COMIRB Protocol

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Protocol #: **16-1404**
Project Title: **Skeletal muscle diacylglycerol and sphingolipids – impact of localization and species on insulin resistance in humans**

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I. **Hypotheses and Specific Aims**: The accumulation of muscle lipids are thought to promote insulin resistance and type 2 diabetes. While initially this was associated with triglycerides, bioactive lipids such as diacylglycerol (DAG) and sphingolipids have emerged as important mediators of insulin resistance. Recent data indicate these lipids are located in mixed membrane fractions, however, the exact membranes are unknown. This limited understanding of where DAG and sphingolipids promote insulin resistance is limiting our ability to prevent and treat lipid-induced insulin resistance. We developed a technique to isolate specific muscle membranes from human biopsies, and found DAG and sphingolipids accumulate in the sarcolemma and mitochondrial/ER in obese people with and without type 2 diabetes. DAG and sphingolipids are assumed to decrease insulin sensitivity in the sarcolemma, by activating protein kinase C (PKC) and protein phosphatase 2A (PP2A), respectively. The influence of DAG and sphingolipids on the function and signaling of the mitochondria and ER, which also regulate insulin sensitivity, is not known. There is a *critical need* to address these gaps in knowledge, as altering the cellular distribution of muscle lipids has the potential to ultimately prevent and treat insulin resistance and type 2 diabetes.

Our *long-term goal* is to identify novel therapeutic targets to combat muscle insulin resistance and type 2 diabetes, filling a need not met by current therapies. The *overall objective* for this project is to determine the impact of changes in subcellular DAG and sphingolipid species on cell signaling and metabolic function, before and after insulin sensitizing lifestyle interventions. Our *central hypothesis* is that DAG and sphingolipids in muscle promote insulin resistance via mechanisms that are unique to location, type of lipid, and species. This hypothesis is based on extensive preliminary data (see *Research Strategy* – Approach), showing insulin resistance is related to sarcolemmal and mitochondrial/ER accumulation of specific DAG and sphingolipids. Our pilot studies revealed changes in specific localized species after an insulin sensitizing lifestyle intervention, and that DAG and sphingolipids alter mitochondrial function in vitro. We propose to measure skeletal muscle DAG and sphingolipid isomers, species, localization, and de novo synthesis before and after diet-induced weight loss or exercise training interventions in obese men and women. Comparing the two interventions will uncover differences in lipid localization, cellular signaling, and mechanisms for improvements in insulin sensitivity. Insulin sensitivity will be measured using hyperinsulinemic/euglycemic clamps, muscle lipids using LC/MS, and mitochondrial function using an Oroboros respirometer. The *rationale* underlying the proposed research is that elucidating changes in localized DAG and sphingolipids predicting insulin sensitivity will reveal specific localized lipids to target in therapeutics to prevent or treat type 2 diabetes.

To attain our overall objective, we propose three *specific aims:*

1. **Identify the influence of sarcolemmal DAG and sphingolipids on cell signaling and insulin sensitivity before and after insulin sensitizing lifestyle interventions.**

Strong preliminary data shape our *working hypothesis* that sarcolemmal 1,2-disaturated DAG and C18:0 ceramide species will decrease after insulin sensitizing lifestyle interventions, leading to less PKC and PP2A activation, and enhanced insulin signaling.

2. **Determine the impact of mitochondrial/ER DAG and sphingolipids on mitochondrial function and ER stress in vivo, before and after insulin sensitizing lifestyle interventions.**

We *hypothesize*, again based on preliminary data, that mitochondrial/ER sphingolipids will decrease, yet DAG will increase after insulin sensitizing lifestyle interventions, and each will associate with increased

insulin sensitivity. Changes in sphingolipids will relate to increased mitochondrial function, less ER stress, ROS, and acyl-carnitine formation, while changes in DAG will associate with increased mitochondrial content and dynamics.

3. **Identify the effect of exogenous DAG and sphingolipids on mitochondrial function in vitro, before and after insulin sensitizing lifestyle interventions.**

Our *working hypothesis* is that DAG and sphingolipids will reduce mitochondrial respiration and increase ROS and acyl-carnitine content, yet the response to DAG will be attenuated after endurance exercise training.

II. **Background and Significance**:

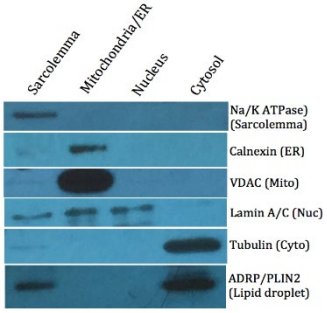
Significance – The intracellular accumulation of DAG and sphingolipids are potential mechanism explaining skeletal muscle insulin resistance in the pathogenesis of type 2 diabetes. DAG and sphingolipids are greater in insulin resistant humans and animal models (1-9), and increase after high fat feeding and lipid infusions that acutely induce insulin resistance (7-9). However, the response of DAG and sphingolipids to insulin sensitizing interventions is variable (10-17), and there are paradoxical studies in the literature dissociating DAG and sphingolipid content from insulin sensitivity (9; 18; 19). These conflicting reports highlight that the current understanding of how and where DAG and sphingolipids induce insulin resistance is incomplete. A weakness of the above studies is analyzing whole muscle concentrations. Recent studies showed that DAG in membranes related to insulin resistance, which is also assumed for sphingolipids (20-23). However, a weakness of these studies is that all membranes were analyzed together and the exact membranes containing lipids promoting insulin resistance are unknown. Sub-membrane localization is important, as a recent study revealed DAG and ceramide in only certain membrane fractions promoted hepatic insulin resistance in mice (24). There are no studies in humans examining what membranes harbor lipids promoting insulin resistance, despite calls in the literature for clarity on this issue (25). Failure to identify bioactive lipid localization related to insulin resistance will prevent understanding how these lipids influence cellular signaling and metabolic function, and limit our ability to intervene in lipid-induced insulin resistance.

