Mechanism by which caloric restriction improves insulin sensitivity in sedentary obese adults

Matthew L. Johnson¹, Klaus Distelmaier¹, Ian R. Lanza¹, Brian A. Irving¹, Matthew M. Robinson¹, Adam R. Konopka¹, Gerald I. Shulman², K. Sreekumaran Nair¹*

¹Division of Endocrinology and Metabolism
Mayo Clinic College of Medicine
Rochester, Minnesota

²Howard Hughes Medical Institute and the Departments of Medicine and Cellular & Molecular Physiology
Yale University School of Medicine
New Haven, CT

*Corresponding author
K. Sreekumaran Nair, M.D., Ph.D.
Professor of Medicine
Division of Endocrinology and Metabolism
Mayo Clinic College of Medicine
200 First St SW
Rochester, MN 55905
Tel: 507-255-2415
Fax: 507-255-4828
Email: nair@mayo.edu

Running Title: Caloric restriction and insulin sensitivity

Keywords: insulin sensitivity, caloric restriction, TXNIP, mitochondria

Conflict of interest: The authors have declared that no conflict of interest exists.
ABSTRACT
Caloric restriction (CR) improves insulin sensitivity and reduces the incidence of diabetes in obese individuals. The underlying mechanisms whereby CR improves insulin sensitivity are not clear. We evaluated the effect of 16-weeks of CR on whole-body insulin sensitivity by pancreatic clamp before and after CR in 11 obese participants (BMI=35 kg/m$^2$) in comparison with a matched control period (CON, N=9, BMI=34 kg/m$^2$). Compared to CON, CR increased the glucose infusion rate needed to maintain euglycemia during hyperinsulinemia indicating enhancement of peripheral insulin sensitivity. This improvement in insulin sensitivity was not accompanied by changes in skeletal muscle mitochondrial oxidative capacity or oxidant emissions, nor was there changes in skeletal muscle ceramide, diacylglycerol, or amino acid metabolite levels. However, CR lowered insulin-stimulated thioredoxin-interacting protein (TXNIP) levels and enhanced non-oxidative glucose disposal. These results support a role for TXNIP in mediating the improvement in peripheral insulin sensitivity after CR.
INTRODUCTION

More than one third of adults and 17% of youth in the US are obese (1). Obesity is associated with reduced insulin sensitivity (insulin resistance) with a high predilection to develop type 2 diabetes (T2D), hypertension, hyperlipidemia and cardiovascular disease. Obesity results from the imbalance between energy intake and energy expenditure. Altered function of skeletal muscle mitochondria (2), the predominant organelle responsible for cellular energy metabolism is reported to occur in obese people. Moreover increased oxidative stress (3,4) and accumulation of lipids, ceramides, and diacylglyceride (DAG) are reported to occur in insulin resistant states including in obesity (5–9). Altered glucose (10), fatty acid (11), and amino acid metabolism (12) are reported in obese people including an inability to adjust to fuel availability (13,14). Together these data support a hypothesis that the failure to safely partition a chronic fuel surplus contributes to insulin resistance. Consistent with the above hypothesis reducing caloric intake is a successful therapeutic strategy to improve insulin sensitivity (15,16).

Caloric restriction (CR) improves insulin sensitivity (17) and reduces the incidence of diabetes and related metabolic disorders. The underlying molecular and cellular mechanisms of improved insulin sensitivity in skeletal muscle however, remain to be fully understood. An investigation of CR on muscle mitochondrial physiology reported that CR enhanced insulin sensitivity without improving mitochondrial function (18). While a 16-week CR intervention reported to decrease total skeletal muscle diacylglycerol (DAG) and ceramide content (17) in obese people. It is not clear however whether these declines in lipid metabolites are related to the dietary differences prior to these measurements. Moreover, the changes in DAG and ceramide after CR did not correlate with improvements in insulin sensitivity, suggesting additional pathways might be involved (17). Emerging evidence suggests a role for thioredoxin-interacting protein (TXNIP), an α-arrestin family member, as a key negative-regulator of insulin-stimulated glucose uptake (19–21) and in cellular fuel substrate partitioning in skeletal muscle (22). TXNIP deficient mice, for example, exhibit hypoglycemia during prolonged fasting (20), maintain skeletal muscle insulin sensitivity when challenged with a high fat diet (19,21), and are unable to utilize lipid fuels (22). Moreover, high levels of TXNIP in vitro decreases insulin-stimulated glucose transport (23) and elevates cellular oxidative stress (24). Furthermore insulin resistant and T2D individuals exhibit elevations in TXNIP mRNA (23). Hence TXNIP represents a potential key regulator of insulin-stimulated glucose transport in skeletal muscle and might be involved in the improvement in metabolic inflexibility and insulin sensitivity imparted by CR.

