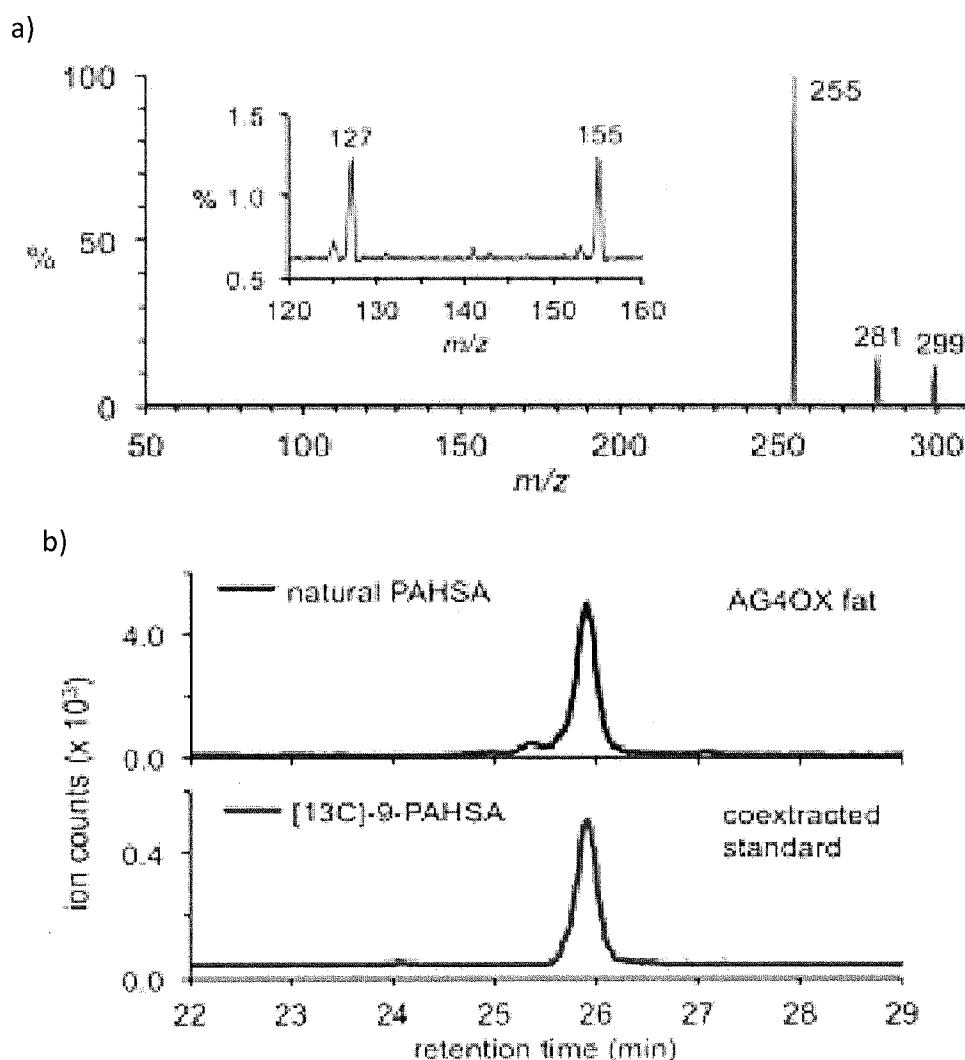


FIG. 6

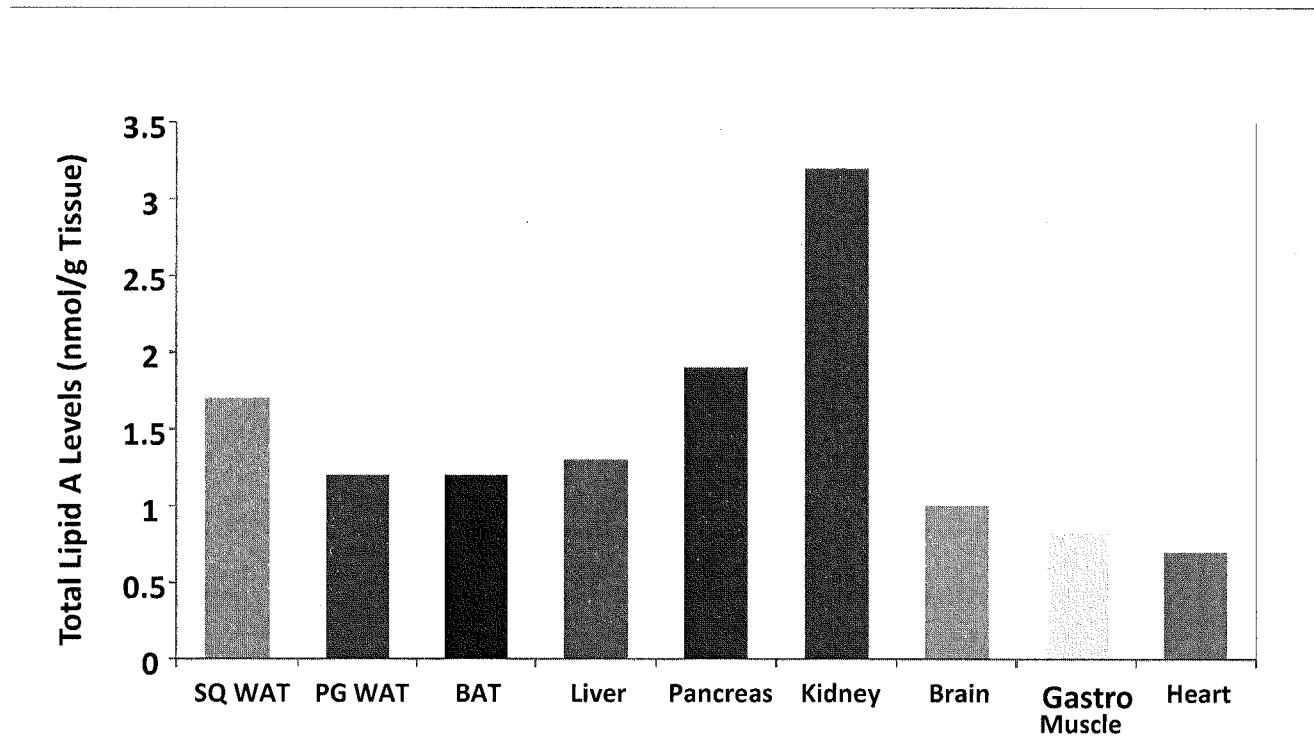


FIG. 7A

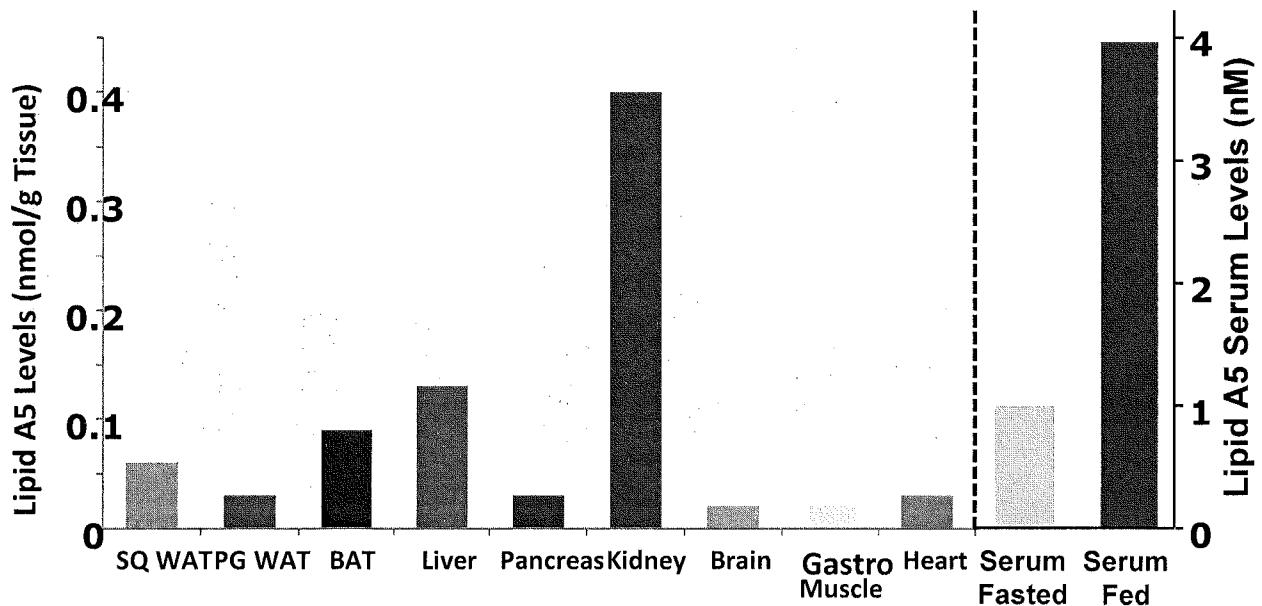


FIG. 7B

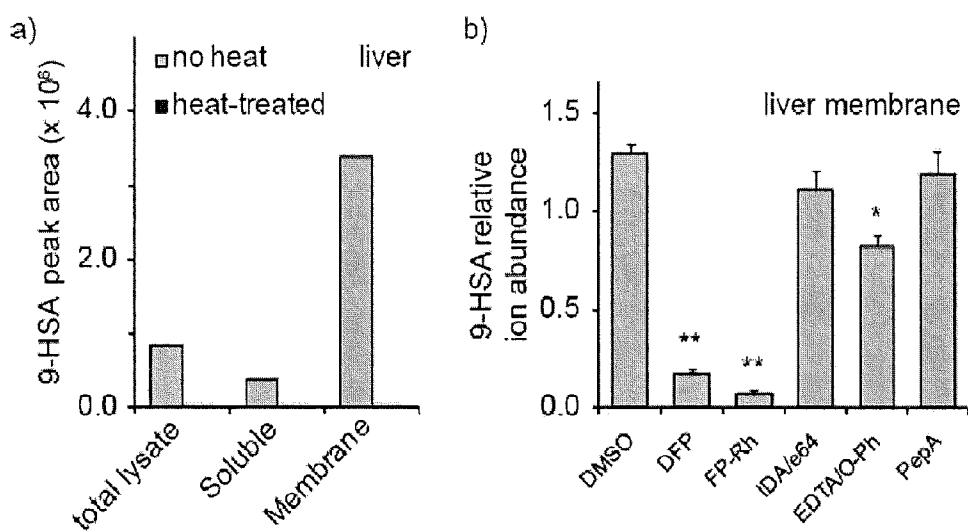


FIG. 8

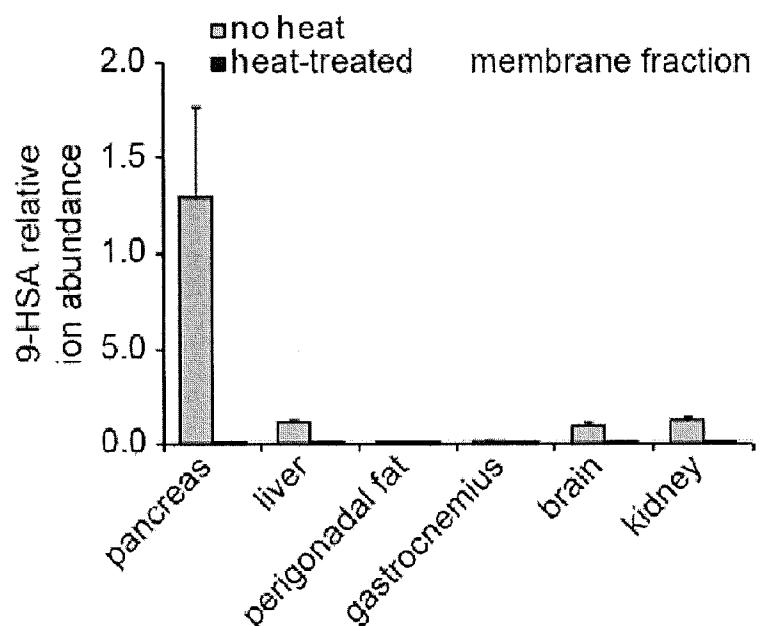


FIG. 9

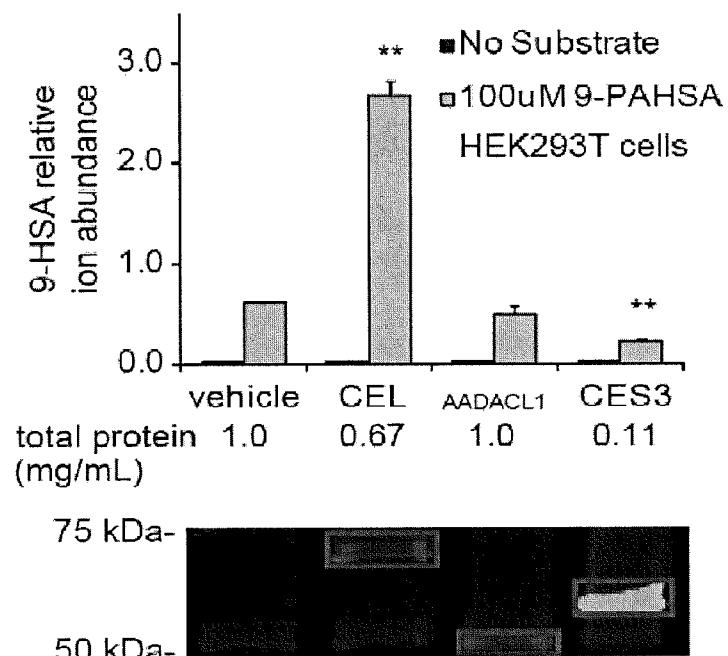


FIG. 10

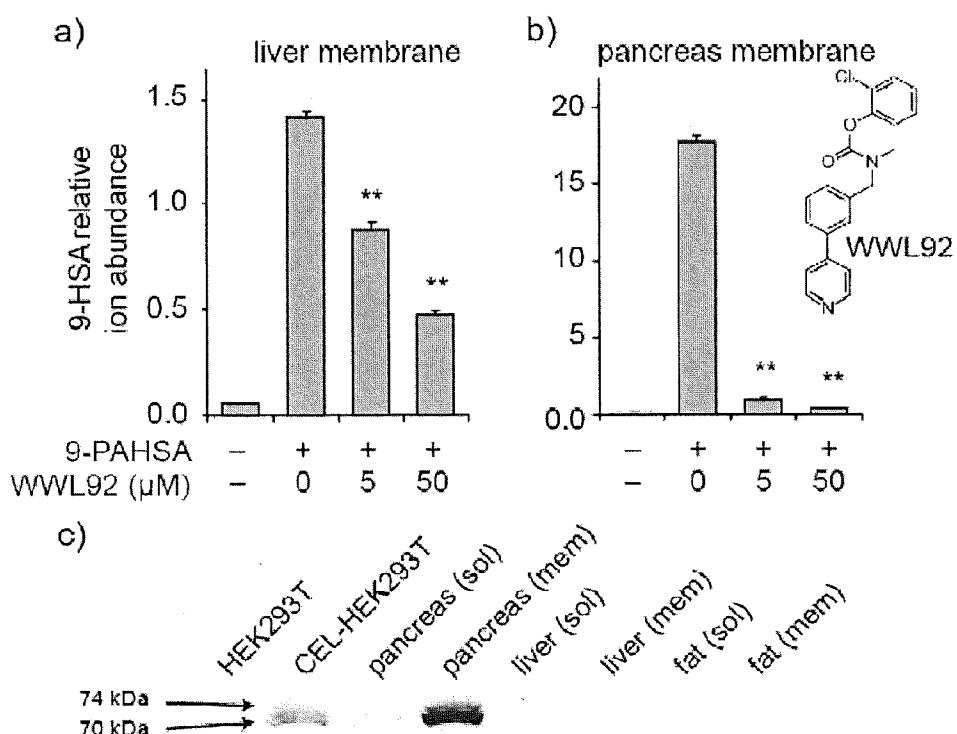


FIG. 11

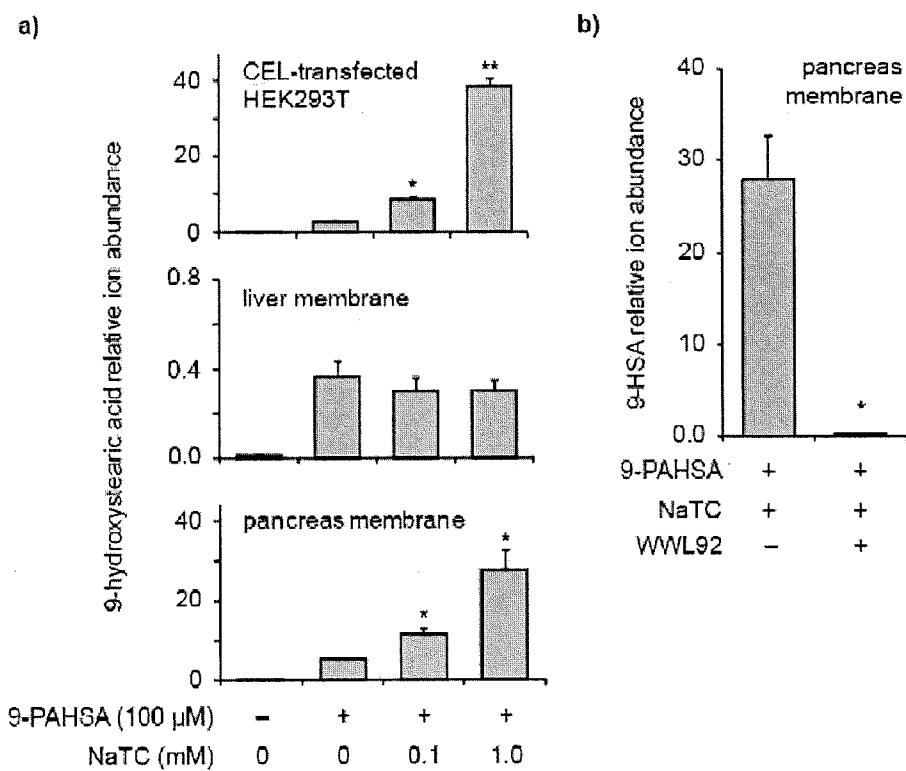


FIG. 12

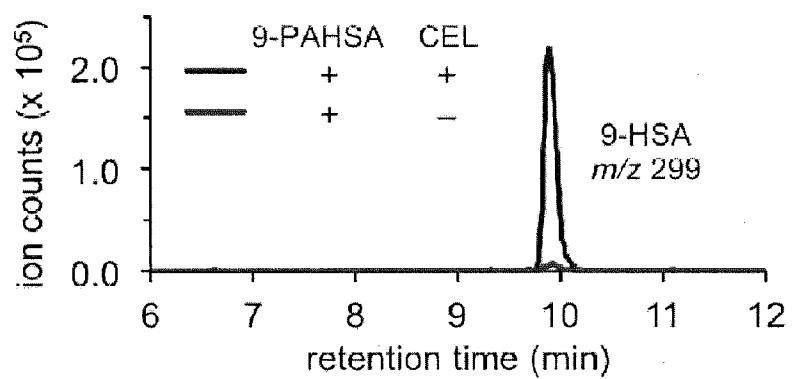


FIG. 13

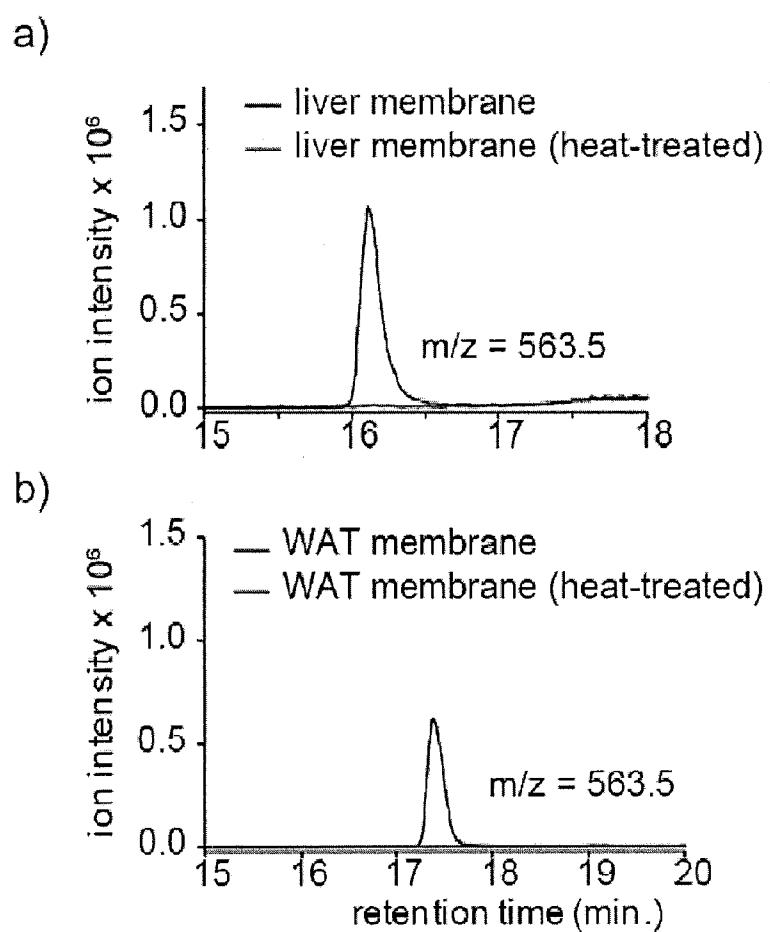


FIG. 14

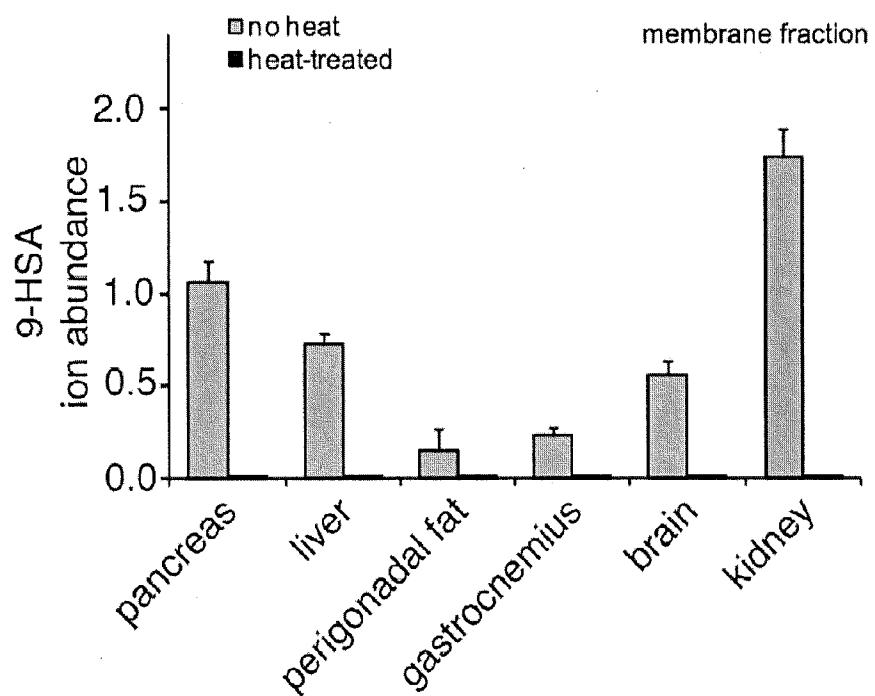


FIG. 15

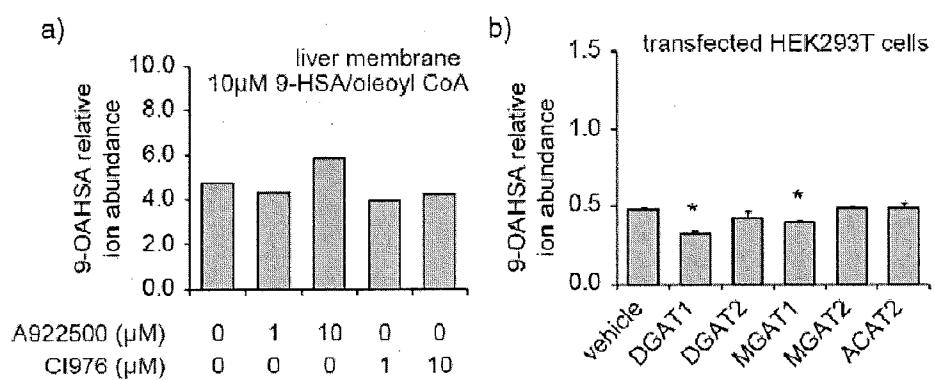


FIG. 16

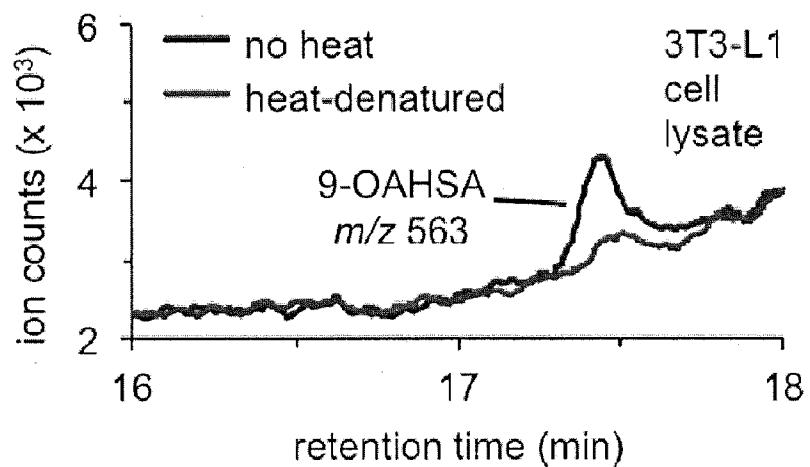


FIG. 17

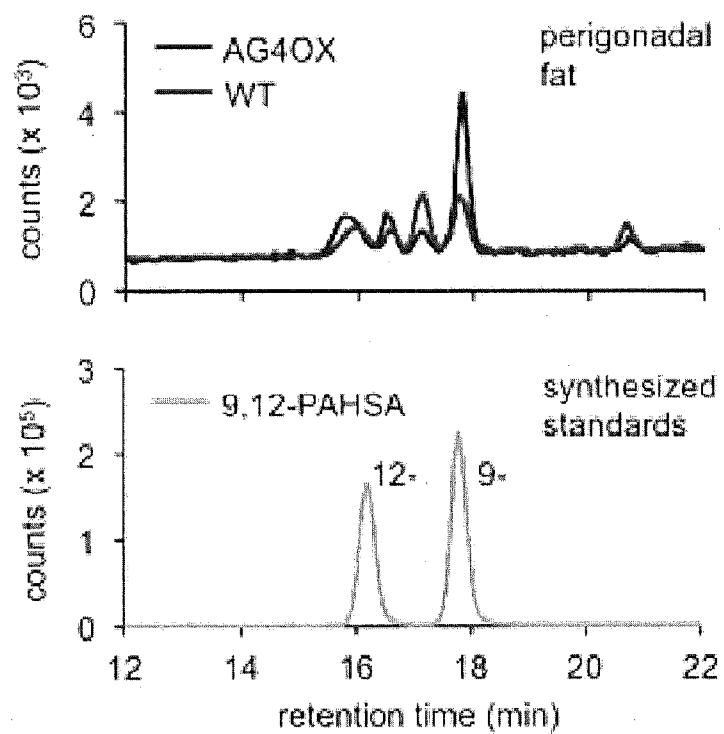


FIG. 18

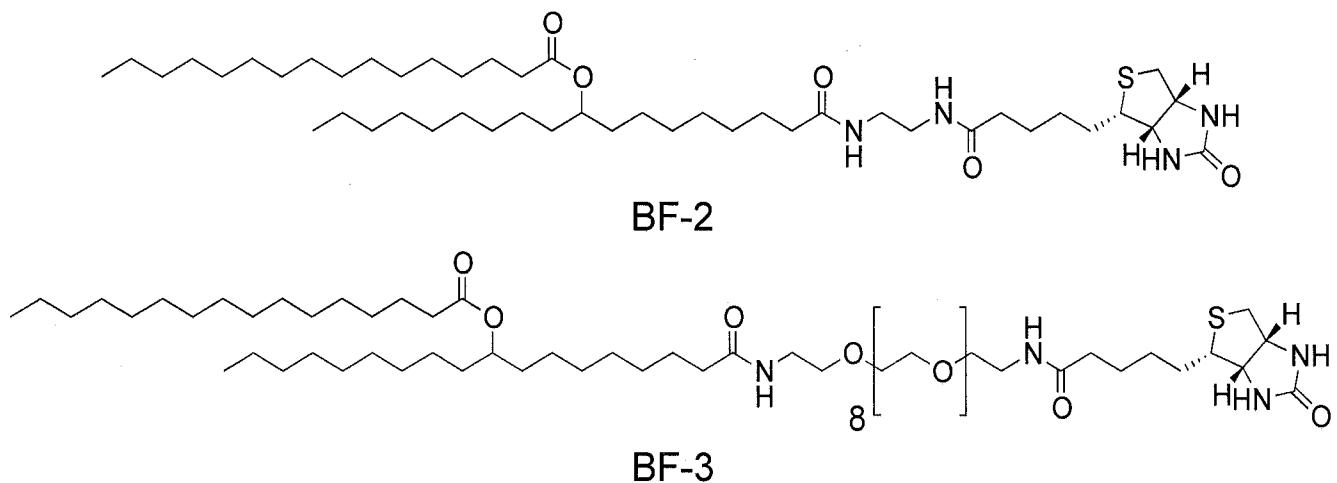


FIG. 19

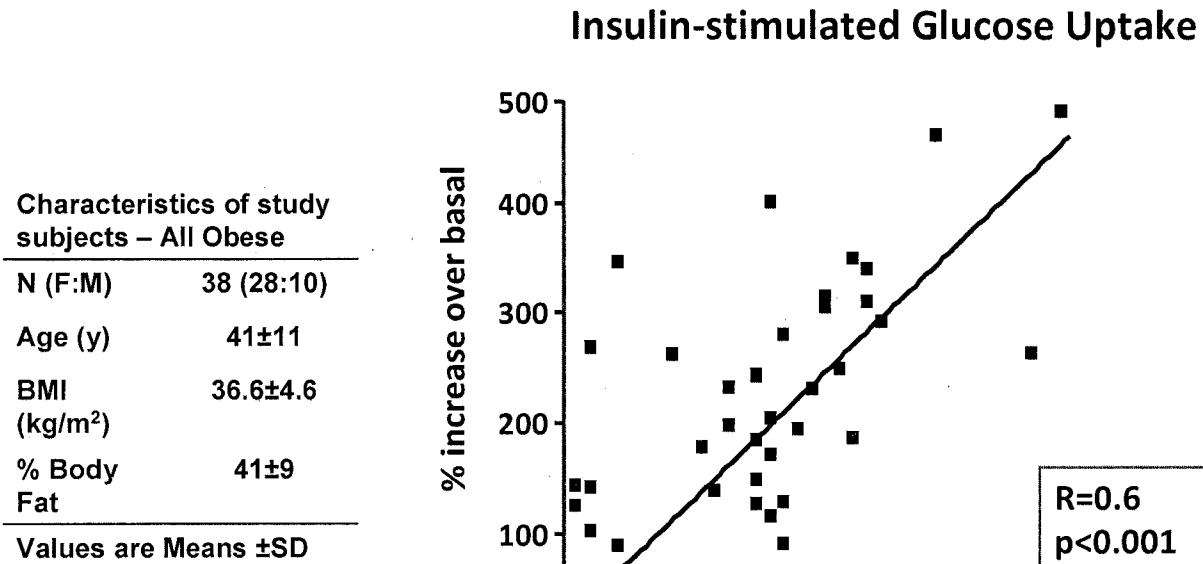