The proposed studies will advance the field by identifying which subcellular membrane compartments of DAG and sphingolipids promote insulin resistance, and how these localized lipid species impact cell signaling and organelle function. *These novel contributions will be significant because they will reveal the key species and locations of DAG and sphingolipids, as well as mechanisms explaining accumulation that could be modified by insulin sensitizing therapeutic interventions.* Identifying specific localized DAG and sphingolipids species contributing to muscle insulin resistance will drive the field of lipid-induced insulin resistance forward in several ways. First, it will lay the foundation for more focused prevention and treatment therapies targeted to the localized species that promote insulin resistance. Second, these results will direct strategies to decrease localized DAG and sphingolipid content by identifying the extent to which de novo synthesis explains membrane accumulation. Third, this project will change the way pharmaceuticals are screened to reduce DAG and sphingolipids by focusing on changes in specific species and locations. This approach is expected to reveal novel therapeutic compounds that cannot be identified by focusing on whole cell content. Together, these advances will address an unmet clinical need to improve muscle insulin sensitivity, and reveal novel treatment and prevention strategies for diabetes.

III. **Preliminary Studies/Progress Report:**

Preliminary Studies

We can fractionate muscle into multiple membrane compartments. We are uniquely suited for this project because we spent 3 years developing a method to separate frozen human skeletal muscle into fractions enriched in sarcolemma, mitochondria/ER, nuclear, and cytosolic compartments (Figure 1). This technique is a substantial advance, as the most recent studies only separate cytosol from mixed membranes, which cannot discriminate specific organelles containing lipids promoting insulin resistance. Lipids are then extracted from these fractions and analyzed using our lipidomic platform.



Sarcolemmal 1,2-Disaturated DAG species relate to insulin resistance. The sum of all sarcolemmal di-saturated 1,2-DAG species were significantly greater in T2D compared to lean volunteers (Figure 2A), and was inversely related to insulin sensitivity (Figure 2B). Notably, 1,3-disaturated DAG content was not different between groups revealing isomer specificity. We previously showed that membrane di-saturated DAG content predicted PKC activation and insulin resistance (26). These data agree and suggest that sarcolemmal DAG containing saturated acyl side chains represent membrane accumulation of DAG that is particularly negative for insulin sensitivity.

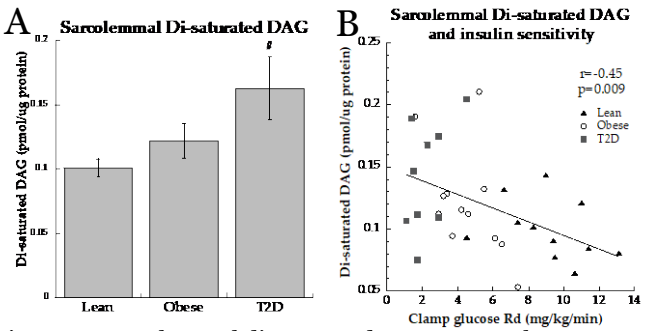


Figure 2. Sarcolemmal di-saturated DAG content by group (A), and by insulin sensitivity (B) in lean, obese, and type 2 diabetics. Values are means +/- SEM. #= significantly different than lean, p<0.05.

PKCε activity is increased in obesity and T2D. We measured the membrane/cytosol ratio of PKCε, which is commonly used as a surrogate for PKC activity. We found significantly greater PKCε activation in obese and T2D subjects compared to lean controls (Figure 3), with no significant differences found for PKCβII, δ, and θ isoforms. These data are consistent with greater PKC activation from sarcolemmal DAG accumulation.

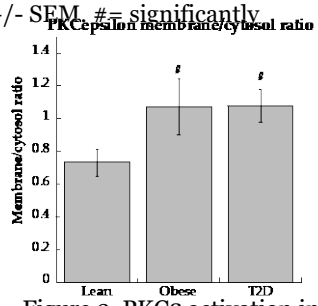


Figure 3. PKCε activation in lean, obese, and type 2 diabetics. Values are means +/- SEM. #= significantly different than lean, p<0.05.

Sarcolemmal C18:0 ceramide accumulates in insulin resistance. Of the 9 sarcolemmal ceramide species measured, only C18:0 ceramide was significantly greater in T2D compared to lean (Figure 4A), and was inversely related to the rate of glucose disappearance during an insulin clamp (Figure 4B). In a pilot study sarcolemmal C18:0 ceramide decreased by 21% after a combined weight loss and exercise training intervention. These data parallel a recent publication by our lab showing C18:0 muscle ceramides significantly related to insulin resistance in a different cohort (27). C18:0 ceramide antagonizes an inhibitor of PP2A, resulting in increased PP2A activity (28). Combined, these data suggest that sarcolemmal C18:0 ceramide may promote insulin resistance, and due to the location may explain the canonical pathways of ceramide induced insulin resistance, including activation of PP2A activity, and inhibition of AKT phosphorylation in response to insulin.

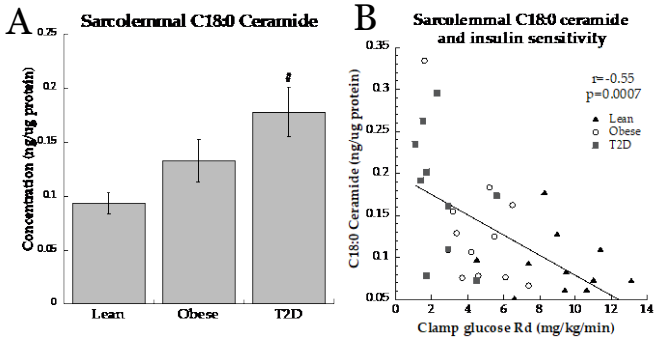
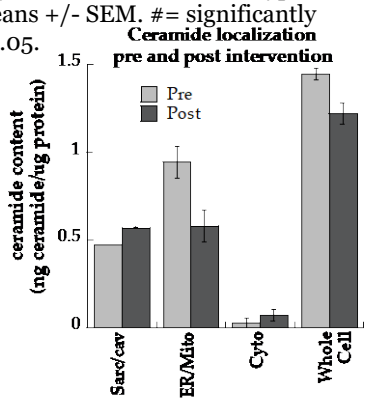


Figure 4. Sarcolemmal C18:0 ceramide content by group (A), and by insulin sensitivity (B) in lean, obese, and type 2 diabetics. Values are means +/- SEM. #= significantly different than lean, p<0.05.