To address these gaps in knowledge, we performed a pilot study and systematically evaluated whole-body insulin sensitivity using the pancreatic clamp technique before and after 16-weeks of CR or control (CON). The CR program was designed to reduce total body weight by approximately 10% without changing physical activity levels. We hypothesized that CR would improve peripheral insulin sensitivity and that the improvement could be explained by
reductions in insulin-stimulated TXNIP expression. We therefore determined skeletal muscle TXNIP mRNA expression and protein content following a hyperinsulinemic-euglycemic clamp in the post-absorptive state. In addition after an overnight fast other purported causes of skeletal muscle insulin resistance were measured including mitochondrial energetics, mtH_2O_2 emissions, whole-body metabolic flexibility, skeletal muscle DAG, ceramide, amino acids, and plasma inflammatory factors to provide a more comprehensive understanding of CR’s effects on skeletal muscle insulin resistance.

MATERIALS AND METHODS

Experimental Procedures

Baseline Studies: A total of 29 participants gave written informed consent for the study, which was registered (Clinicaltrials.gov identifier NCT01497106) and the informed consent was approved by the Mayo Foundation Institutional Review Board. Participants were included if their BMI was \( \geq 30 \text{kg/m}^2 \) and were between the ages of 45 and 65 years old at the time of screening. Exclusion criteria were smoking, participation in a structured exercise program more than 2 times per week for 30 minutes or longer, fasting blood glucose value \( \geq 7 \text{ mM} \), or taking medications known to affect energy metabolism or insulin sensitivity, renal failure (serum creatinine > 1.5mg/dL), chronic active liver disease (AST and ALT > 3 times normal), anti-coagulant therapy, or active coronary artery disease.

Before and after 16-weeks of CR or CON, two outpatient visits and one inpatient visit were scheduled. Before both outpatient visits participants were instructed to fast overnight from 10:00pm the evening before and to avoid strenuous exercise for 24 hours preceding visits. One outpatient visit consisted of magnetic resonance imaging to measure subcutaneous and visceral fat distribution, and magnetic resonance spectroscopy to measure skeletal muscle oxidative capacity (25). The second outpatient visit was for measurements of resting energy expenditure (REE, for calculation of a weight maintenance diet, Parvomedics TrueOne 2400 Canopy system), DEXA scan (Lunar DPX-L; Lunar Radiation, Madison, WI), and maximal oxygen uptake (VO_2peak) test on a bicycle ergometer (Figure 1).

Participants were admitted to the Clinical Research Unit (CRU) on the evening of the fifth day of weight-maintaining diet provided by the CRU metabolic kitchen (Supplemental Figure 1). The weight maintenance meals (diet composition: 20% protein, 30% fat, 50% carbohydrate) were monitored daily to assure that the correct calorie level was achieved. Upon admission to the CRU no calories were consumed after 2100 hours to achieve a ten hour fast before the two-stage insulin euglycemic pancreatic clamp the following morning as previously published (26) with modifications as follows: the following morning at 0400 hours a primed [6,6^2H_2]glucose bolus (6 mg·kgFFM^{-1}) was administered followed by a nine-hour continuous infusion of [6,6^2H_2]glucose (started at 4 mg·kgFFM^{-1}·h^{-1} then titrated downward over the infusion time period to match anticipated changes in endogenous glucose production). At 0600 hours gas exchange was measured by indirect calorimetry for 30 minute for REE determination. Then at 0700 hours glucagon (0.001 mcg·kgFFM^{-1}·min^{-1}),
somatostatin (0.093 mcg·kgFFM⁻¹·min⁻¹), and growth hormone (0.0047 mcg·kgFFM⁻¹·min⁻¹) were infused for six-hours. Insulin was infused from 0700 to 1000 hours at (0.62 mU·kgFFM⁻¹·min⁻¹) and then from 1000 to 1300 hours at 2.3 mU·kgFFM⁻¹·min⁻¹. A 40% dextrose with 2% enrichment of [6,6²H₂]glucose was infused as needed to maintain blood glucose above 4.7 mM from 0700 to 1000 hours and then between 4.7 and 5.3 mM from 1000 to 1300 hours. Blood samples were collected in a heated hotbox (131°F) through a retrograde intravenous catheter at baseline for glucose, hormone levels, and every 10 minutes during the clamp to maintain euglycemia. In addition blood samples were collected every 20 minutes from 0600 to 0700, 0900 to 1000, and 1200 to 1300 for plasma [6,6²H₂]glucose. At 1330 hours, a percutaneous needle muscle biopsy (350-400mg) was obtained from the vastus lateralis muscle under local anesthesia and immediately frozen in liquid nitrogen and stored at -80°F for future analysis (27). This biopsy sample was used for analysis of TXNIP mRNA and protein content. The participant remained in the CRU through the remainder of the day and was given a weight maintenance diet until 2200 hours.