FIG. 20

<i>Human Serum</i>	mg lipid	Novel Lipid (nM)
serum-1	1.6	50.7
serum-2	3.1	83.3
serum-3	1.6	48.7

<i>Human Fat</i>	mg lipid	Novel Lipid (pmol/mg lipid)
fat-1 (fed)	123.4	1.12
fat-2 (fasted)	100.1	0.97

FIG. 21

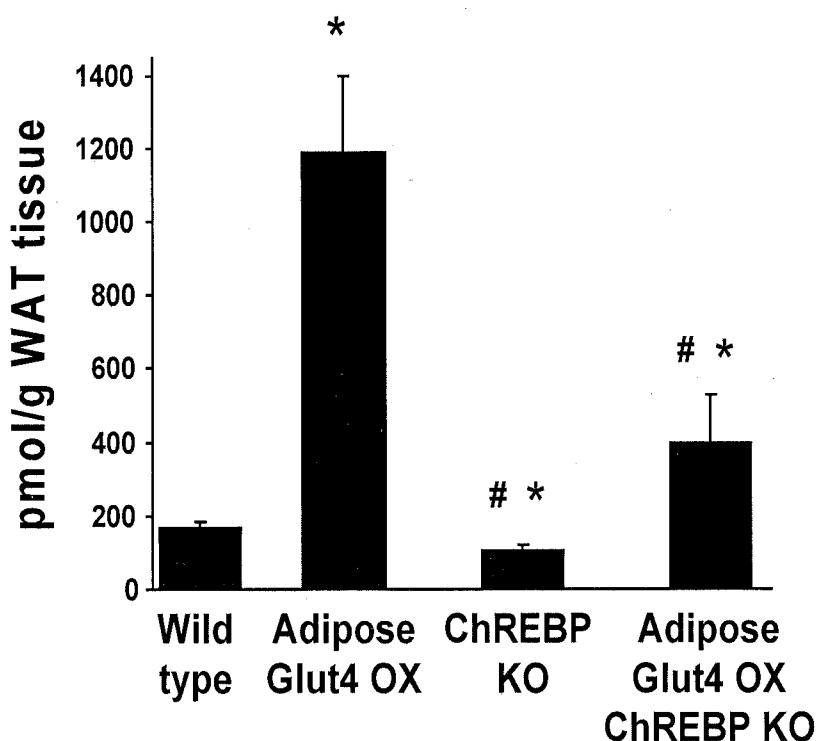


FIG. 22

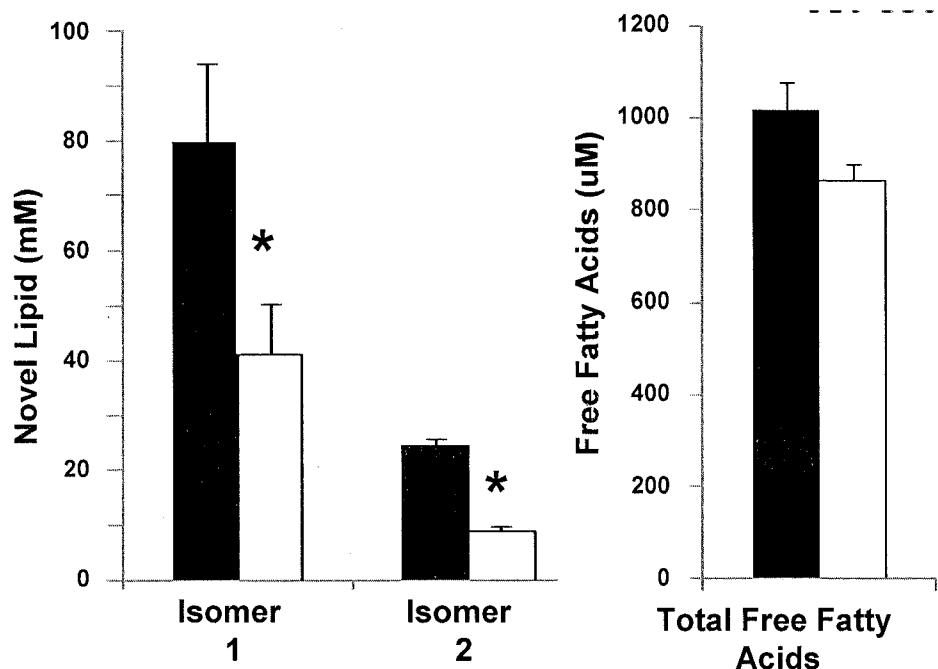


FIG. 23

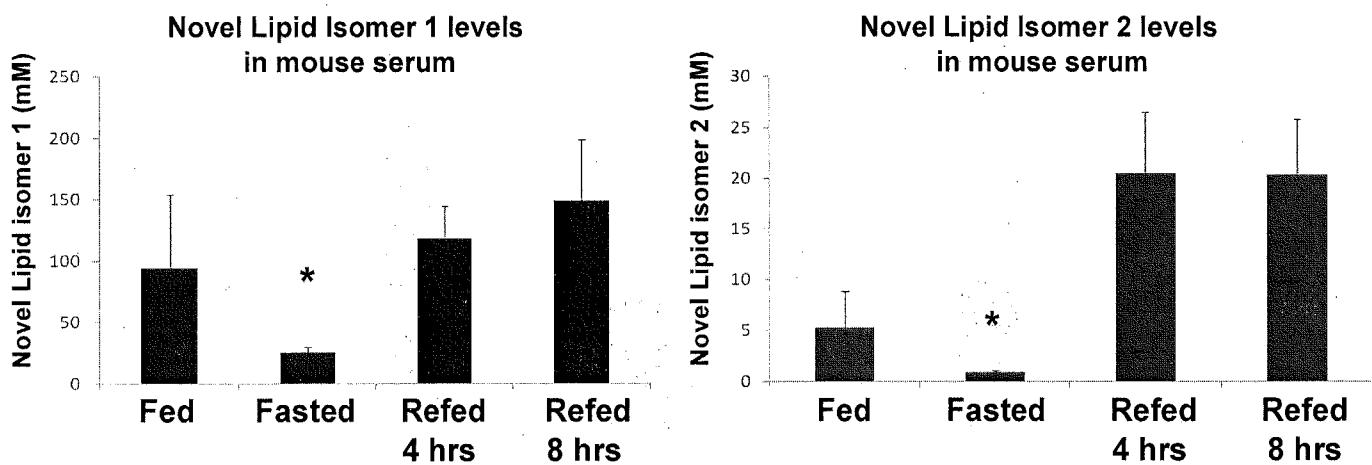


FIG. 24

Characteristic	Lean, insulin-sensitive (N = 8)	Lean, insulin-resistant (N = 8)	Obese, insulin-resistant (N = 8)	P-value (L-IS vs L-IR)	P-value (L-IR vs O-IR)
Sex (male/female)	4/4	4/4	4/4		
Age (yrs)	42.2 ± 2.4	36.6 ± 2.3	39.0 ± 2.6		
HbA1c (%)	4.14 ± 0.07	4.18 ± 0.05	4.09 ± 0.12		
Body mass index (kg/m ²)	22.3 ± 1.3	22.4 ± 1.3	31.1 ± 0.9	0.94	< 0.001
Glucose infusion rate (mg/kg/min)	19.3 ± 2.0	8.3 ± 1.3	8.1 ± 2.2	< 0.001	0.92