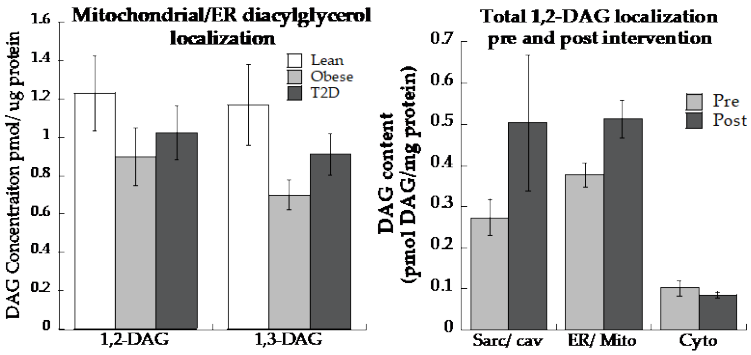
Mitochondrial/ER ceramide accumulates in insulin resistance. Mitochondrial ceramide content was greater in T2D compared to lean individuals, which is largely explained by C22:0 and C24:0 species (Figure 7A). Total ceramide content was inversely related to insulin sensitivity (Figure 7B). These data are consistent with the hypothesis that mitochondrial ceramides promote insulin resistance.

Mitochondrial/ER ceramides decrease after insulin sensitizing lifestyle intervention - Pilot data for a combined weight loss/exercise training intervention show decreased whole cell ceramide content, as shown before (11; 15) (Figure 8). However, the decrease in whole cell ceramide content comes only from a decrease in mitochondrial/ER ceramide. These data point to an important role of mitochondrial/ER ceramides on insulin sensitivity in vivo.

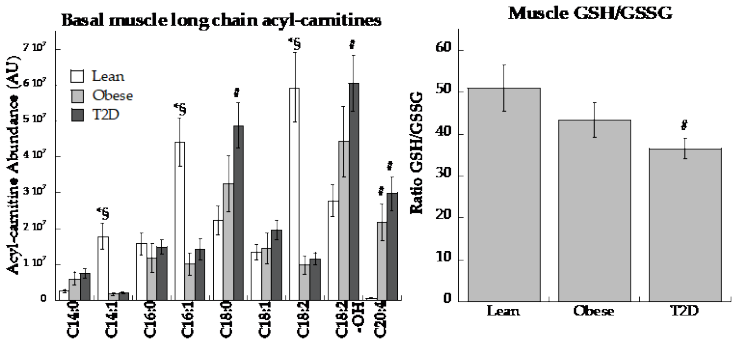


Mitochondrial/ER DAG accumulates in lean insulin sensitive individuals. Preliminary data show mitochondrial/ER 1,2- and 1,3-DAG were

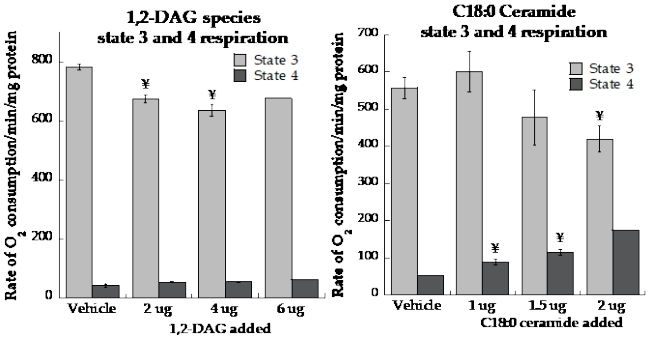
higher in lean individuals compared to obese or type 2 diabetic volunteers (Figure 9), with the greater DAG content distributed over most species. These data are counterintuitive, but may reflect the greater content of mitochondria in lean individuals compared to obese volunteers with and without diabetes, and/or increased mitochondrial fission/fusion (29; 30).



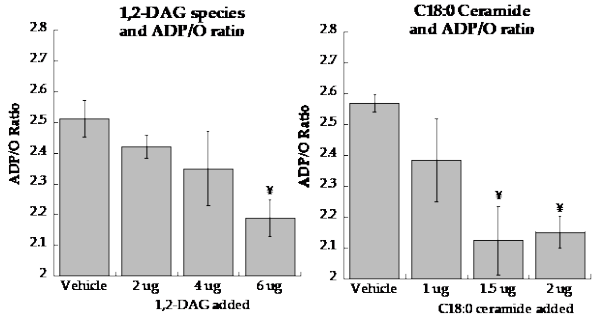
Mitochondrial/ER DAG increase after weight loss and exercise training. Preliminary data from a weight loss and exercise training intervention show an increase in mitochondrial/ER 1,2-DAG after the intervention (Figure 10). These data are consistent with some reports in the literature where DAG concentration was increased in endurance trained athletes (13). These data agree with Figure 9 and suggest mitochondrial/ER DAG accumulation may be a previously unrecognized adaptation to exercise training and/or weight loss.



Insulin resistant muscle accumulates long chain acyl-carnitines and shows oxidative stress. Basal acyl-carnitines revealed accumulation of many long chain acyl-CoA's in type 2 diabetics including C18:0, C18:2-OH, and C20:4, which are thought to promote insulin resistance (Figure 11A). A significantly decreased GSH/GSSG ratio in type 2 diabetes individuals compared to lean volunteers suggest a more oxidized state, consistent with increased oxidative stress in insulin resistance (Figure 11B).



DAG and ceramide decrease mitochondrial function. We administered 1,2-DAG, ceramide, dihydroceramide, and vehicle controls to isolated rat mitochondria in vitro to evaluate the impact on mitochondrial respiration using pyruvate and malate as substrates. DAG and ceramide decreased state 3 respiration, with no changes observed with dihydroceramide administration (Figure 12A and B). State 4 respiration did not change for 1,2-DAG and dihydroceramide, but increased significantly with increasing doses of ceramide. Both 1,2-DAG and ceramide decreased ADP/O ratios (Figure 13A and B), while dihydroceramide had no effect. These data indicate 1,2-DAG and ceramide reduced mitochondrial respiration and coupling, and are consistent with accumulation of mitochondrial lipids altering mitochondrial function.