At 0700 hours the following morning a second muscle biopsy was obtained under local anesthesia, approximately 100mg was used immediately for mitochondrial function measurements of isolated mitochondria and mTH₂O₂ emissions (28). The remainder was immediately frozen in liquid nitrogen and stored at -80°F for future analysis including DAG, ceramide, and amino acid measurements (Figure 1).

**Study Intervention**

Following the baseline study visits, participants were randomly assigned to CR or CON for 16-weeks. The CR program consisted of removing 1,000kcal from participant’s daily allowance from fat and carbohydrate. Protein content (grams per day) remained constant. To assist in achieving a 1,000kcal daily deficit, participants were provided meals from the Mayo Clinic CRU kitchen for the first 5 days and met with a registered dietitian weekly or more frequently if needed throughout the entire intervention to monitor weight loss and adherence to the CR diet. Portioned meal replacement products (Newlifestylediet Inc., San Ramon CA) were also provided as needed by the dietitians throughout the intervention to assist in adherence. If a participant failed to lose weight on two consecutive weeks with the dietitians they were provided with meals from the metabolic kitchen for another 5 days together with additional weekly meetings to appropriately address adherence. The CON group was instructed to maintain their normal eating and activities of daily living. Both groups were instructed to wear an accelerometer throughout the 16-week period to ensure daily physical activity level did not differ between groups or vary throughout the time of the intervention (data not shown).

**Mitochondrial energetics**

Respiration of isolated mitochondria with glutamate and malate substrates were performed as previously described (28) on the biopsy sample taken after an overnight fast. Briefly, mitochondria were isolated from fresh tissue by differential
centrifugation. Respiration of isolated mitochondria was measured by high-resolution respirometry (Oxygraph, Oroboros Instruments, Innsbruck, Austria) using a stepwise protocol to evaluate various components of the electron transport system. Protein content of the mitochondrial suspension was measured using a colorimetric assay (Pierce 660-nm Protein Assay). Oxygen flux rates are expressed per tissue-wet weight and per milligram of mitochondrial protein.

**mtH$_2$O$_2$ Emissions**

The reactive oxygen species emitting potential (mtH$_2$O$_2$) of isolated mitochondria were evaluated under state 2 conditions as described previously (25) on the second biopsy sample (taken after an overnight fast). Briefly, a Fluorolog 3 (Horiba Jobin Yvon) spectrofluorometer with temperature control and continuous stirring was used to monitor Amplex Red (Invitrogen) oxidation in a freshly isolated mitochondrial suspension. Amplex Red oxidation was measured in the presence of glutamate (10 mmol/L), malate (2 mmol/L), and succinate (10 mmol/L). The fluorescent signal was corrected for background auto-oxidation and calibrated to a standard curve. H$_2$O$_2$ production rates were expressed relative to mitochondrial protein.

**Glucose kinetic calculations**

Glucose concentration was measured every 10 minutes during the insulin clamp with an Analox glucose analyzer (Analox Instruments, London, UK). [6,6-$^2$H$_2$]-D-glucose enrichment in the plasma and infusates was measured using gas chromatography mass spectrometry. As described previously, the steady-state equations of Steele (29) were used to calculate glucose appearance (Ra) and disappearance (Rd). Endogenous glucose production (EGP) was calculated as the difference between total glucose rate of appearance and exogenous glucose infusion rate, peripheral insulin sensitivity was assessed from the rate of glucose infusion required to maintain euglycemia during the high dose insulin clamp, while hepatic insulin sensitivity was assessed by the extent to which EGP was suppressed from baseline to low-dose hyperinsulinemia (26).

**Metabolic Flexibility**

Rate of energy expenditure, lipid and carbohydrate oxidation were calculated from pulmonary gas exchange using the equations of Lusk (30) for which was assumed that the rate of amino acid oxidation over the inpatient study periods was unchanged within participants (31). Nonoxidative glucose disposal was calculated from glucose Rd in the last 30 minutes of the insulin clamp minus total carbohydrate oxidation.