FIG. 25

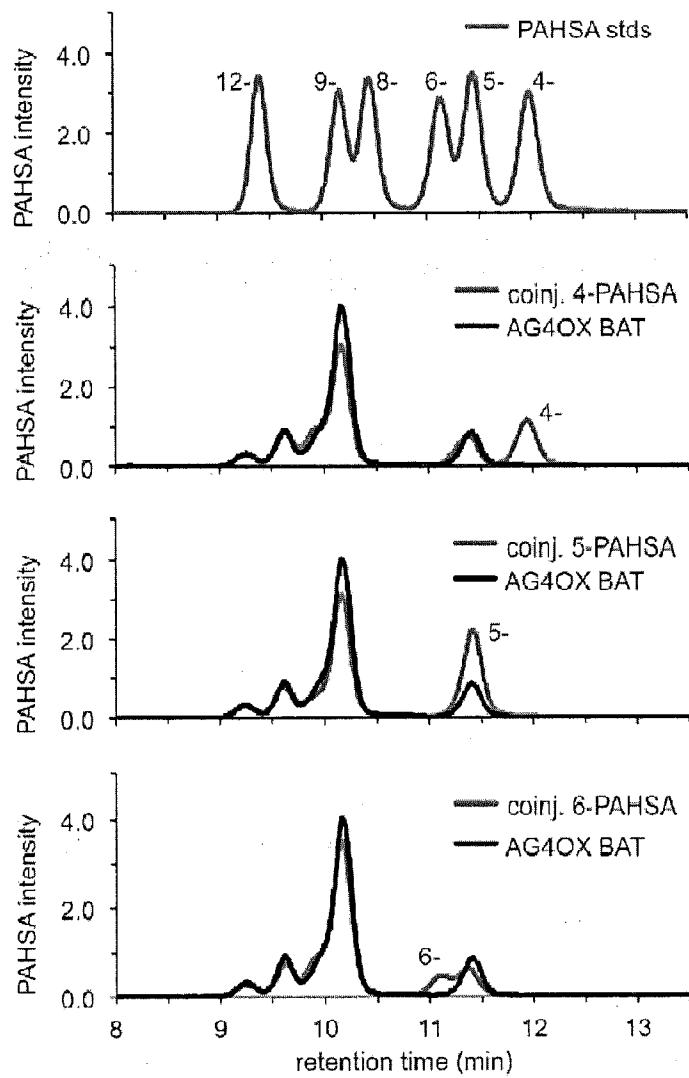


FIG. 26

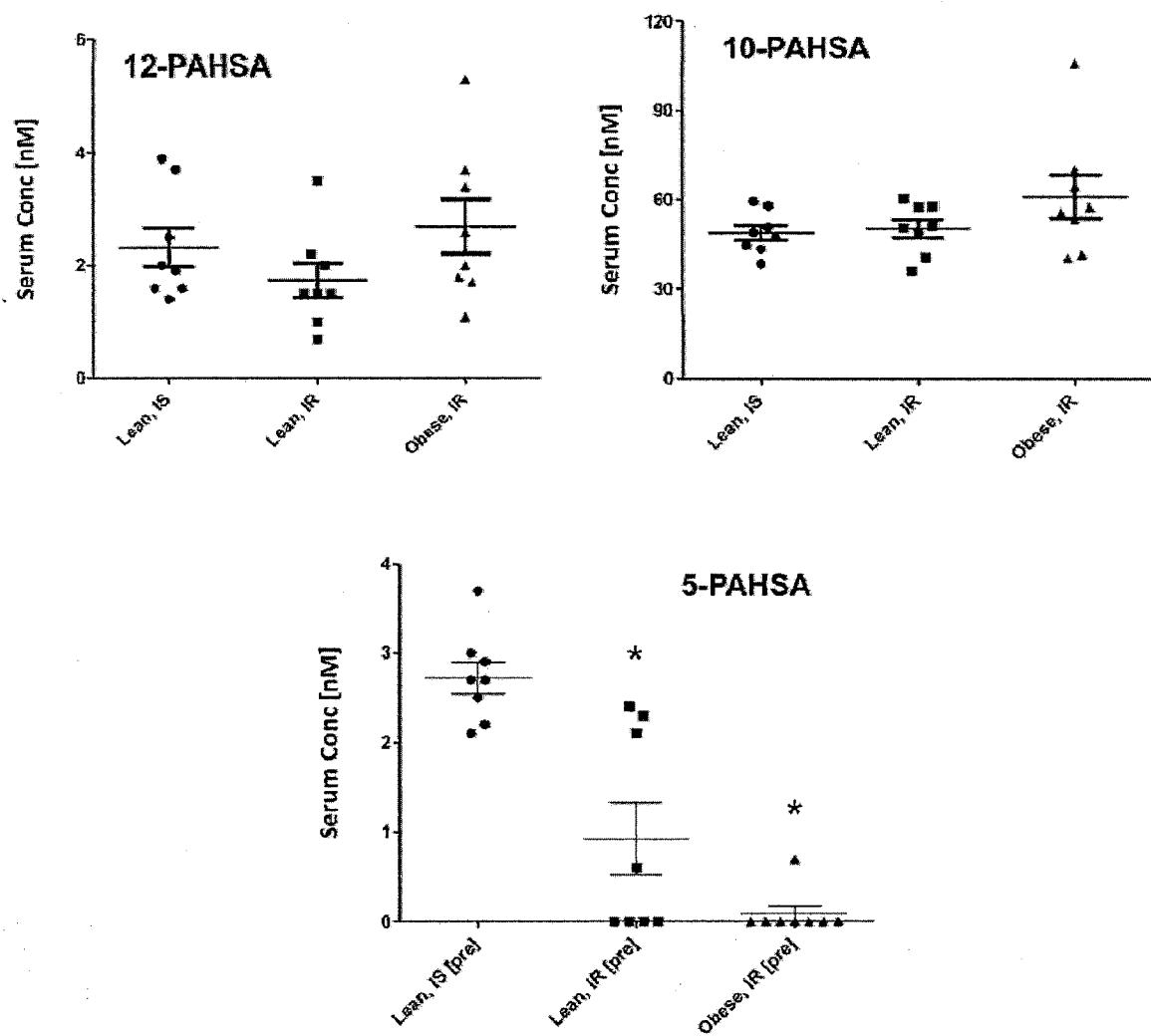


FIG. 27

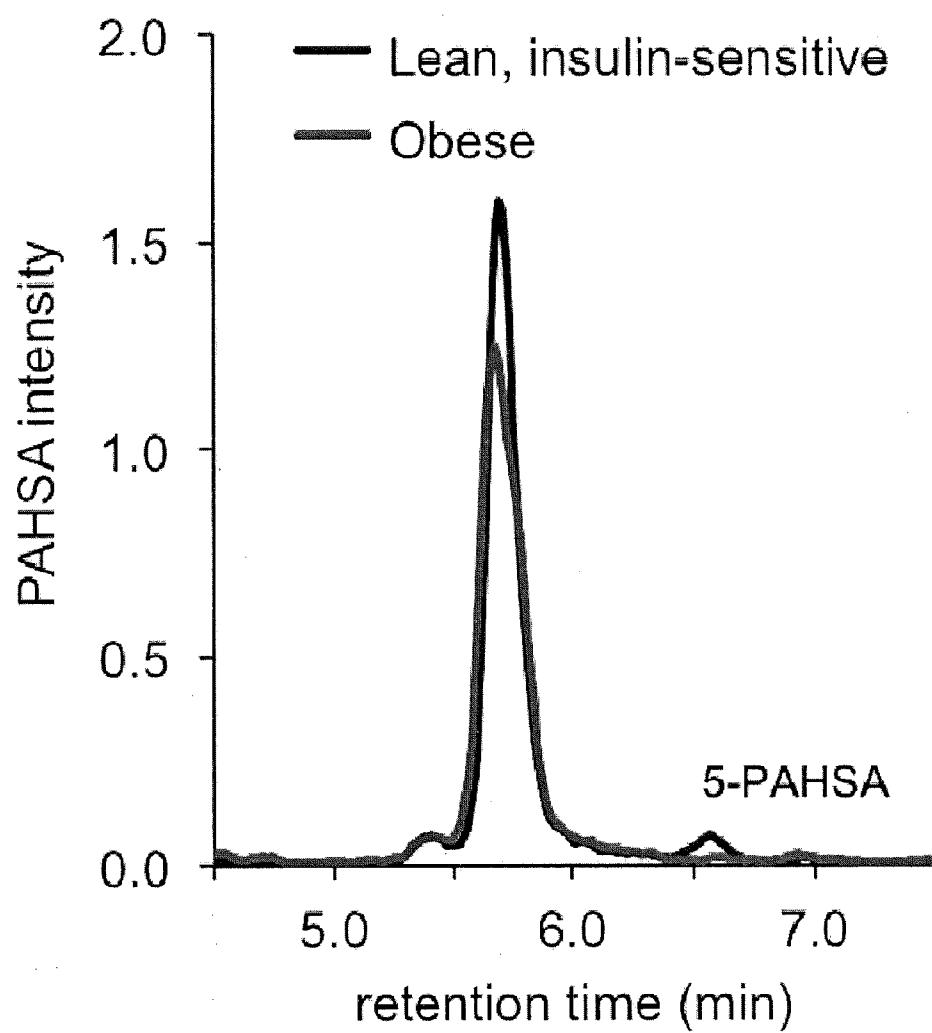


FIG. 28

5- PAHSA, All Subjects vs Glucose Infusion Rate (mg/min/kgBW)

FIG. 29

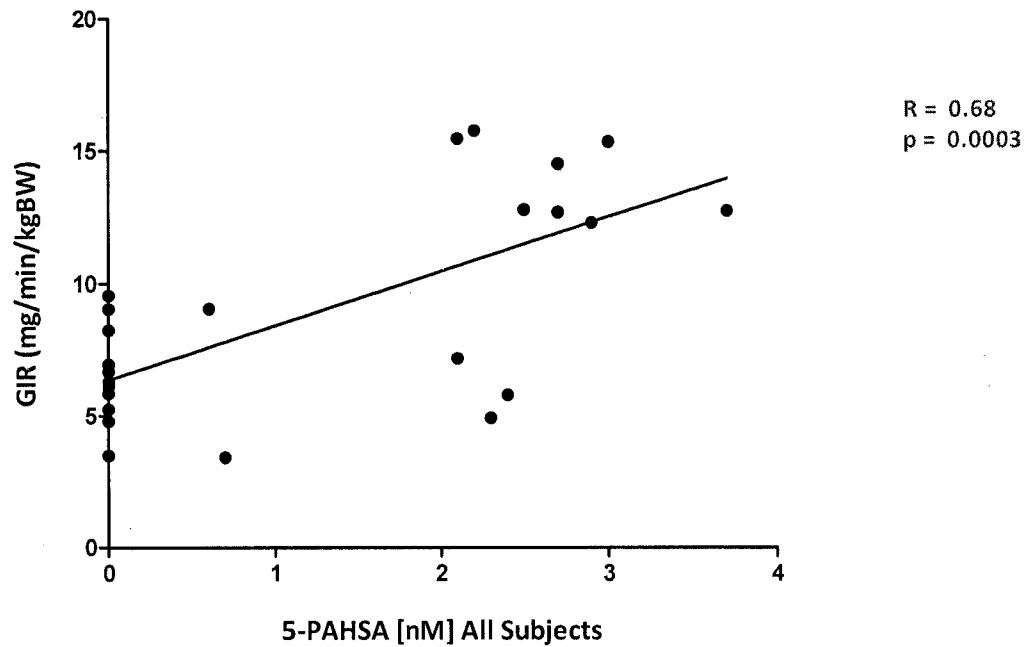
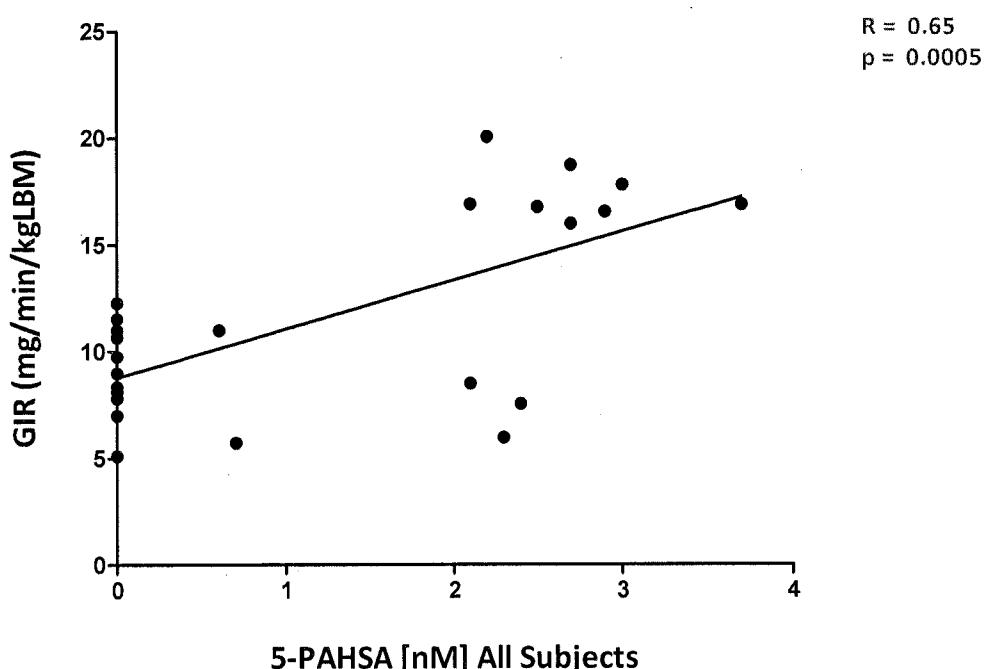
**5- PAHSA, All Subjects vs Glucose Infusion Rate [mg/min/KgLBM]**