IV. Research Methods

A. Outcome Measure(s):

Primary outcomes:

Aim 1: Change in insulin sensitivity, change in sarcolemmal Di-C18:0 DAG and C18:0 ceramide, change in PKCepsilon and PP2A activity

Aim 2: Change in insulin sensitivity, change in mitochondrial/ER total DAG and ceramide content, change in state 3 mitochondrial respiration, change in GSH/GSSG, change in C18:0, C18:2-OH, and C20:4 acylcarnitines, change in GRP78

Aim 3: Change in mitochondrial state 3 and state 4 respiration in response to DAG and ceramide before and after lifestyle interventions

Secondary outcomes: Changes in other localized lipids and intracellular responses will be exploratory in nature. In addition, we will collect subjective and objective information about sleep and an exploratory analysis.

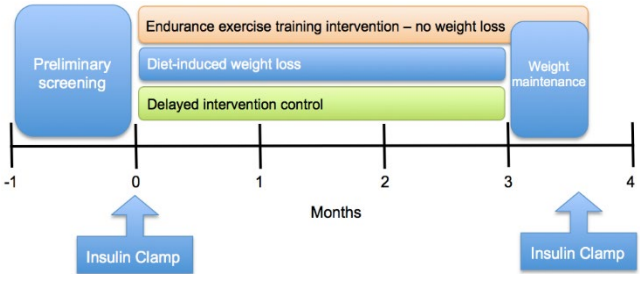
B. Description of Population to be Enrolled:

Obese men and women (BMI 30-40 kg/m^2) will be recruited for this study who are between 30-50 years of age and sedentary (<2 hours/week of planned physical activity). Individuals with normal glucose tolerance (NGT), pre-diabetes, and newly diagnosed type 2 diabetes will be enrolled. Normal glucose tolerance is defined as a HbA1c of <5.7%, pre-diabetes as a HbA1c of 5.7-6.4%, and type 2 diabetes as a HbA1c of ≥6.5% (31). Oral contraceptive use is allowed as long as women don't change their use, or lack thereof, during the course of the study. Exclusion criteria include: regular exercise more than 2 hours/week, medications that could affect glucose, lipid metabolism, thyroid disease, a history of lung disease, pregnancy, active cigarette smoking and severe plasma lipid disorders.

Inclusion criteria:

BMI: 30-40 kg/m^2
Age: 30-50 years
Planned physical activity: <2 hrs/week
Glucose tolerance: NGT, pre-diabetes, and Type 2 diabetes
Normal glucose tolerance (NGT) is defined as HbA1c of <5.7%, pre-diabetes as HbA1c of 5.7-6.4%, and type 2 diabetes as HbA1c of ≥6.5% (31).
Oral contraceptive use: Yes or No as long as there is no change during the study
Medications: not taking TZD's, insulin
Thyroid status: TSH between 0.5-5.0 mU/L
Pregnancy status: Not pregnant
Nonsmoker (tobacco and any form of marijuana use)
Fasting TG <400mg/dl
FSH < 20 IU/L

C. Study Design and Research Methods



Ninety-two individuals (30-50 years old) will be screened to recruit sixty-nine obese men and women into the study. Individuals with and without pre-diabetes will be recruited to achieve a range of starting insulin sensitivities without confounding effects of medications used to treat type 2 diabetes. Volunteers will undergo an insulin clamp before and after block randomization by sex and pre-diabetes strata equally across intervention groups, into a three month intervention consisting of: 1) endurance exercise training without weight loss, 2) diet-induced weight loss, or 3) delayed intervention control (Figure 5). The three month intervention will result in significant gains in fitness, weight loss, and improvements in insulin sensitivity, but is short enough to maximize retention.

Exercise training and weight loss will differentially change DAG and sphingolipid content (11), revealing distinct changes in lipid localization, cellular signaling, metabolic function, and improvements in insulin sensitivity. We are expecting a 25% dropout rate, and are powered to complete testing on 17 individuals per group (51 total individuals). Subjects will be tested 48 hours after the last exercise bout to minimize acute exercise effects on insulin sensitivity. Women will be pre-menopausal and tested during the follicular phase (days 1-10) of the menstrual cycle to control for effects on insulin sensitivity.

Preliminary screening- Subjects will arrive after an overnight fast to the outpatient Clinical Translational Research Center (CTRC), and a fasting blood sample will be taken for a standard lipid panel, fasting glucose, TSH, and HbA1c. Women will take a pregnancy test. A physician will perform a history and physical, and subjects will fill out a dietary questionnaire, the NIH Dietary History Questionnaire II (DHQII), and the IPAQ physical activity questionnaire (33).

Strength and Exercise testing, body composition, maximal mitochondrial respiration, and MRI – Eligible subjects will undergo a Kin-Kom isokinetic dynamometer quadriceps strength test, Balke treadmill test to measure maximal work capacity (VO_2max) (34), a DEXA scan to determine body composition, a non-invasive test of maximal mitochondrial respiration, and MRI scan to measure leg muscle and fat mass, and liver fat content. During the maximal mitochondrial respiration test, volunteers will lie down on a table, and we will have a near infrared spectrometer measurement unit strapped to their leg. This unit uses a laser to measure the oxygen levels in the muscle. A blood pressure cuff will be wrapped around their upper thigh. To start the test, we will ask volunteers to make a muscle contraction for 20 seconds, after which we will intermittently inflate and deflate the blood pressure cuff every 5-10 seconds over a 5 minute period. This test will then be repeated. The total time for this test is 30 minutes.

Dietary monitoring – We will provide food for all volunteers for 3 days prior to each insulin clamp study. The diet will be designed to replicate their typical diet. An estimate of habitual fat consumption will be obtained from red blood cell phospholipid composition (35; 36), and the DHQII. Saturated fat intake will be used to determine if there is a relationship to DAG and sphingolipid saturation between individuals.