**Skeletal muscle amino acid and lipid metabolite measurements**

All measurements were made on the second biopsy sample taken after an overnight fast. Concentrations of amino acids and metabolites were determined using MassTrak Amino Acid Solution (Waters) modified for mass spectrometry as previously described (32). Muscle samples were spiked with internal standards for amino acids and metabolites, deproteinized using cold methanol, and
centrifuged. An aliquot of the supernatant was derivatized using 6-aminoquinolyl-N-hydroxysuccinimidy carbamate and separated with an Acquity ultraperformance liquid chromatograph. Mass detection was performed using a TSQ Ultra 182 Quantum mass spectrometer (Thermo Finnigan) in electrospray ionization positive mode. Ceramide, cytosolic and membrane DAG were measured as previously reported (33).

qPCR
Approximately ~20 mg of muscle was powered in liquid nitrogen then total RNA extracted using a kit (RNeasy Fibrous Tissue, Qiagen) with DNAse treatment. RNA concentration and purity (A_{260}/A_{280}>2.0 for all samples) were determined by spectrophotometry (Nanodrop), then two micrograms of RNA were converted to cDNA according to manufacturers instructions (Applied Biosystems). Quantitative real-time polymerase chain reaction (qPCR) was performed in 384 well clear plates with 20µl reaction volume using 20 ng cDNA. Amplification conditions were 10 minutes at 60°C followed by 40 cycles of denaturing (95°C for 15 s) and annealing (60°C for 60 s) using a ViiA7 thermocycler (Applied Biosystems). Samples were amplified with multiplex conditions in triplicate on a single plate with a no template control, internal repeated control and 7 point relative standard curve spanning 4 log dilutions. Primers and probes were commercially produced (Applied Biosystems) for TXNIP (Assay ID# Hs01006900_g1, context sequence TTATACTGAGGTGGATCCCTGCATC) and reference gene β-2 microglobulin (Assay ID# 4326319E). Efficiencies of the target and reference genes were similar (~95-100%) from the standard curve.

Western Blots
Frozen muscle tissue was pulverized in liquid nitrogen and homogenized on ice in a lysis buffer containing 100mM NaCl, 20mM Tris-HCl, 0.5mM EDTA, 0.5% (v/v) Nonidet P40, and phosphatase and protease inhibitors. Homogenates were incubated on ice for 20 minutes, followed by centrifugation at 10,000g to remove insolubilized fragments. An aliquot of the supernatant containing solubilized proteins was used for protein estimation using a detergent and reducing agent-compatible protein assay kit (Pierce 660, Thermo&Fisher Scientific, Rockford, IL). Based on results from the protein estimate, samples were prepared in a lithium dodecyl sulfate sample buffer (NuPAGE LDS Sample Buffer, Invitrogen, Carlsbad, CA) with 5% 2-mercaptoethanol to achieve a final concentration of 2µg/µl. Samples were heated at 70°C for 10 minutes and 20µg protein were added to each well of precast gels (NuPAGE Novex Bis-Tris Mini Gels, Invitrogen, Carlsbad, CA). Proteins were separated by electrophoresis and blotted to PVDF membranes. Membranes were then blocked with LiCor blocking buffer before incubating overnight with primary antibodies for TXNIP (Abcam ab114981) and vinculin protein (CP74, Calbiochem, EMD Millipore Corporation, Billerica, MA). Proteins were detected using infrared fluorescent detection (LiCor Odyssey, Lincoln, NE) using anti-mouse and rabbit secondary antibodies. Signal intensity was determined using LI-COR imaging software (version 3.0.3).
Statistical analyses
Statistical analysis was performed using PRISM v6.0e (GraphPad Software Inc, La Jolla, CA). Differences between group (CR vs CON) and time were compared using a repeated measures two-way analysis of variance. When a significant interaction was detected, post hoc analysis was performed with the Sidak procedure. Age and VO_{2peak} at baseline were compared using an unpaired Student’s t-test. For outcomes where a significant change was found due to the intervention a Pearson correlation was performed between the change in glucose infusion rate (Δ GINF) and change in the outcome variable (i.e., Δ TXNIP). Significance was set at p < 0.05. Data are presented as means ± SEM.