FIG. 30



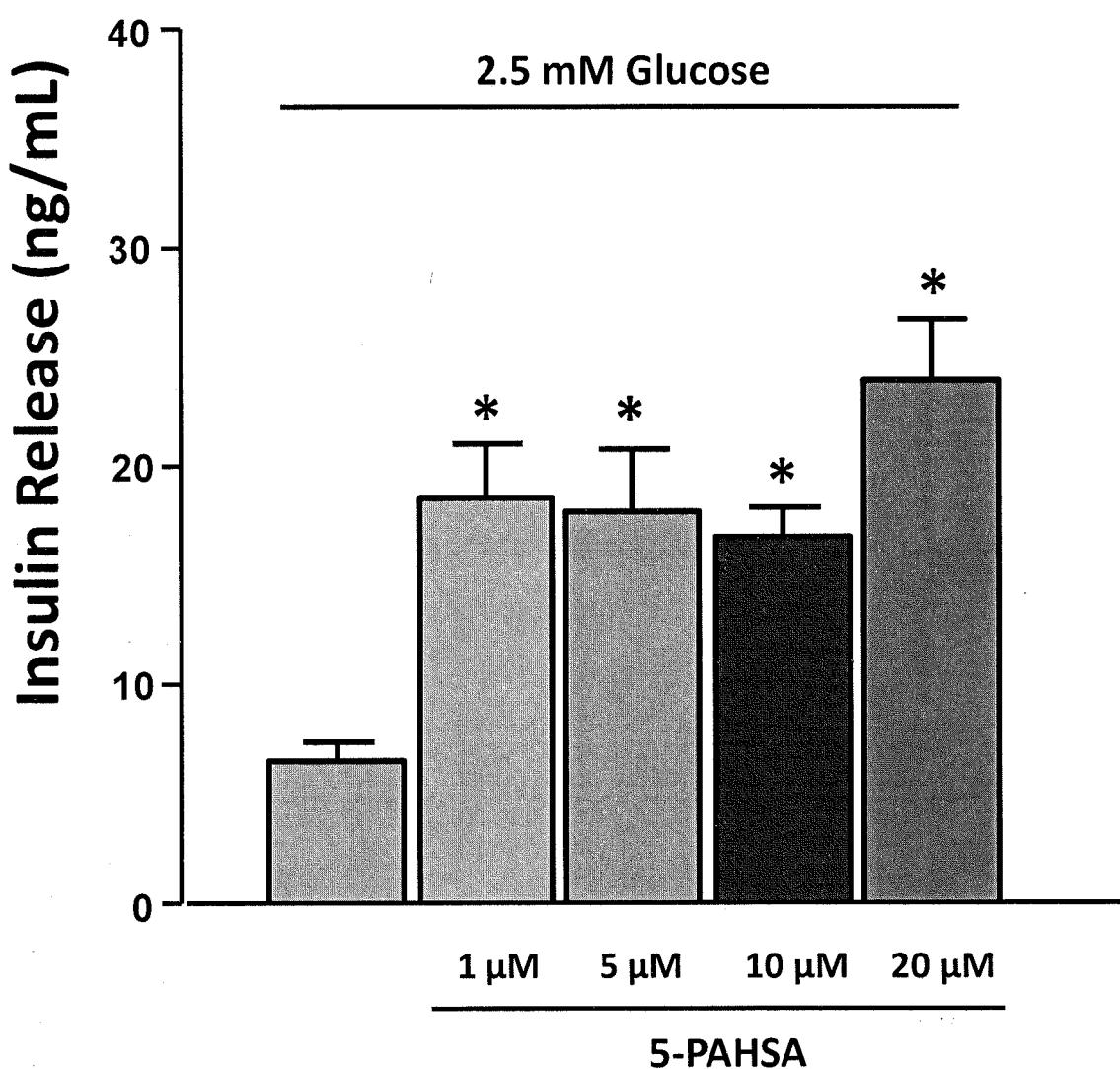


FIG. 31A

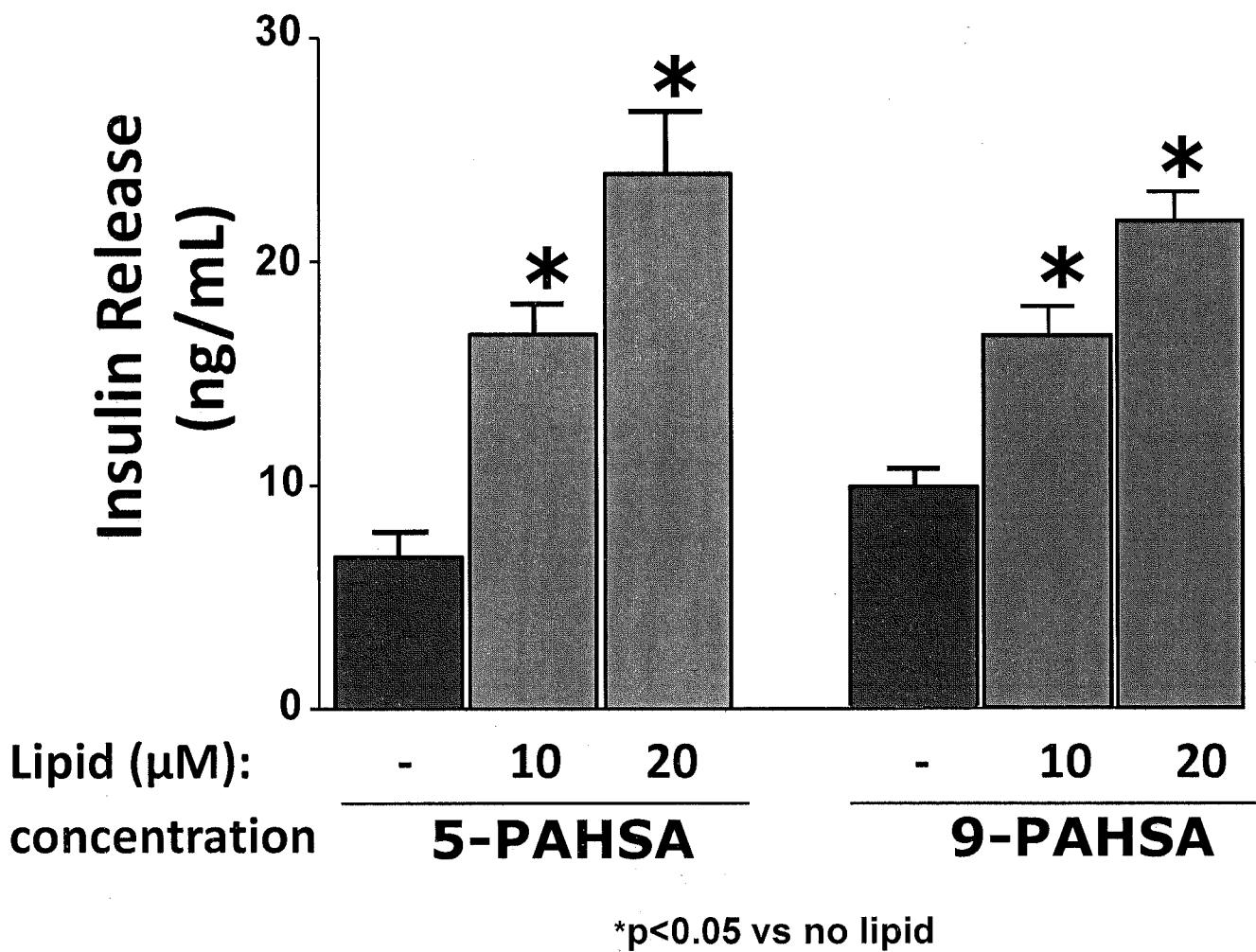


FIG. 31B

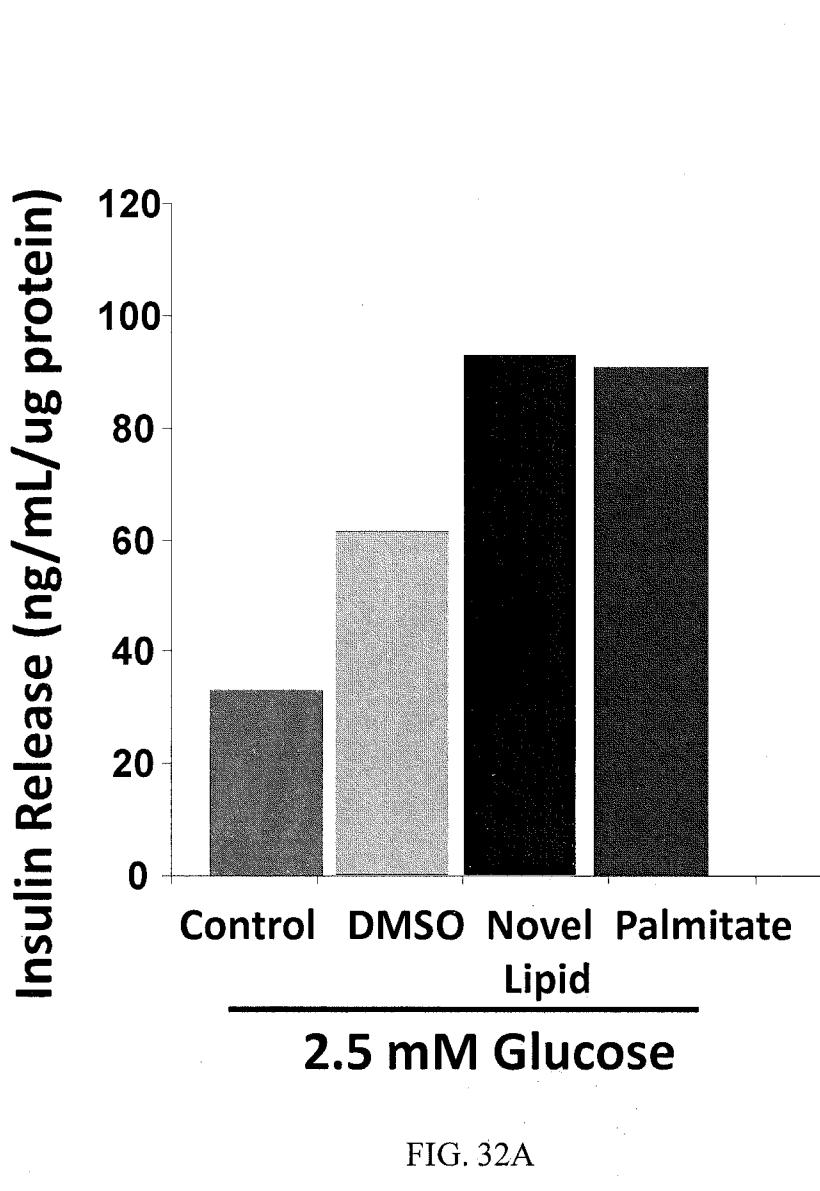


FIG. 32A

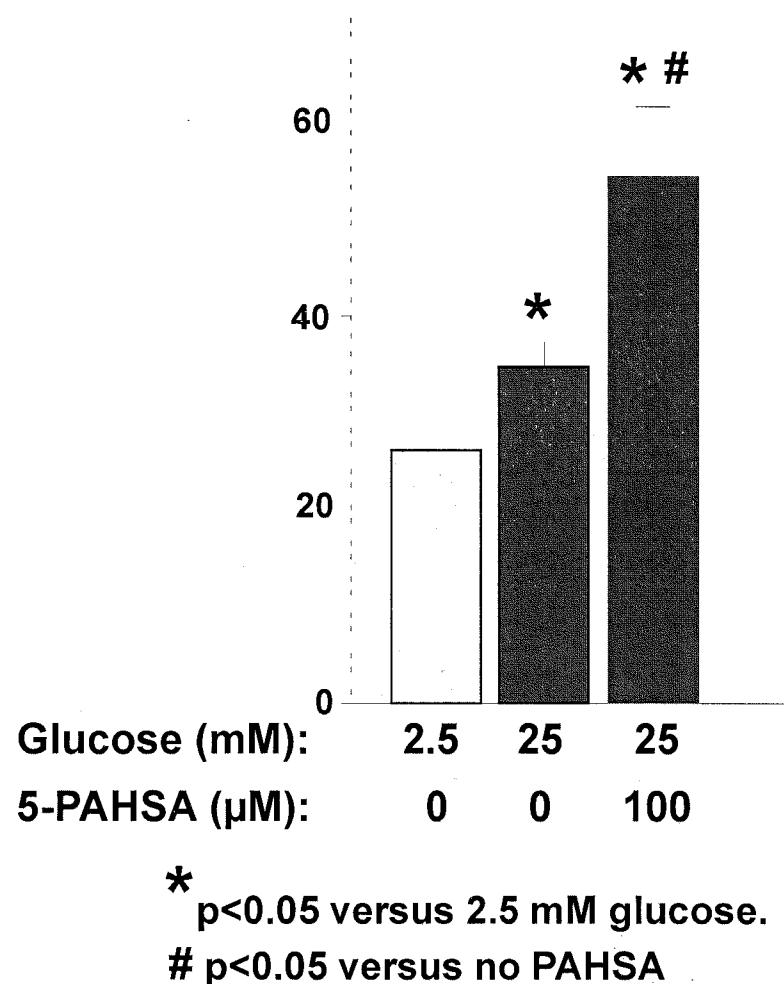


FIG. 32B

5-PAHSA in HepG2 Cells

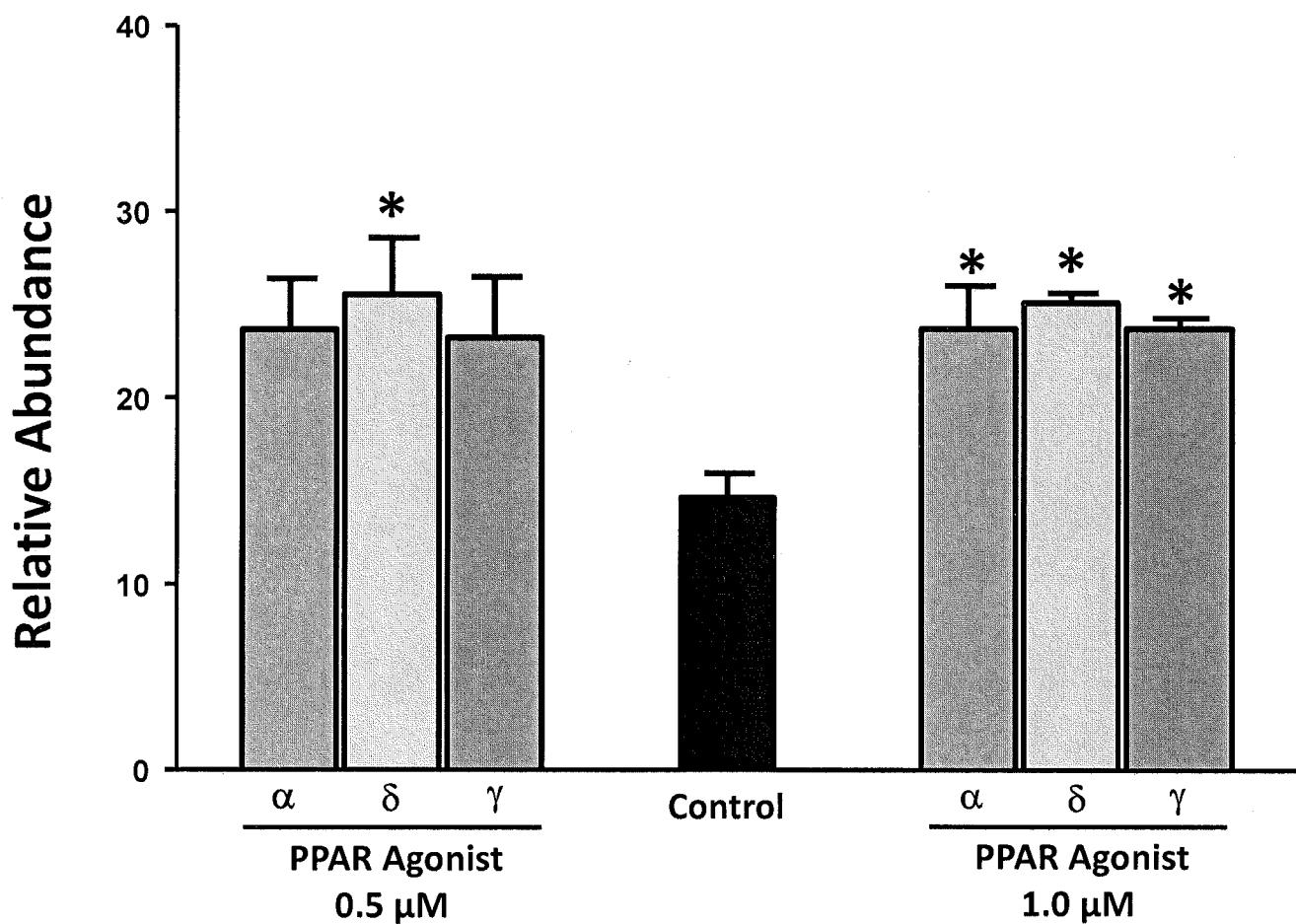


FIG. 33

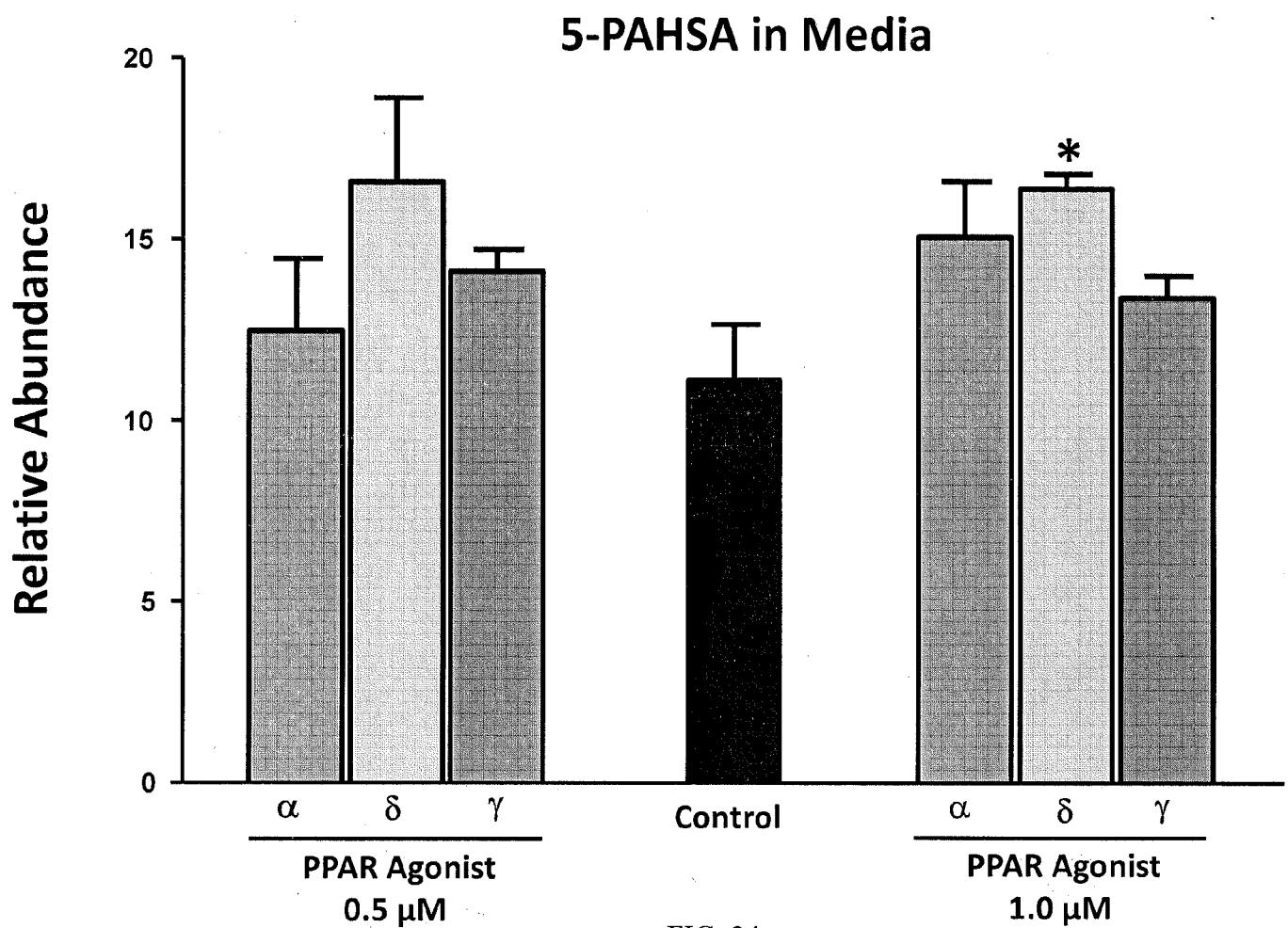


FIG. 34

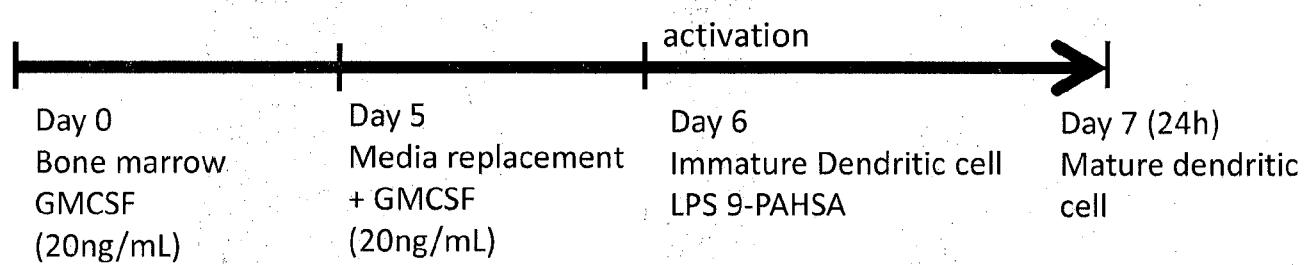
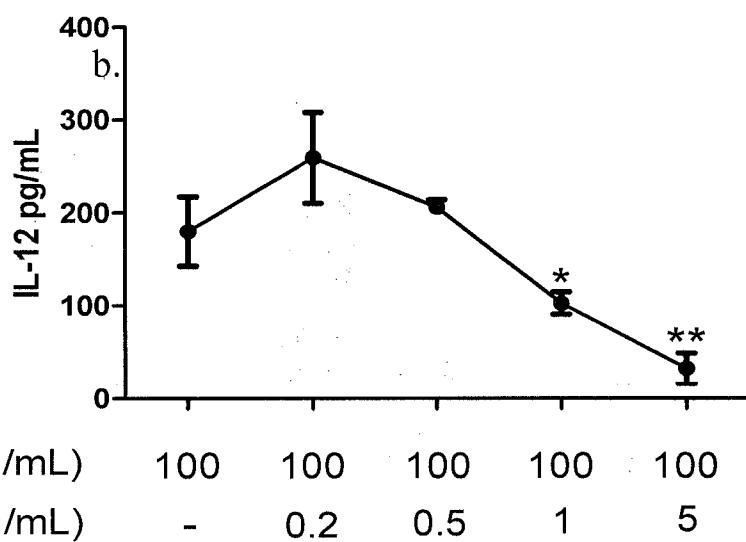
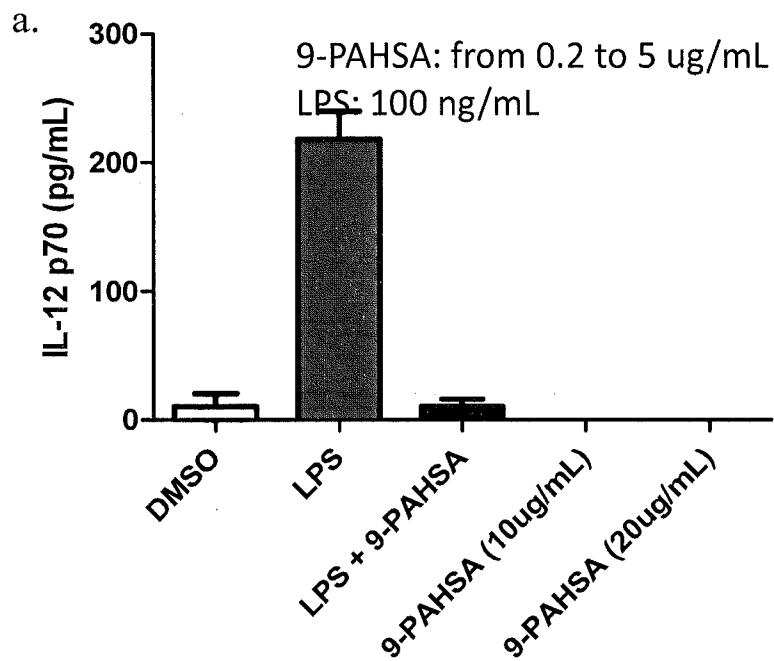