Sleep monitoring – Subjects will wear small wrist activity and light exposure recorder (Actiwatch, Mini Mitter, Bend, OR) throughout the study. The Actiwatch is a small and light device about the size of a watch that uses accelerometer-derived information to estimate sleep and wake times. It should not be worn swimming or in the shower.

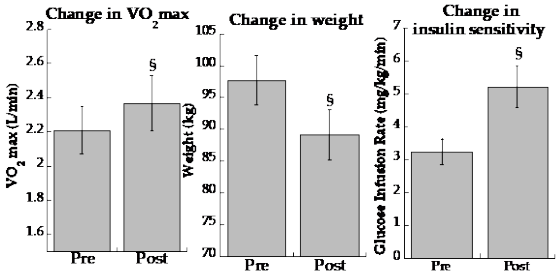
Insulin clamp study - Subjects will spend the night on the inpatient CTRC to ensure compliance with the overnight fast. During the overnight stay we will administer standardized sleep questionnaires that ask about subjective sleep quality, sleep latency, number of awakenings, sleep duration, factors that may have disturbed sleep and a sleepiness rating. We will use Compumedics Inc Siesta digital sleep recorders to assess polysomnographic (PSG) data. Data will be stored and sampled at a rate of 256 samples per second per channel with a 12-bit A-D board. High and low pass digital filters for electroencephalogram (EEG) and electroocculogram (EOG) will be set at 0.25 and 35 Hz. Monopolar EEGs-International 10-20 system using current guidelines (F3xM2, C3xM2, F4xM2, C4xM1, and O2xM1 O1xM2). Sleep will be scored according to standard guidelines. Impedances below 5 kohms will be obtained. Sleep data will be scored to describe how the treatments influence sleep. Oronasal thermal sensor, nasal pressure, pulse oximiter, thorasic and abdominal plethysmography, ECG, and bilateral bipolar anterior tibialis electromyogram (EMG) recordings will be used to measure sleep disturbances (Apnea Hypopnea Index (AHI), Periodic Leg Movements (PLM)).

The following morning, an antecubital catheter will be placed in one arm for infusions, and another placed in a distal hand vein in the contra-lateral arm for blood draws using the heated hand vein technique (37). After 30 minutes of rest, a baseline blood sample will be taken for metabolic substrates, hormones, and cytokines. Thereafter, a primed (0.03mg/kg), constant infusion (0.04mg/kg/min) of [6,6-$^{2}H_2$]glucose and [U-^{13}C]glucose, as well as a constant infusion (0.03μmol/kg/min) of [U-^{13}C]palmitate will begin. The glucose and palmitate stable isotope infusions will continue through the end of the study and will be used to determine hepatic glucose production, peripheral glucose disposal, and muscle *de novo* DAG and sphingolipid synthesis. After 2 hours of infusion, a percutaneous needle biopsy will be taken midway

between the greater trochanter of the femur and patella. Then a standard 3 hour hyperinsulinemic/euglycemic clamp will follow as described by DeFronzo with insulin infused at 40 mU/m^2/min, and glucose clamped at 90mg/dl with a variable dextrose infusion (38). Dextrose infused to maintain euglycemia will be "spiked" with [6,6-$^2H_2$]glucose to minimize changes in isotopic enrichment. Blood will be sampled every 5 minutes to determine glucose concentration and the dextrose infusion adjusted as necessary. A second muscle biopsy will be taken from the contra-lateral limb 1 hour into the insulin clamp to measure insulin-stimulated insulin signaling. Blood samples will be taken over the final 30 minutes for substrate, hormone, and cytokine analyses. Isotopically measured glucose rate of disappearance will be measured during the last 30 minutes of the 3 hour clamp, normalized to plasma insulin concentration, and used as a measure of whole body insulin sensitivity (39).

Muscle biopsy – Subcellular fractionation will be performed as described in Figure 1. Diacylglycerol (1,2- and 1,3- isomers), sphingolipids (dihydroceramide, ceramide, glucosylceramide, lactosylceramide, sphingosine, sphingosine-1-phosphate, sphingomyelin), triacylglycerol, phosphatidic acid, and long chain acyl-CoA species will be analyzed by Dr. Kathleen Harrison using the lipidomics platform in our laboratory. Subcellular fractions are fortified with internal standards (ISs), lipid extracted, and analyzed by an Agilent 1100 HPLC connected to an API 2000 triple quadrupole mass spectrometer (40). The 1,3- and 1,2- DAG isomers are separated chromatographically using a Hilic 2.1 micron, 3x100mm column. Concentration is determined by comparing ratios of unknowns to odd chain or deuterated internal standards. Incorporation of [U-^{13}C]glucose into DAG glycerol, and [U-^{13}C] palmitate into the sphingolipid backbone will be used to estimate *de novo* synthesis of DAG and sphingolipids (41; 42). Western blot analysis will quantify PKC translocation of θ, ε, βII, and δ isoforms in basal biopsies, as well as insulin signaling in basal and insulin stimulated biopsies including AKT^{ser473} /total, IRS-1^{Tyr612}/total, and AS160^{Thr642}/total using standard techniques (43). AKT^{Thr34} phosphorylation will be used to estimate PKCζ activation by ceramide. PP2A activity will be measured using manufacturer instructions (Ser/Thr Phosphatase Assay Kit 1, Billerica, MA).

After the baseline clamp, subjects will be randomized into one of three interventions for three months:



1. Endurance exercise training - Participants will undergo supervised endurance exercise training administered by the Nutrition Obesity Research Center (NORC) Energy Balance Core (see attached letter of support from Dr. Kohrt) using well-established methods (44-48). Participants will perform supervised exercise 4x/week as well as exercise 1x/week on their own. Each 60 min session will include a short warm-up, 40 to 50 min of endurance exercise, and a cool-down. The exercise program will consist primarily of brisk walking or jogging, and will be supplemented with rowing, stepping, or elliptical exercise. The initial exercise prescription will be 30 minutes at 65% of maximal HR. During the first 2 weeks of training, exercise duration and intensity will be gradually increased to 45 min at 80 to 85% of maximal HR. This exercise training program resulted in a 9% increase in VO_2max in our pilot study (Figure 6A). Participants will be weighed weekly, and will be instructed to consume more food to prevent weight loss during this intervention. Maximal exercise testing will be performed half way through the intervention, and exercise prescriptions updated every 2 weeks.

2. Diet-Induced Weight loss - The weight loss program will be administered by the Clinical Core of the NORC (see attached letter of support from Dr. Bessesen). Participants will receive a low calorie diet consisting of 4-5 servings of vegetables per day and 4-5 meal replacements (Health Nutrition Technology Inc., Carmel California). No other food intake will be allowed. To reduce the risk of gallstone formation subjects will consume 2 teaspoons (10g) per day of vegetable oil. This diet will provide 890 kcal/d, 75 g of protein, 15 g fat and 110 g of carbohydrate and 100% of the DRI of all vitamins, minerals and micronutrients. This meal replacement program was used in our pilot study with an average 9% weight loss and 62% increase in insulin sensitivity in in 12 weeks (Figures 6B and 6C). Subjects will be weighed

weekly and will meet bi-weekly in group sessions to receive nutritional/behavioral counseling. After the 3-month intervention, subjects will transition to their normal diet for 2 weeks of weight maintenance, supervised by research dieticians with extensive experience helping subjects maintain a weight-reduced state.

3. Delayed intervention control – This group of individuals will serve as our free-living control group, and ensure none of the changes observed in the diet-induced weight loss or exercise training interventions are due to receipt of health information, exercise testing, or contact with investigators. After finishing the study, they can choose to undergo the weight loss, exercise training, or combination intervention.

After finishing the intervention, participants will repeat the maximal exercise testing, MRI scan, DEXA measurement of body composition, sleep recording and the insulin clamp study after 2 weeks of weight stability on their typical diet.

Muscle biopsy specimens from specific aims 1 and 2 will be used for this aim. Fresh muscle biopsies samples will be permeabilized for mitochondrial respiration studies, as well as homogenized for mitochondrial respiratory chain complex activity. Measurements shown in Table 2A will be performed with and without the addition of increasing doses of specific 1,2- and 1,3-DAG and sphingolipid species, as shown in Table 2B, before and after each intervention. Ceramide and dihydroceramide will be administered as an equal mix of C18:0, C24:0, and C24:1 species, while 1,2- and 1,3-DAG will be an equal mix of Di-C18:0, C16:0/C18:0, and C16:0/C18:1 species. In addition to vehicle controls, we will also administer triolein to ensure responses observed are not due to a general effect of lipids on mitochondrial function.

Mitochondrial Respiration - All experimental measurements of oxygen consumption will be determined by a high-resolution respirometry Oxygraph-2K (OROBOROS Instruments, Innsbruck, Austria, see attached letter of support from Dr. MacLean). Measurements will be performed at 37 °C and respiration rates expressed in nmolO2·min−1 ·mg·dry wt−1. Respiration will be normalized to vehicle control, since basal respiration rates will vary based on training status and quality of muscle fibers from the biopsy. Permeabilization will be carried out directly in an oxygraph chamber with saponin (20 µg/mL) for 5 min before starting the measurements as previously described (49). To evaluate the in vivo condition, mitochondrial respiration in permeabilized muscle will be determined with glutamate + malate (GM), octanoyl carnitine + malate(OM), and succinate as respiratory substrates (Table 2A). In the presence of either GM or OM, respiration is initiated with a saturating concentration of ADP (2 mM) and the Vmax of maximal ADP-stimulated respiration rate or state 3 respiration rate is determined. To evaluate the influence of DAG and sphingolipids on mitochondrial respiration, sphingolipids in isopropyl alcohol (1ul in 2ml respiration buffer), DAG in ethanol (1ul in 2ml respiration buffer), or vehicle controls are then added serially to measure affects of low, medium, and high lipid content. The intactness of mitochondrial membranes will then be validated by cytochrome c tests for each experiment. Only samples passing this quality test will be used further. Oligomycin is then given to measure mitochondrial proton leak. FCCP will then be titrated to uncouple the mitochondria and a maximal rate of the electron transport system rate determined. Antimycin A (10 µM) is then added to determine the rate of residual oxygen consumption (Rox). Respiration rates are then corrected for Rox. Respiration kinetics of GM and OM will also be determined by adding ADP

Table 2A. Mitochondrial respiratory substrate plan for permeabilized fibers

Respiratory substrates + ADP	Respiratory substrates - ADP
• Glutamate + malate • Octanoyl carnitine + malate • Succinate	• Succinate +H_2O_2 production

Table 2B. Treatments for permeabilized fibers

No treatment
Vehicle control
Lipid control -Triolein
Low (1 ug/ml)
Medium (2 ug/ml)
High (4 ug/ml)
Ceramide
Low (1 ug/ml)
Medium (2 ug/ml)
High (3 ug/ml)
1,2-DAG
Low (1 ug/ml)
Medium (2 ug/ml)
High (4 ug/ml)
1,3-DAG
Low (1 ug/ml)
Medium (2 ug/ml)
High (4 ug/ml)
Dihydroceramide
Low (0.1 ug/ml)
Medium (0.2ug/ml)
High (0.3 ug/ml)

cumulatively and the maximum rates of respiration (Vmax) and values for apparent KmADP determined using Michelis-Menton kinetics.
Mitochondrial Complex Activity – Respiratory chain enzyme activity for complexes I, II, II+III, III, IV, and citrate synthase will be assayed from muscle homogenates as previously described, modified for 96 well plate format (50). Respiratory chain activity assays will be performed with addition of DAG and sphingolipids as shown in Table 2B to parallel mitochondrial respiration studies.
GSH/GSSG and acyl-carnitines – These will be measured as described in specific aim 2.