FIG. 35

9-PAHSA inhibits LPS-induced IL-12 p70 production in dendritic cells



FIGs. 36A and 36B

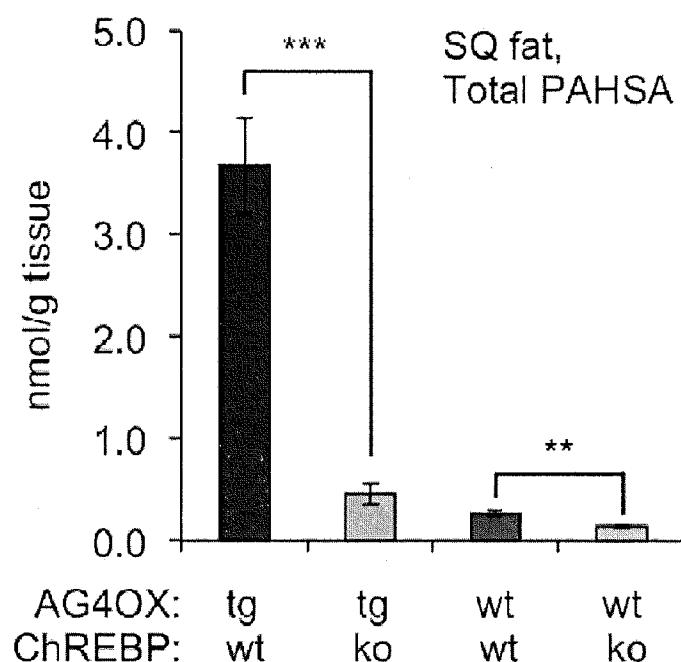


FIG. 37

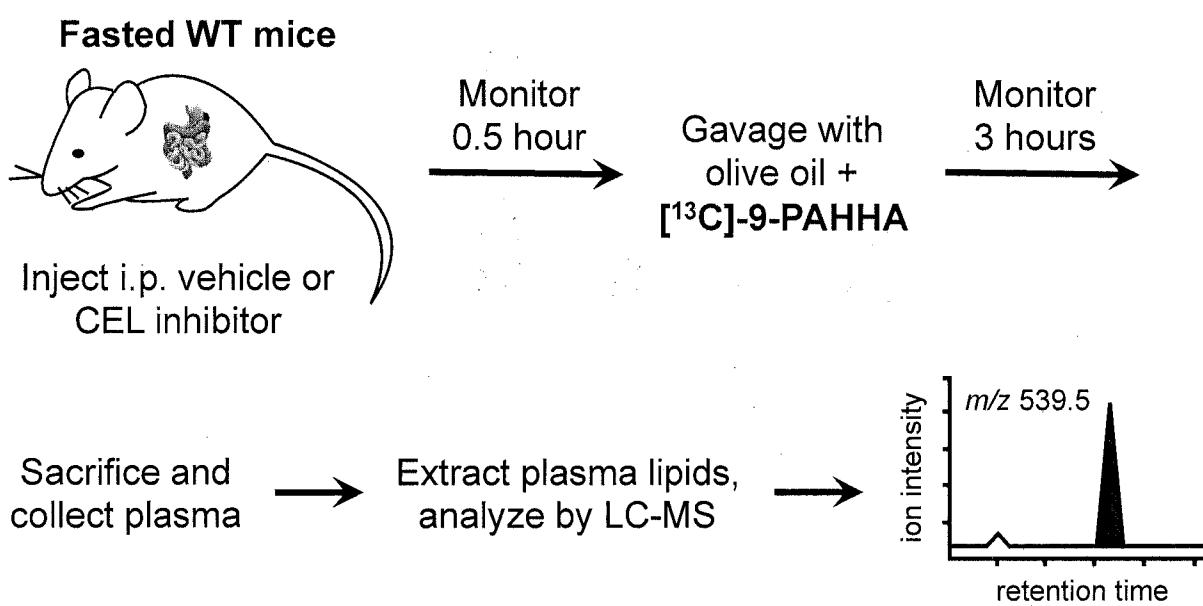


FIG. 38

Pancreas membrane lysate

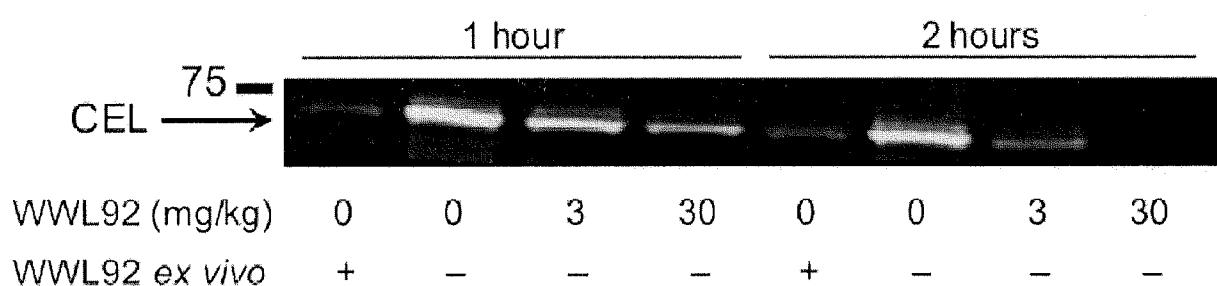


FIG. 39

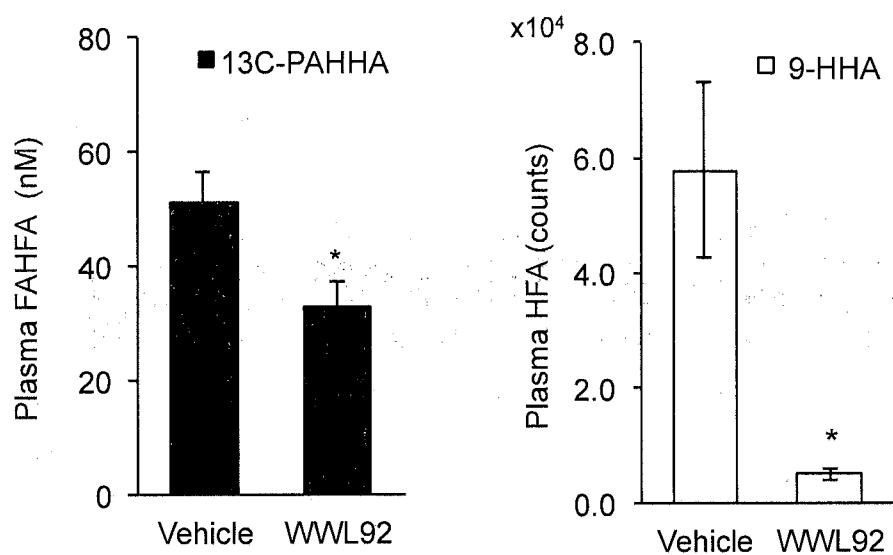


FIG. 40

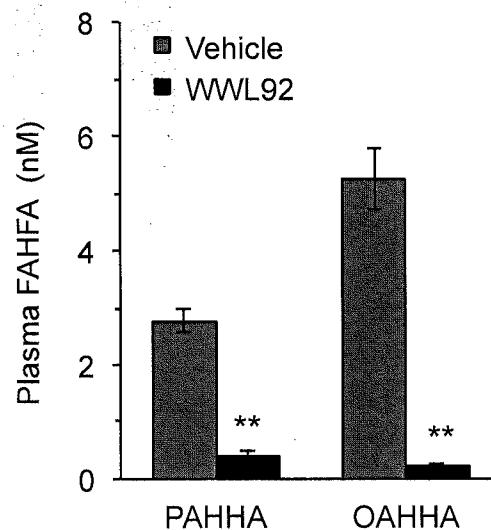


FIG. 41