D. Description, Risks and Justification of Procedures and Data Collection Tools:

Potential Risks

Radiation exposure. The DEXA measurement involves a very low dose of radiation (approximately 0.03mRem total equivalent dose per measurement) which is less than 1% of the radiation received from a chest x-ray. There is no known risk from receiving this dose of radiation.

IV risks. A hollow needle/plastic tube will be placed for obtaining blood samples or giving fluids. This will be left in for 5-8 hours. When the needle goes in the vein it may hurt for a short time, and there may be discomfort from having the hollow needle/plastic tube in the subjects' arm. There is a 1/10 chance a small bruise forming in the area, 1/100 chance of a clot forming in the vein, and 1/1000 chance of infection or significant blood loss. A total of ~250ml of blood will be taken from the subjects during the study in aims 1 and 2. This is approximately 5% of total blood volume and is not associated with a risk of anemia.

Venipuncture. Subjects will feel pain when the needle goes into the vein. Approximately 5% of people may faint, feel nauseous or dizzy. Not uncommonly a bruise may form at the puncture site.

Muscle strength testing. Subjects may feel muscular discomfort during the muscle strength test. They may have sore muscles the day after the test.

Risk of maximal mitochondrial respiration test. You will experience frequent tightening of the blood pressure cuff on your leg, which may be uncomfortable.

Risks of sleep recording. Measurement of the electrocardiogram (ECG-heart tracing) may cause some skin irritation from the sensors. Measurement of the electroencephalogram (EEG-brain wave activity), Electroocculogram (EOG-eye movement activity) Electromyogram (EMG-muscle activity on the chin and legs), nasal-oral air thermister (breathing in and out of the nose & mouth), and respiratory bands placed over the chest and abdomen, may cause some minor discomfort and/or skin irritation due to the paste used to attach the sensors. In addition, the paste used to hold sensors to the scalp may leave a flaky residue for several days. The physiological recording device is electrically isolated and complies with hospital standards for electrical safety.

MRI. Subjects may feel claustrophobic during the MRI. They may also experience flashing light in their eyes, and temporary warmth and reddening of the skin.

Insulin clamp. The hyperinsulinemic/euglycemic clamp carries a 1 in 10 risk for symptoms of hypoglycemia (neuroglycopenic or autonomic), and less than 1 in 10,000 of serious sequellae of hypoglycemia such as seizure, coma or death.

Muscle biopsies. Muscle biopsies are frequently uncomfortable and result is a bruise over the site. Risks of bleeding, infection, damage to nerves or vessels is ~1 in 100 or less. There may be discomfort following the biopsies after the anesthetic wears off. The CTRC will provide pain relievers to minimize potential discomfort.

Weight loss. During the weight loss phase, subjects could experience one or more of the following: bowel changes, cold sensation, and menstrual irregularities. During the weight loss phase,

subjects could experience one or more of the following: dizziness or lightheadedness, fatigue, dry skin, and some temporary hair loss. Not everyone will have these side effects. Eating high fat foods while on the low calorie diet has additional risk of triggering an acute gallbladder attack. Some research studies have found that during a low calorie diet, there is an increased risk of gallstone formation or gallbladder disease.

Exercise testing and training. During the exercise testing and training subjects may experience a fall, as well as discomforts such as pain in muscles or joints. During the exercise testing and training subjects may feel dizzy or faint as well as experience irregular heartbeats. During the exercise testing and training subjects may experience a stroke or heart attack. The risk of death during or immediately after an exercise test is less than 1 in 10,000. The risk of a heart attack during or immediately after an exercise test is less than 4 in 10,000. The risk of a problem that would require hospitalization, such as chest pain, is less than 2 in 1000.

Risks of losing confidentiality. Violation of privacy and loss of confidentiality are both risks to which research participants are exposed. The possibility of these risks increases when protected health information is collected.

Alternative treatments. We see no psychological, social, or legal risks beyond those of participation in health-related research. There are no alternative methods available that would permit the collection of the required information for this research study.

ADEQUACY OF PROTECTION AGAINST RISKS

a. Recruitment and informed consent

A member of the investigative team will obtain informed consent and HIPAA authorization in tandem. Subjects will have a copy of the COMIRB-approved consent form sent to them my mail or email for their review prior to meeting with a team member. Potential subjects will talk with a team member for a full explanation of the study logistics, rationale, risks and benefits, and also to ask/answer questions. Informed consent will be obtained at the start of the preliminary screening visit. This will be done in a private, quiet, and unhurried environment. Volunteers will be asked to explain the consent form in their own words to ensure understanding of the study and what is expected of them. No tests or procedures will be performed prior to the subject signing the informed consent. Only volunteers capable of providing informed consent may participate in this research. Informed consent will be obtained by a member of the research team who has been through COMIRB 101 and 102 training (from our local institutional review board). Subjects will receive a copy of their signed consent.

b. Protection against risk

Every attempt will be made to minimize the risks involved to patients in this study. Subjects privacy, autonomy and confidentiality will be protected with redundant IRB & HIPAA-compliant measures. Subjects may withdraw from the study at any time. Only state-of-the-art techniques will be used in a NIH-funded, JCAHO accredited CTRC facility. CTRC personnel are exquisitely trained, and emergency equipment is easily accessible on-site. Universal precautions will be taken to protect everyone involved with subject testing. Sterile technique is invoked where appropriate. All infusion are prepared and stored by a licensed pharmacist and tested for sterility and pyrogenicity. We will also have an independent safety officer who will evaluate subject risk annually. Taken together, these measures have proven successful in the minimization of risk to subjects. Protected and de-identified information will be kept on password-protected computer systems with hard copies in locked filing cabinets in locked offices.