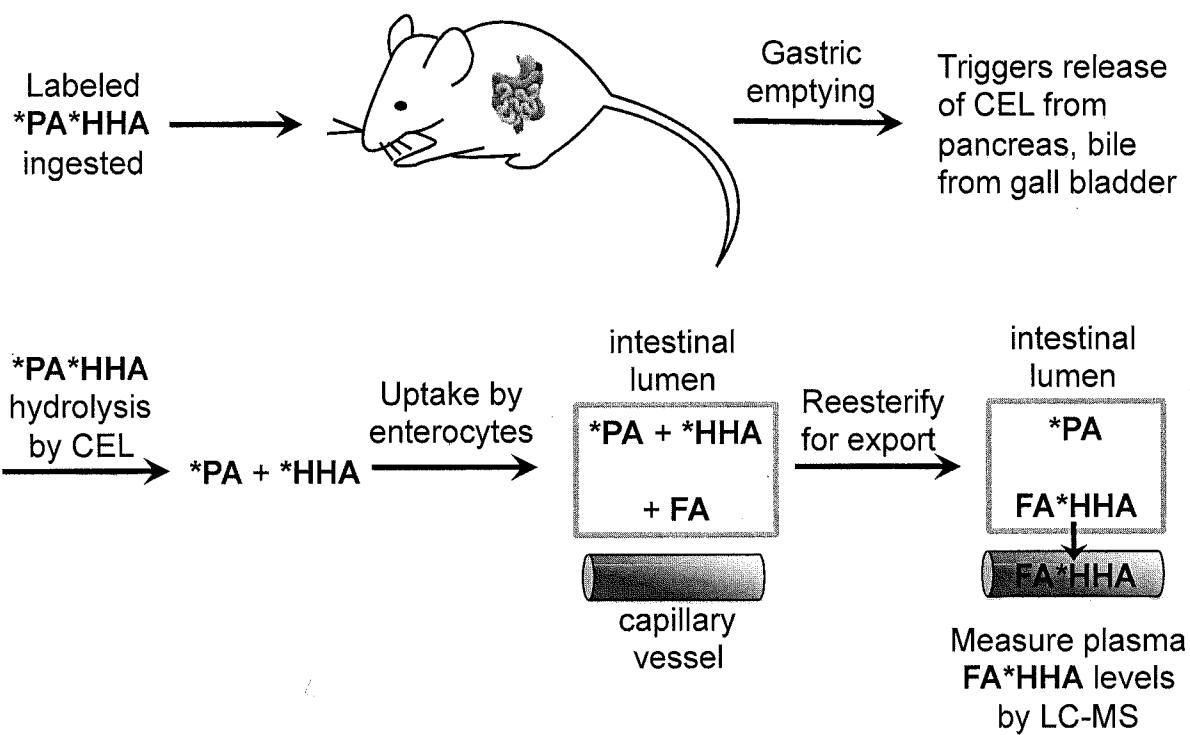


FIG. 42

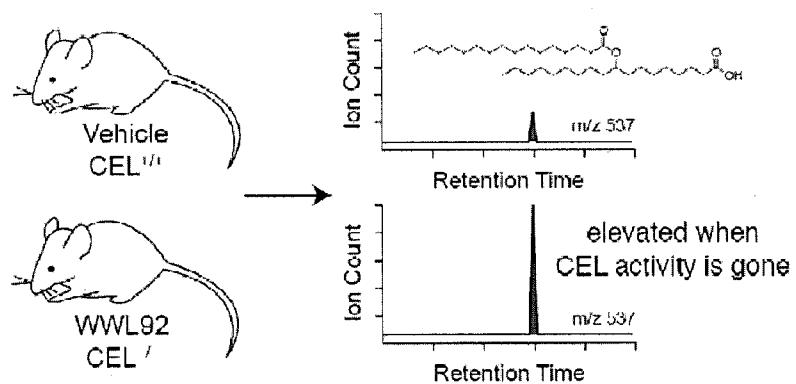


FIG. 43

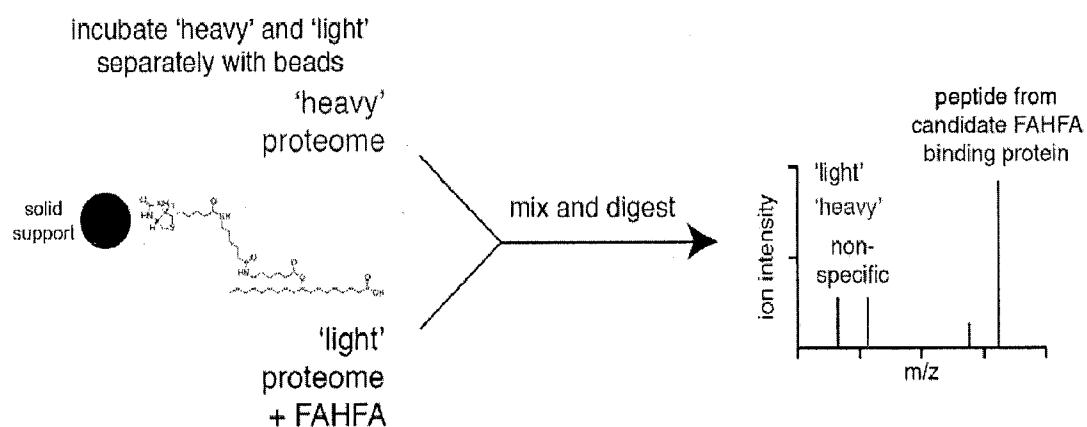


FIG. 44

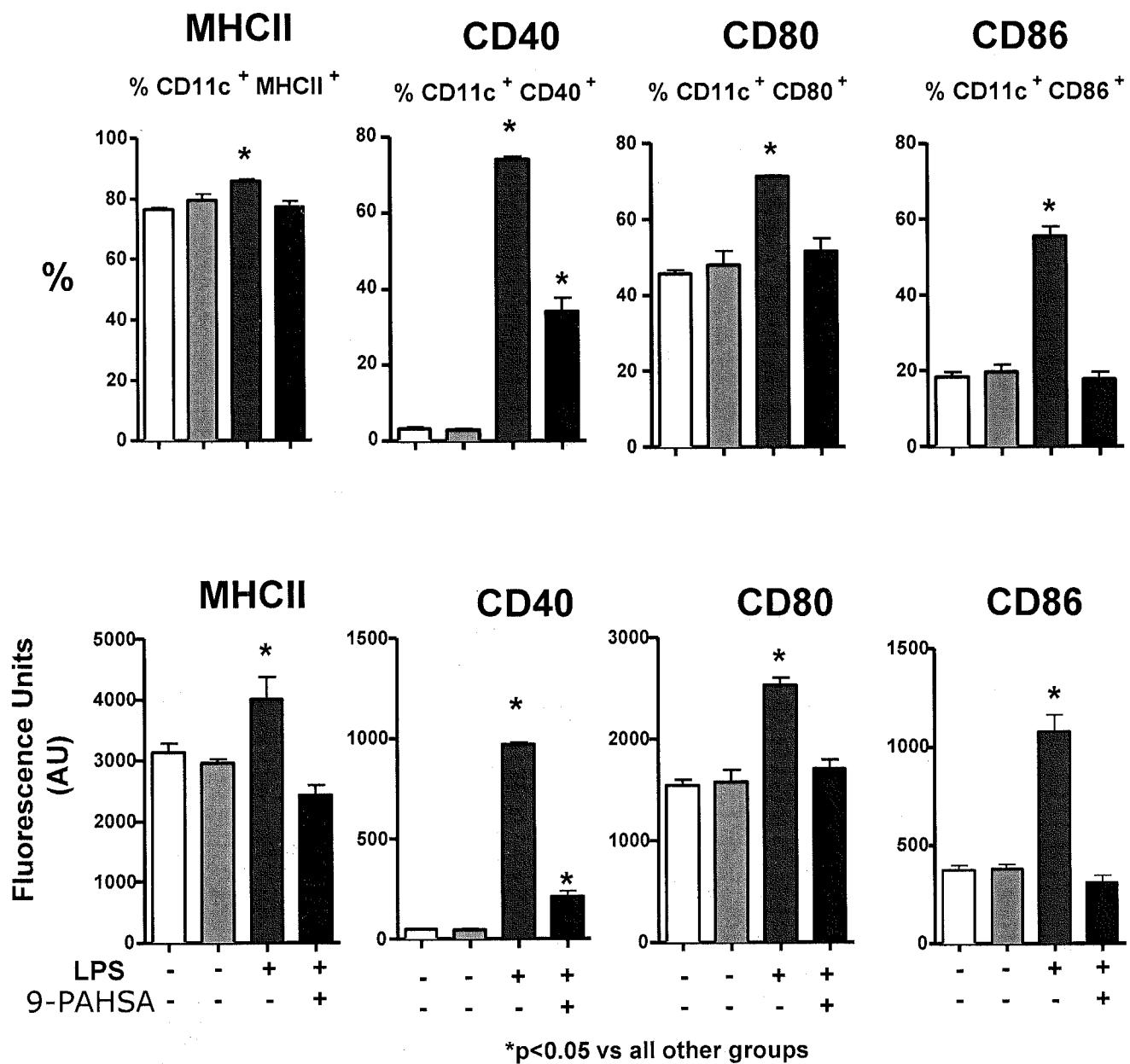


FIG. 45

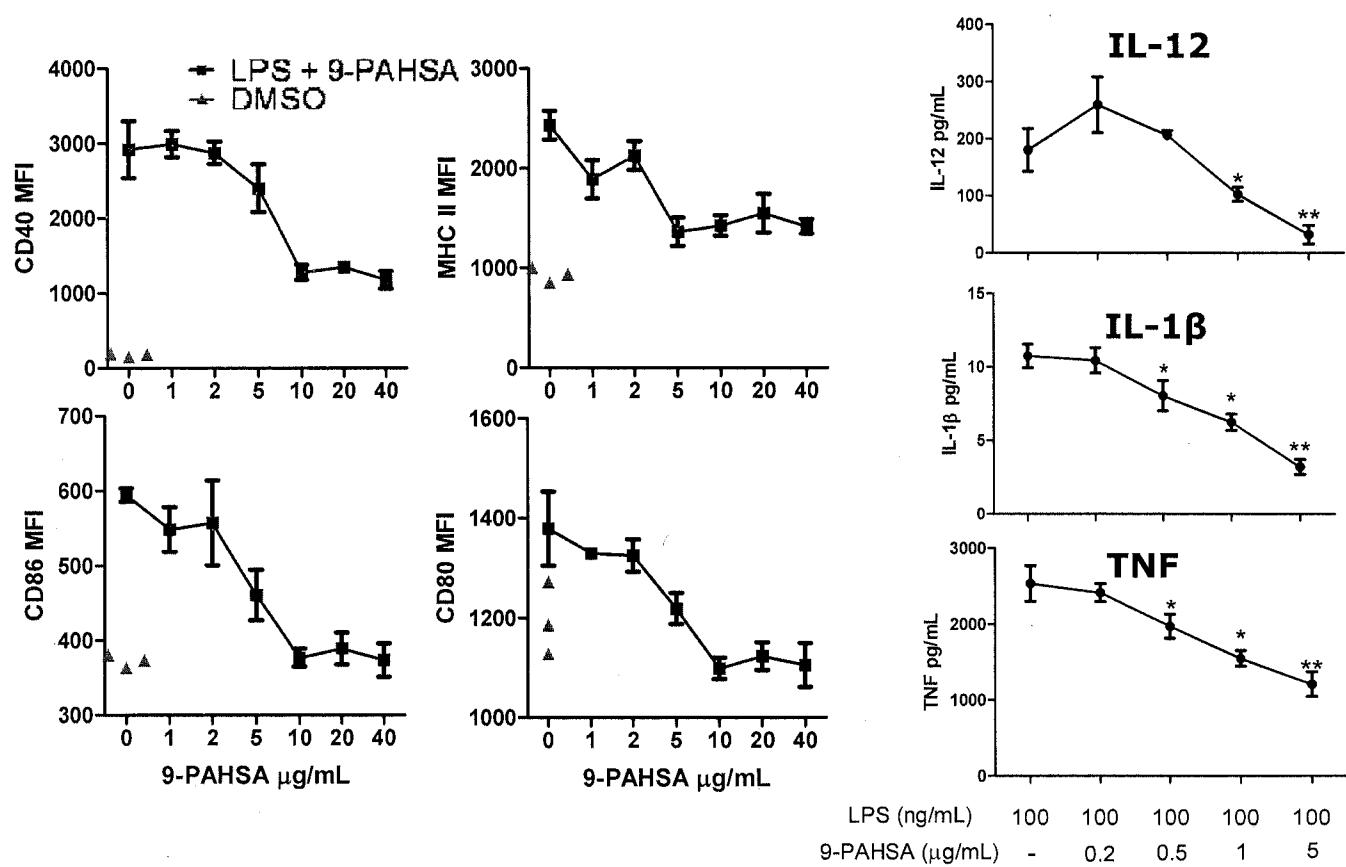


FIG. 46

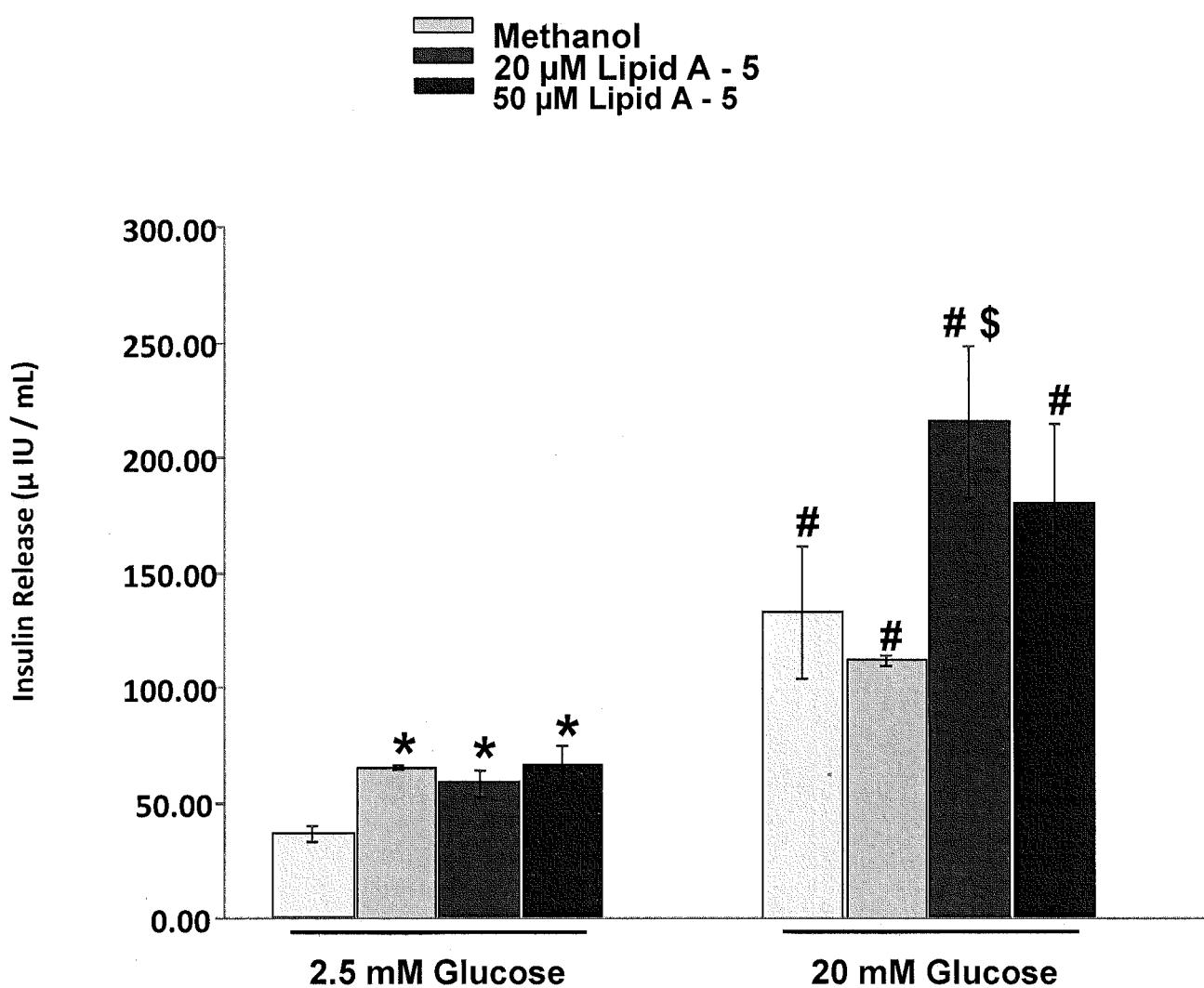


FIG. 47

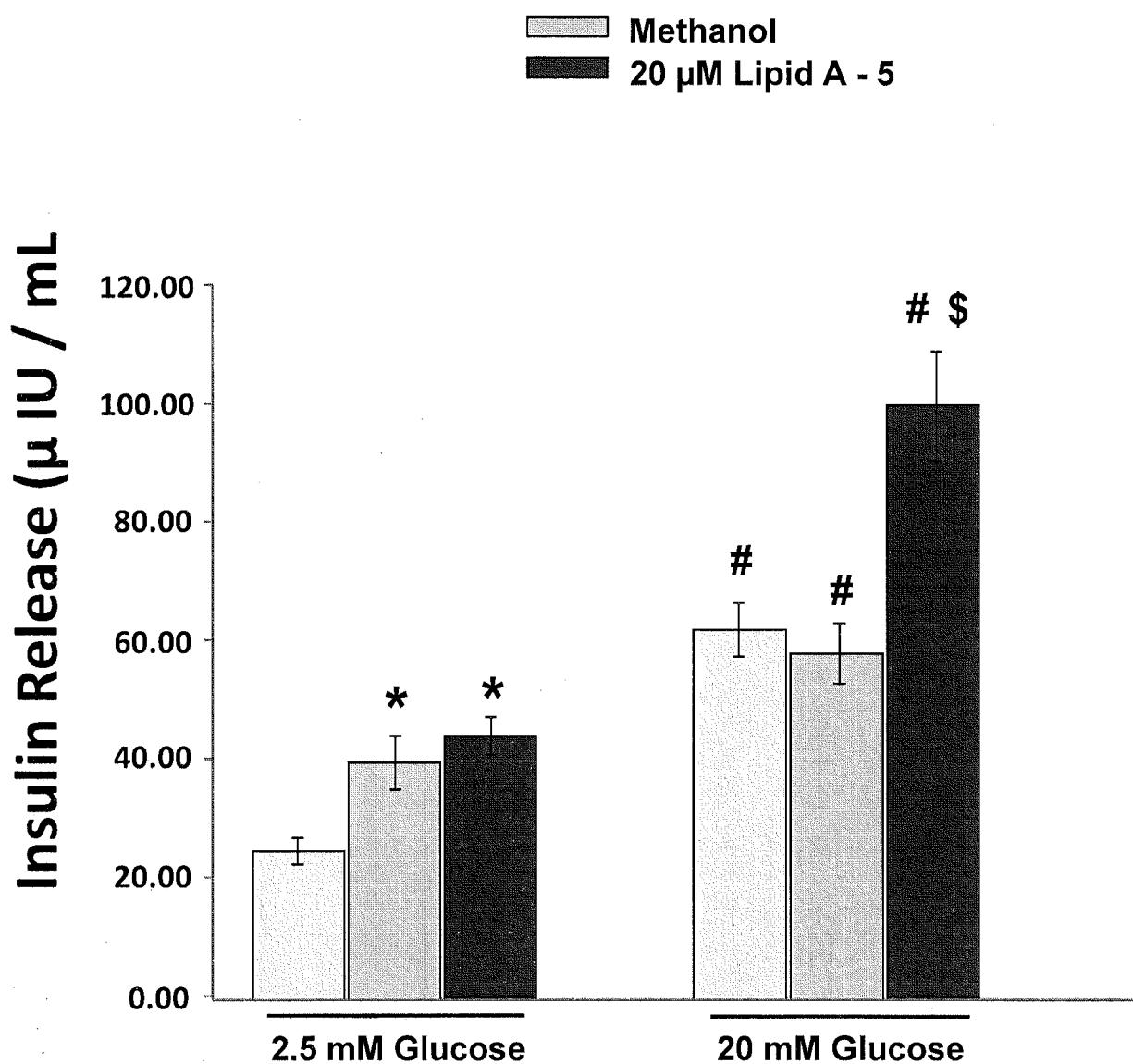


FIG. 48

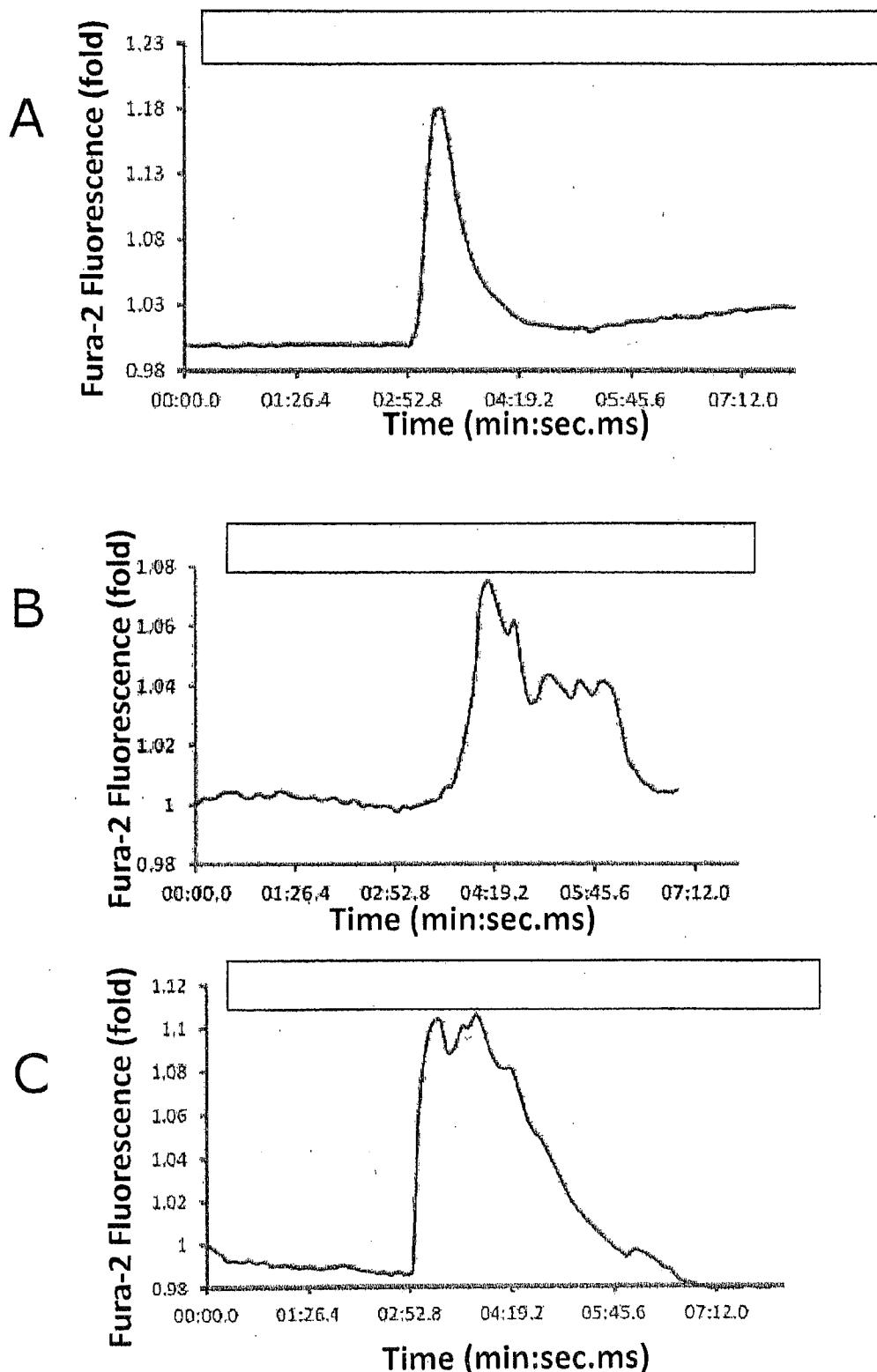


FIG. 49A-C

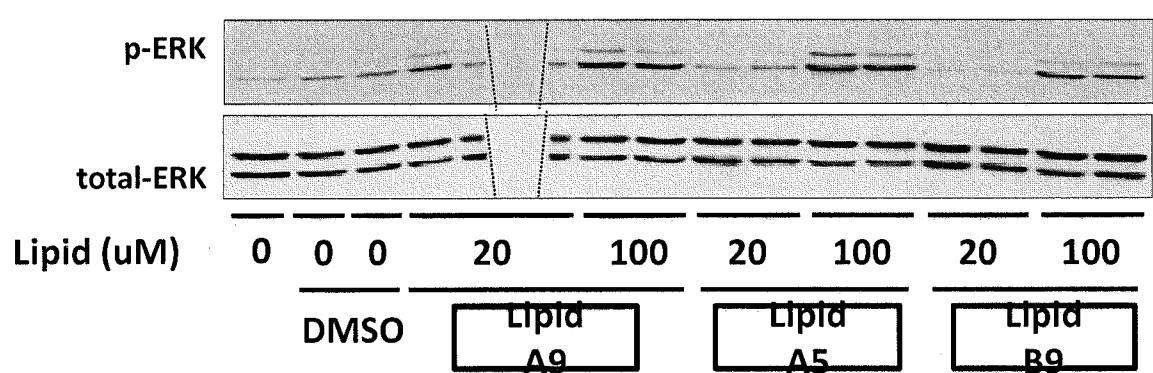


FIG. 50

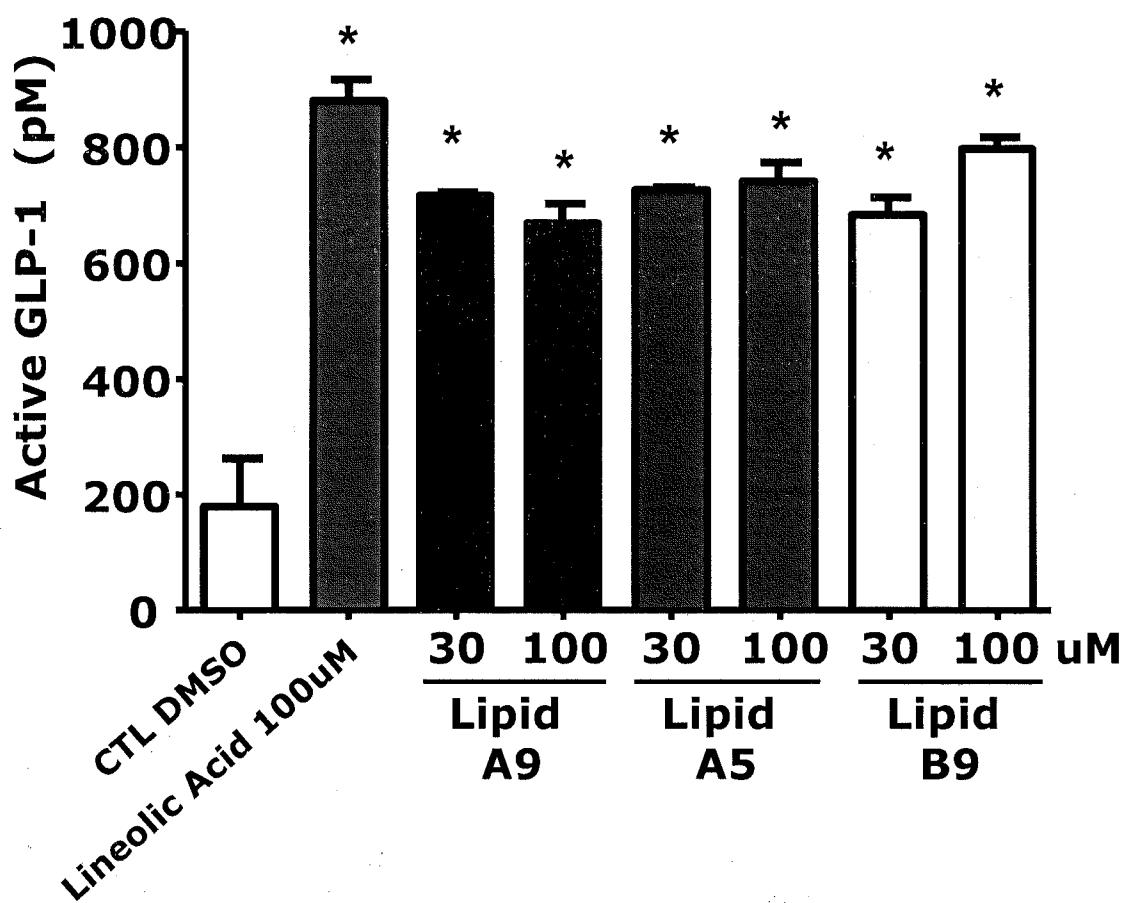


FIG. 51

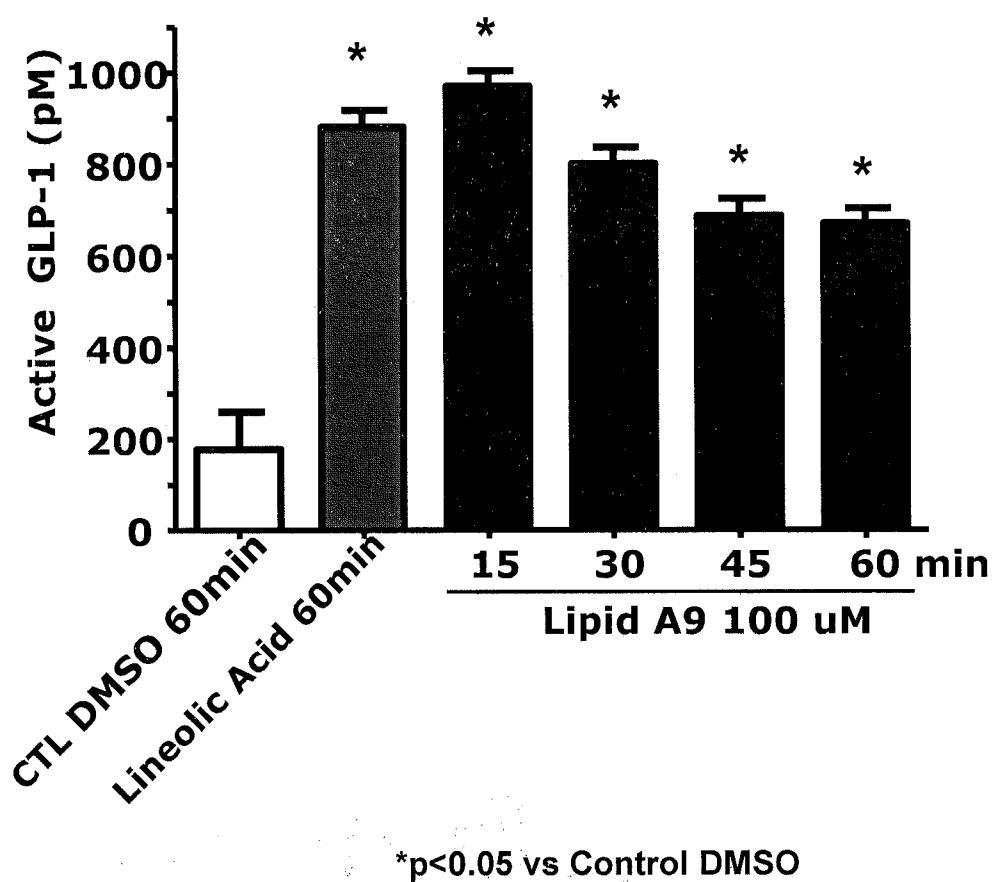


FIG. 52

CHO-GPR120/Beta Arrestin Cells

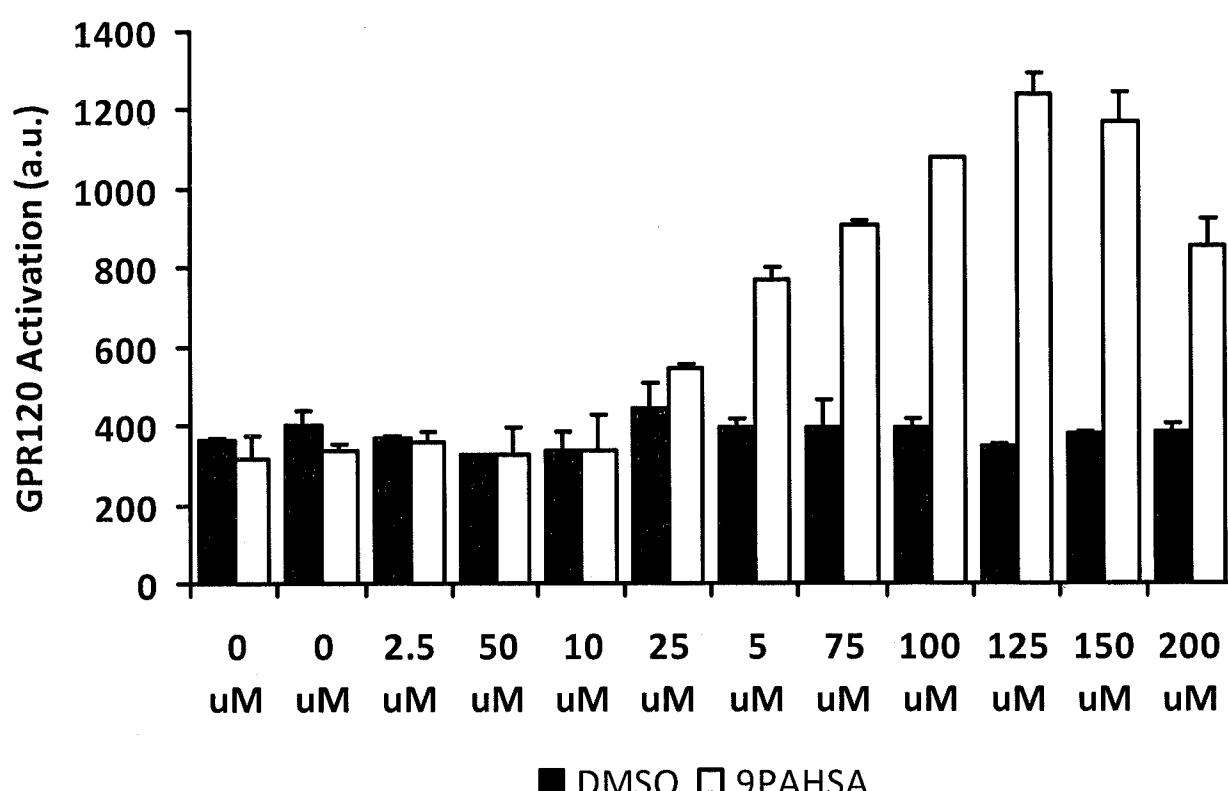


FIG. 53

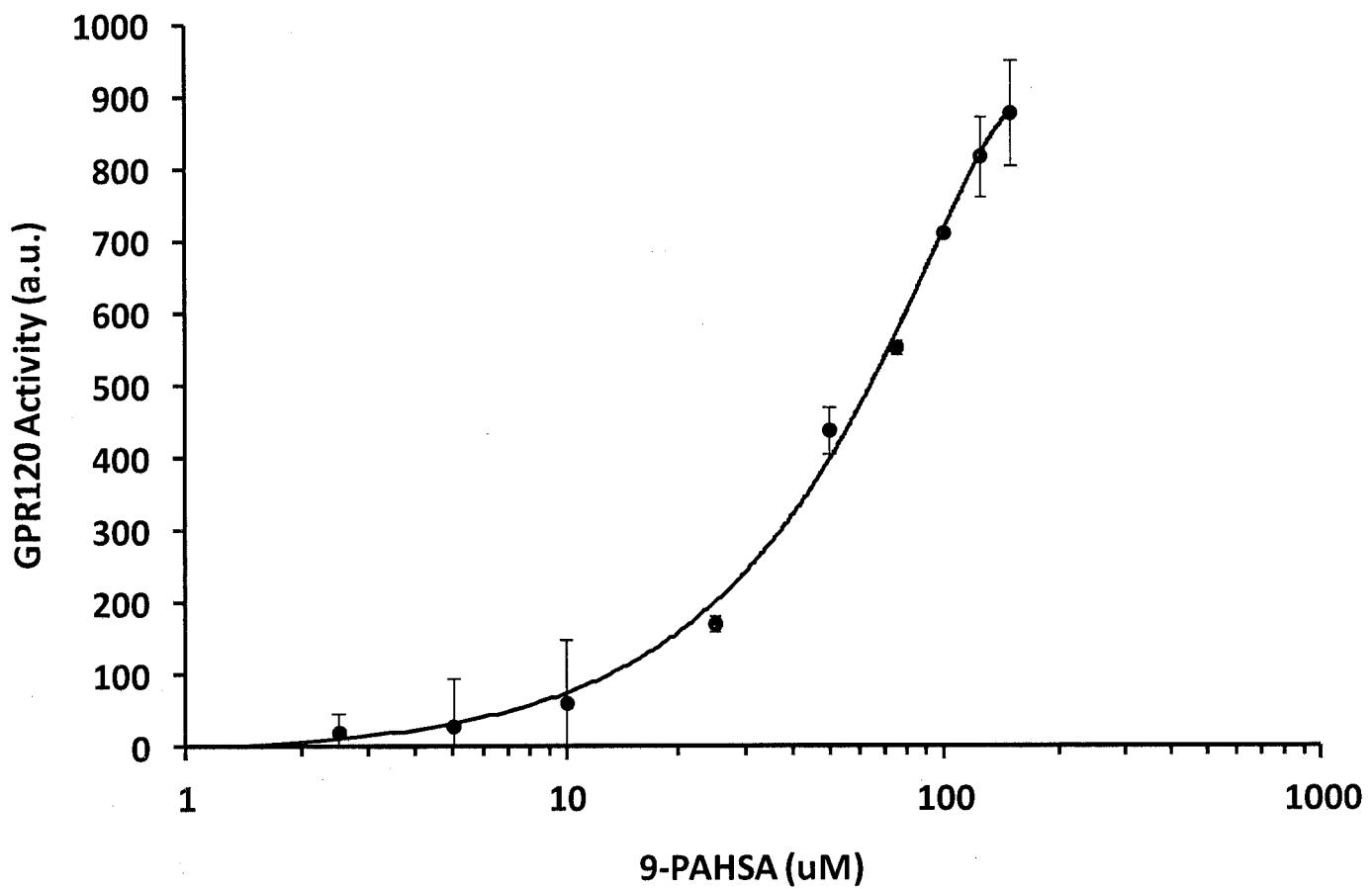


FIG. 54

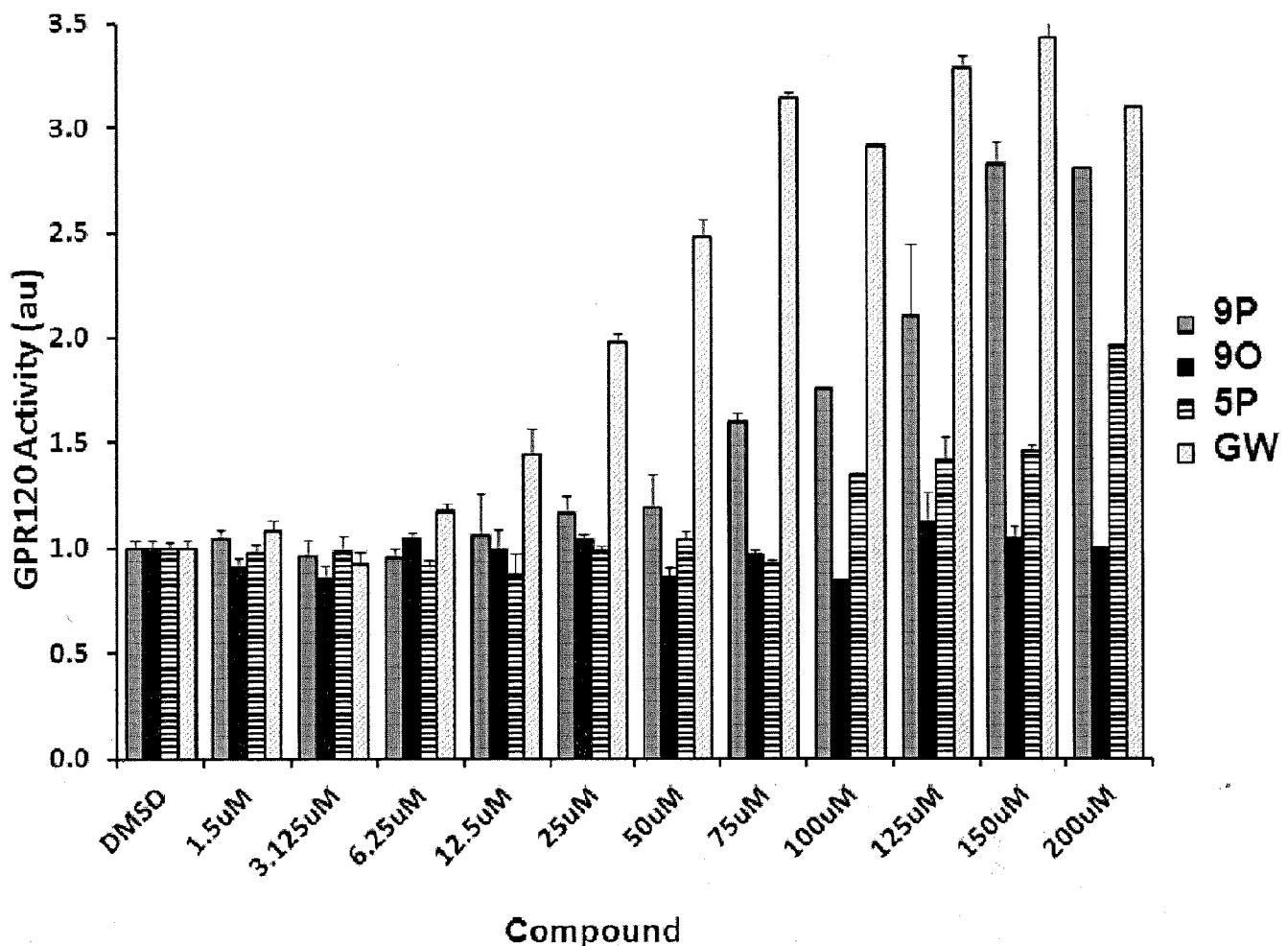
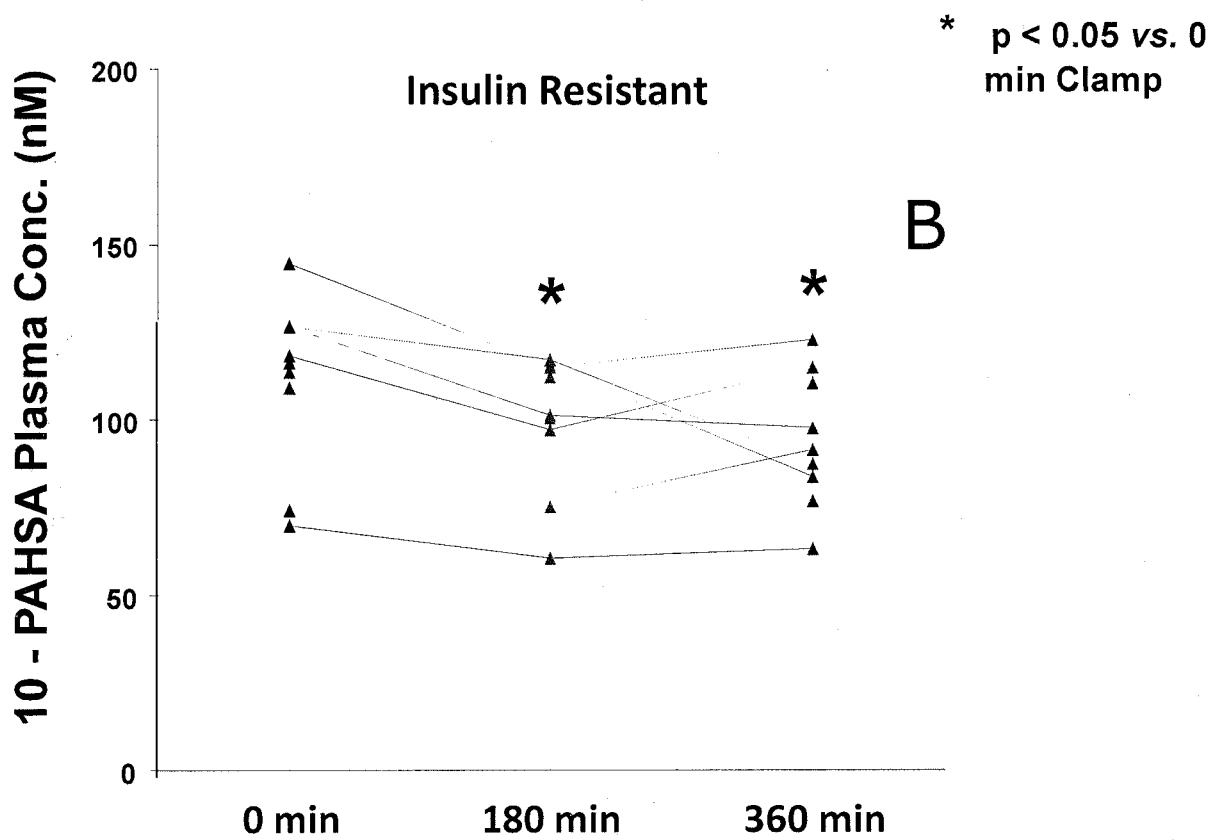
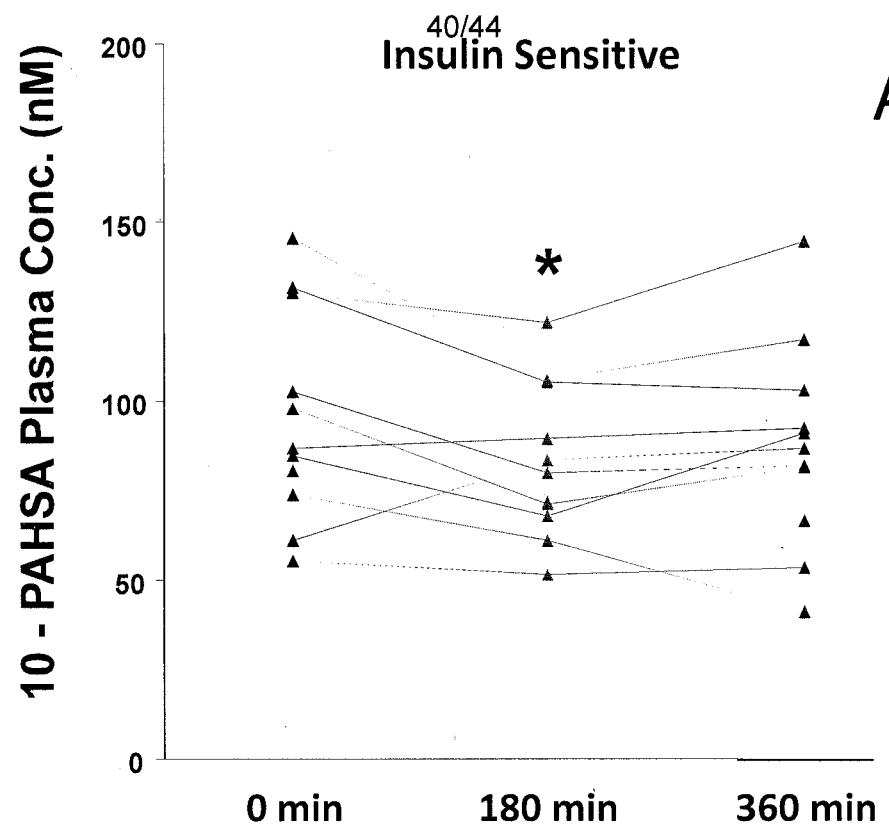