E. Potential Scientific Problems:

We have extensive experience recruiting individuals across the metabolic spectrum (51-55), as well as performing insulin clamps (39; 51; 56; 57), and muscle biopsies (26; 43; 51; 53; 58-61) and therefore no problems are expected with the proposed studies. An alternative approach to our proposal would be to use a combined exercise and weight loss intervention, which may have more dramatic results compared

to exercise or weight loss only interventions. If we find minimal changes in insulin sensitivity with either intervention used in isolation, we will move to a combined intervention. Different degrees of success with the interventions are important to provide a spread in the data for changes in localized lipids and insulin sensitivity. An alternative approach would be to provide food for subjects to consume prior to both insulin clamps. We excluded this design because of the possibility of changing muscle lipid species and localization with changing dietary patterns. If individuals cannot maintain their weight within 2kg of starting weight in the exercise only intervention, they will be removed from the study. Our focus in this proposal is on DAG and sphingolipids, however, we will also measure changes in triglyceride, long chain acyl-CoA, and phosphatidic acid to determine other lipids related to changes in insulin sensitivity and/or cellular signaling. If there are no changes in PKC, we will evaluate changes in non-PKC DAG targets such as PKD and RasGRP1/4 to determine if these less studied DAG targets relate to insulin resistance.

Our typical muscle biopsy yield after removal of extra-muscular lipid, is approximately 160mg wet weight, and the tissue required for the assays in this project is 135mg wet weight, allowing all assays to be performed on the majority of biopsies. If muscle yield is limiting, we will prioritize analysis in the following order: subcellular fractionation (50mg) >western blot (15mg) >mitochondrial respiration (25mg) >PP2A activity (10mg) >PKC activity (35mg). We will also evaluate changes in other mitochondrial/ER lipids related to changes in mitochondrial function. In addition to those lipids listed under aim 1, we will also measure cardiolipin content as well as composition.

F. Data Analysis Plan:

Aim 1: Our statistical methods are rigorous with an *a priori* statistical analysis plan to test our hypothesis in an unbiased manner, and power analysis based on our own preliminary data. Prior to analysis, data will be analyzed for normality, and any non-normally distributed variables will be log-transformed for use in analytic models. Correlations between the change in sarcolemmal DAG and sphingolipid species and the change in insulin sensitivity will be performed using Pearson's correlation coefficients in the entire study group (n=51). Then, data comparing changes in sarcolemmal DAG and sphingolipid species to changes in insulin sensitivity before and after the intervention will be analyzed using a one-way Analysis of Variance (ANOVA) to examine overall differences between the intervention groups, and using Tukey's procedure to make pair-wise comparisons (e.g control vs. exercise, control vs. diet only) while adjusting for multiple comparisons. Changes in individual DAG and ceramide species will be examined using the Benjamini Hochberg procedure to limit false discoveries (62), and then Principal Component Analysis (PCA) will be used to examine groups of DAG and ceramide species (13). Analyses will be conducted using SAS statistical software ver. 9.4 (Cary, NC). The power analysis is based on changes in sarcolemmal lipids assuming there will be no significant changes in the control intervention. With an overall n of 51, we will have 92% power to detect a Pearson correlation coefficient similar to our preliminary data (Figure 2B, r= -0.45), and 80% power to detect a correlation coefficient as small as -0.38. Pilot data showed sarcolemmal C18:0 ceramide decreased 0.041 ng/ug protein with a SD of .02 after the combined intervention. With an n of 17/group we will have 84.6% power to measure a similar change in sarcolemmal DAG and sphingolipids for individual group comparisons using the Tukey procedure. The type 1 error rate (alpha) will be set at 5% for all comparisons.

Aim 2: Our statistical methods are rigorous with an *a priori* statistical analysis plan to test our hypothesis in an unbiased manner, and power analysis based on our own preliminary data. Data will be analyzed for normality, and any non-normally distributed variables will be log-transformed for use in analytic models. Correlations between the change in mitochondrial/ER DAG and sphingolipid species and the change in insulin sensitivity, mitochondrial respiration, ROS production, acyl-carnitine species, and ER stress will be performed using Pearson's correlations coefficient in the overall study group. Data comparing changes in mitochondrial/ER DAG and sphingolipid species to changes in insulin sensitivity and parameters of mitochondrial respiration between groups before and after the intervention will be analyzed using a one-way ANOVA (SAS statistical software ver. 9.4, Cary, NC). If overall differences are observed by group then individual group comparisons will be made using Tukey's procedure, which corrects for family-wise error rates. Changes in individual DAG and ceramide species will be examined using the Benjamini Hochberg procedure to limit false discoveries, and changes in groups of DAG and ceramide species will be examined using PCA as described in Aim #1. Our power analysis is based on our preliminary data for

the change in mitochondrial ceramide and DAG species after the pilot intervention shown in Figures 8 and 10, and assuming there will be no significant changes in the control intervention. With an overall n of 51, we will have 99% power to detect a Pearson correlation coefficient as large as that observed in our preliminary data (Figure 7B, r= -0.44), and we will have 80% power to detect a correlation coefficient as small as -0.38. With an n of 17/group, we will have 95% power to measure a similar change in mitochondrial DAG and sphingolipids as we have observed in our pilot data after the weight loss or exercise interventions using pairwise comparisons in ANOVA models using the Tukey procedure. The type 1 error rate (alpha) will be set at 5% for all comparisons.

Data analysis plan for secondary data points regarding sleep component of the study:
Sleep duration and markers of sleep quality will be obtained from Actiwatch recordings and scored using the provided Actiware Software. Data are collected from one week preceding the baseline clamp study visit and again from one week preceding the post-intervention clamp study visit. Actigraphy data will be averaged across 7 days to create average sleep duration and sleep quality scores for each participant before and after intervention. We will then examine the relationship between changes in sleep variables and changes in insulin sensitivity assessed by the clamp study.

G. Summarize Knowledge to be Gained:

Achievement of these aims will yield the following *expected outcomes*: 1) sarcolemmal 1,2-disaturated DAG and C18:0 ceramide promote insulin resistance through PKC and PP2A activation; 2) mitochondrial/ER sphingolipids correlate with mitochondrial function, ER stress, ROS, acyl-carnitine content, and insulin resistance; 3) DAG and sphingolipids reduce mitochondrial function and increase ROS and acyl-carnitine generation in vitro consistent with a cause and effect relationship. These outcomes will be *clinically significant* by identifying signaling pathways and mechanisms of accumulation of specific localized DAG and ceramide species which can ultimately be targeted for the prevention and treatment of diabetes.

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