FIG. 55



FIGs. 56A-B

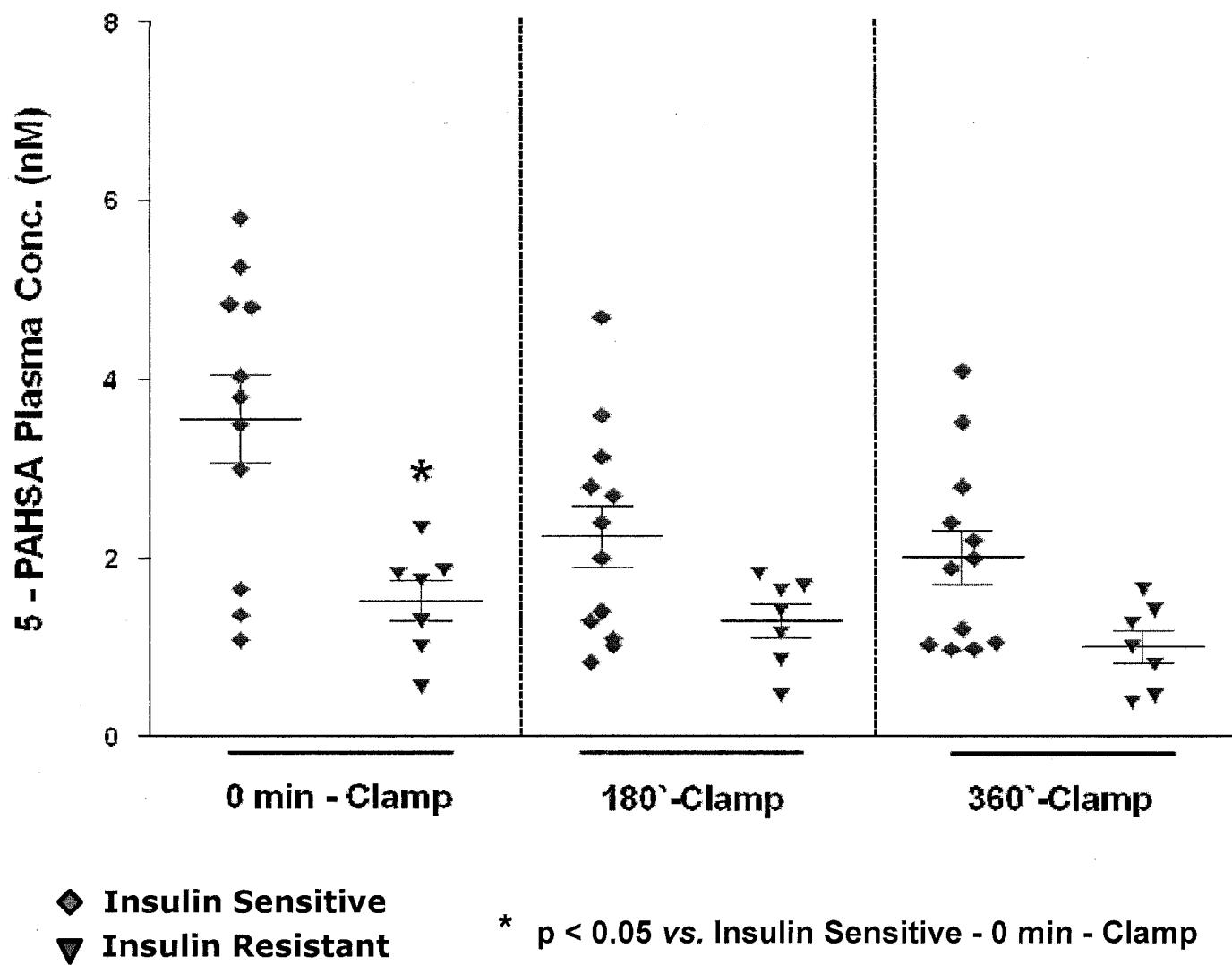
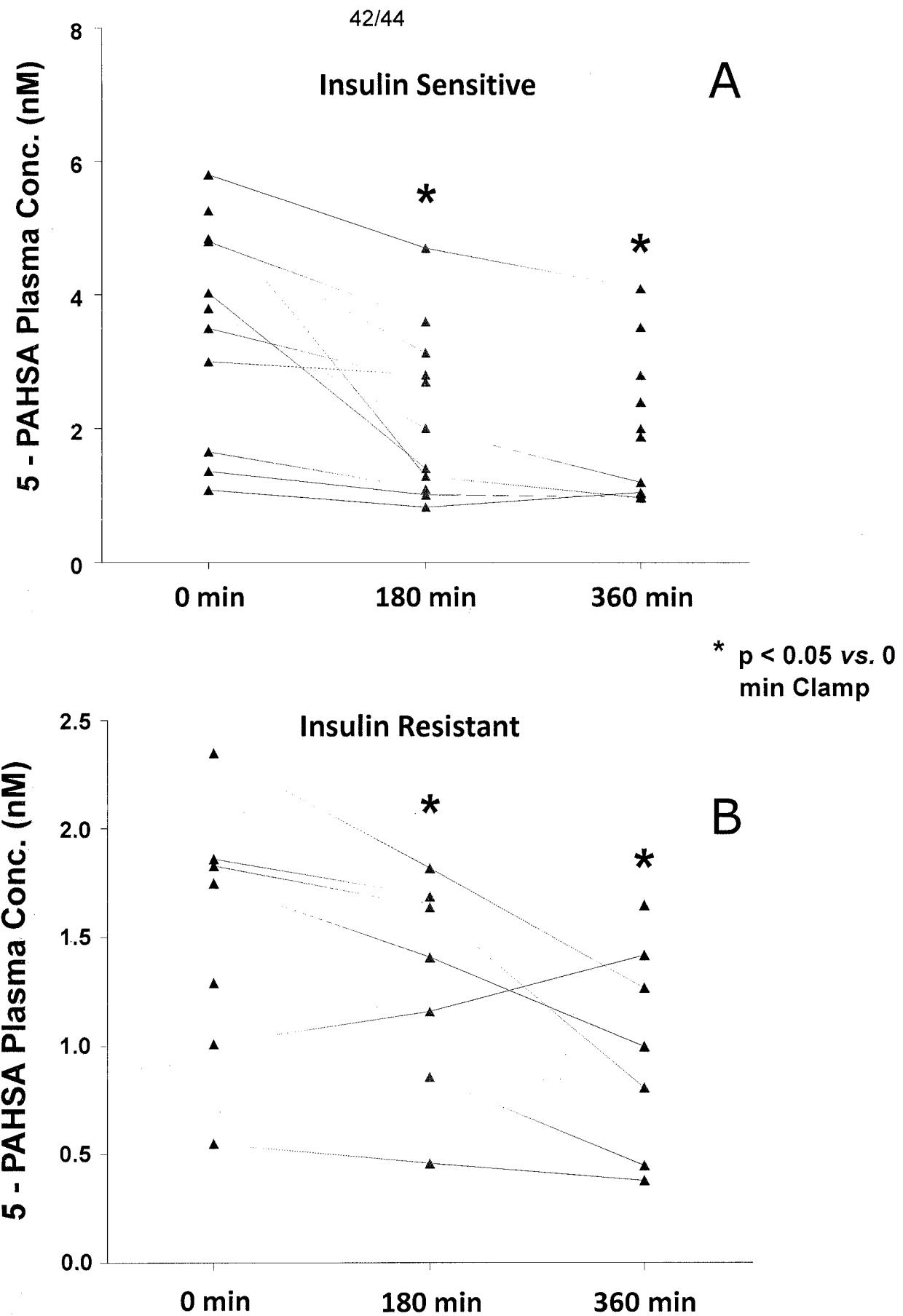


FIG. 57



FIGs. 58A-B

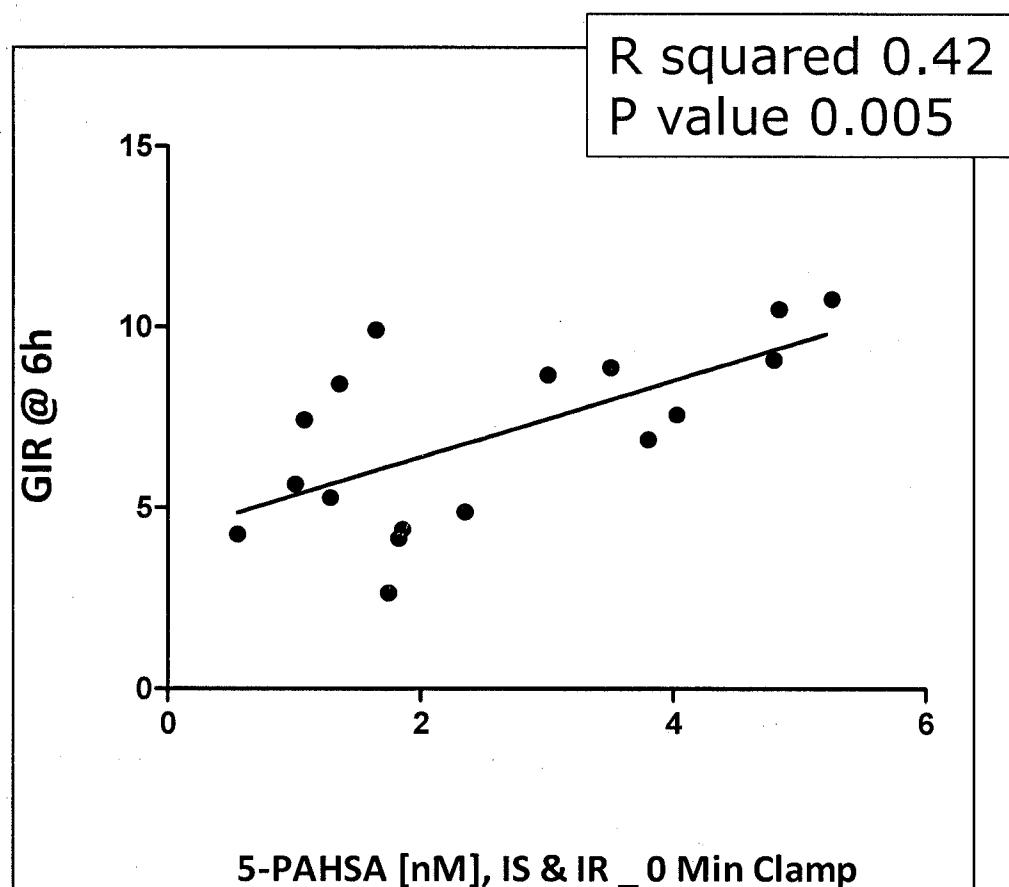


FIG. 59

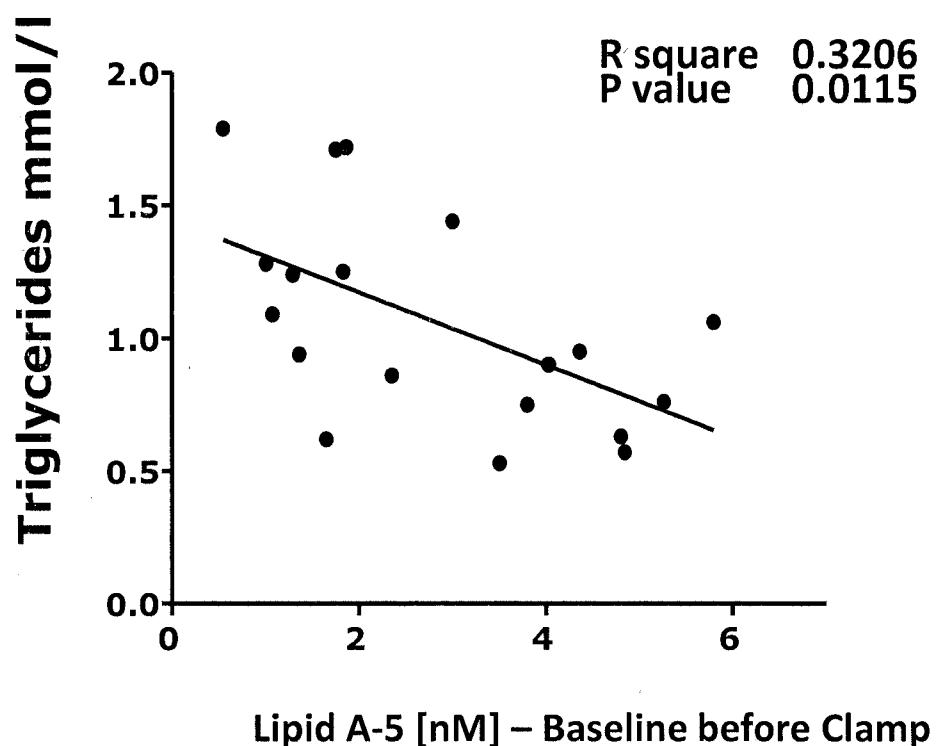


FIG. 60

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference 144 0 . 207 0 0 0 2	FOR FURTHER ACTION <small>see FORM PCT/ISA/220 as well as where applicable, item 5 below.</small>	
International application No. PCT/US 2013 / 039532	International filing date (day/month/year) 03/05/2013	(Earliest) Priority Date (day/month/year) 03/05/2012
Applicant BETH ISRAEL DEACONESS MEDICAL CENTER, INC.		

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 07 sheets.

It is also accompanied by a copy of each prior art document cited in this report.

1. Basis of the report

- a. With regard to the **language**, the international search was carried out on the basis of:
 - the international application in the language in which it was filed
 - a translation of the international application into _____, which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1 (b))
- b. This international search report has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43.6E>s(a)).
- c. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, see Box No. I.
- 2. **Certain claims were found unsearchable** (See Box No. II)
- 3. **Unity of invention is lacking** (see Box No III)
- 4. With regard to the **title**,
 - the text is approved as submitted by the applicant
 - the text has been established by this Authority to read as follows:

5. With regard to the **abstract**,

- the text is approved as submitted by the applicant
- the text has been established, according to Rule 38.2, by this Authority as it appears in Box No. IV. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority

6. With regard to the **drawings**,

- a. the figure of the **drawings** to be published with the abstract is Figure No. _____
 - as suggested by the applicant
 - as selected by this Authority, because the applicant failed to suggest a figure
 - as selected by this Authority, because this figure better characterizes the invention
- b. none of the figures is to be published with the abstract

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2013/039532

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K49/00 A61K31/22 C07C69/22 G01N33/48
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07C A61K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal , CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 993 861 A (FOGEL ARNOLD W [US]) 30 November 1999 (1999-11-30) claim 1 column 9; example 1 ----- JP 2000 226397 A (OTSUKA PHARMA FACTORY INC) 15 August 2000 (2000-08-15) paragraph [0038] ; compound (4) ----- US 4 639 369 A (CIAUDELLI JOSEPH P [US]) 27 January 1987 (1987-01-27) claim 1 column 2; examples 1-3 ----- -/- -	1,5,6 1,2,5,6 1,2,5,6
X		

Further documents are listed in the continuation of Box C.

See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
18 July 2013	02/08/2013

Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Authorized officer Guazzel 1i , Gi udi tta
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INTERNATIONAL SEARCH REPORT

International application No PCT/US2013/039532	
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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HARRY-OKURU R E ET AL: "Synthesis of estolides from cis-9-octadecenoic acid estolides", JOURNAL OF THE AMERICAN OIL CHEMISTS' SOCIETY, SPRINGER, DE, vol. 78, no. 3, 1 January 2001 (2001-01-01), pages 219-222, XP001526490, ISSN: 0003-021X Scheme 1; page 221; compound 1	1-3, 5, 6
X	----- US 2007/092475 AI (WOHLMAN ALAN [US]) 26 April 2007 (2007-04-26) claim 1	1, 5, 6
X	----- US 6 290 973 B1 (HAWKINS LYNN D [US] ET AL) 18 September 2001 (2001-09-18) columns 78-80; example 7 columns 85-86; compound 112048 reactant for reaction 12 to 13; columns 15-16 claim 1	1, 5, 6, 15-18, 20, 21, 23
X	----- WO 94/06014 AI (KLEINFELD ALAN M [US]) 17 March 1994 (1994-03-17) claim 1 page 10, lines 12-23	7, 9, 10, 12, 13
X	----- K. IIZUKA ET AL: "Deficiency of carbohydrate-activated transcription factor ChREBP prevents obesity and improves plasma glucose control in leptin-deficient (ob/ob) mice", AJP: ENDOCRINOLOGY AND METABOLISM, vol. 291, no. 2, 1 August 2006 (2006-08-01), pages E358-E364, XP055060819, ISSN: 0193-1849, DOI: 10.1152/ajpendo.00027.2006 paragraph "Research design and methods"; page E359; table 1	7, 9, 11-19, 24-30, 32-36, 38, 39, 41, 42
X	----- US 2008/015227 AI (KYM PHILIP R [US] ET AL) 17 January 2008 (2008-01-17) pages 9-10, paragraph 0154 - paragraph 0160 page 17; example 10 claim 1	15-17, 19, 24-29, 32-35, 38, 39, 41, 42
	----- -/-	

INTERNATIONAL SEARCH REPORT

International application No	
PCT/US2013/039532	

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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