RESULTS
Anthropometric characteristics
29 participants underwent randomization after enrollment into the study with 13 assigned to CR and 16 assigned to CON. 2 CR participants were lost to follow-up and 7 participants were lost to follow-up in the CON group. Therefore a total of 11 completed the CR and 9 completed the CON group. Main clinical characteristics at baseline and post-intervention are shown in Table 1. A total of 3 participants, 2 in CR and 1 in CON were on statin therapy at baseline. At baseline both groups were similar in age, body composition, body mass index (BMI), free fatty acid (FFA) concentrations, β-hydroxybutyrate concentrations and cardiorespiratory fitness (VO_{2peak}). Bodyweight, lean mass, and fat mass were higher in the CON group at baseline. After the 16-week intervention period total bodyweight, visceral fat, subcutaneous fat, BMI, body fatness, and fasting insulin concentrations all decreased in the CR group while there was no change in CON. While free fatty acid concentrations did not change, fasting β-hydroxybutyrate concentrations increased in the CR group. Participants in the CR group lost on average 10.1 ± 1.2% of total bodyweight over the 16-week period.

Insulin sensitivity and indirect calorimetry
The integrated area under the curve for the glucose infusion rate (GIR) required to maintain euglycemia during the last one-hour of the clamp increased from baseline to follow-up (P < 0.05, Figure 2 A-C) with area under the curve (AUC) for the last hour equal to 431±73.7 to 629±61.7 mg/kgFFM/min in the CR group, indicating that CR increased insulin sensitivity, while no change was found in the CON group (600±94.3 to 610±104.6 mg/kgFFM/min). This corresponded to a glucose rate of disposal (Rd) on high-dose insulin of 7±1.1 to 11±0.9 (P < 0.05) and 9±1.4 to 9±1.5 mg/kgFFM/min in CR and CON groups respectively. Because randomization resulted in the CON group having significantly higher area under the curve at baseline compared to the CR group and that CR increased the AUC to a similar level to that of the CON group we compared the AUC for the last hour from a reference population of nine lean but sedentary participants that underwent an identical clamp procedure (66±0.4 years, BMI 27±1.1) and found the lean participants exhibited a significantly higher AUC (P < 0.05, 848±68.7
mg/kgFFM/min) compared to both the CON or CR group in the current study demonstrating that indeed both groups were insulin resistant. Endogenous glucose production (EGP) did not change after CR (3±0.3 to 3±0.1 mg/kgFFM/min) and did not change in CON (3±0.8 to 3±0.4 mg/kgFFM/min), while the percent suppression of EGP from fasting to low dose insulin slightly increased but did not reach statistical significance after CR (Figure 2C&E). No change in percent suppression of EGP was found in CON (Figure 2C&E). Insulin, glucagon, and growth hormone levels were effectively clamped, and c-peptide levels were repressed throughout the six-hour clamp (Supplemental Figure 2). Indirect calorimetry measurements after an overnight fast (basal), showed a significant decrease in the respiratory exchange ratio (RER) after CR in comparison to CON (P < 0.05). That decrease in fasting RER drove increased lipid oxidation during basal conditions and the significant increase in delta RER from basal to clamp conditions after CR (P < 0.05, Table 2). CR significantly increased non-oxidative glucose disposal under clamp conditions (P < 0.05, Table 2).

**Skeletal muscle mitochondrial function**

*Ex-vivo* mitochondrial function did not change in either group before and after the intervention. State 3 respiration was unchanged from baseline in either group whether expressed per tissue weight or when normalized to mitochondrial protein (Figure 3A - D). These results were evident under experimental conditions where substrates were provided through respiratory chain complex I (CI, glutamate + malate), complex I and II together (CI+II, glutamate+malate+succinate), and complex II (CII, succinate+rotenone). The absence of any change in mitochondrial capacity was confirmed *in vivo* using NMR spectroscopy (Figure 3E). In addition there was no change in mitochondrial efficiency (i.e., decreased proton leak), evident from the respiratory control ratio (RCR, state 3/state 4), or mtH2O2 emissions under state 2 conditions (Figure 3E, F, & G). Together, these results demonstrate that 16-weeks of CR in obese individuals maintains, but does not enhance mitochondrial function.

**Lipid metabolite levels**

Skeletal muscle analysis of total skeletal muscle ceramide, cytosolic DAG and membrane DAG species showed no changes. In addition no changes were found in skeletal muscle amino acid concentrations (Supplemental Figures 3, & 4, Supplemental Table & 1).

**Plasma inflammatory markers**

Circulating c-reactive protein (CRP), interleukin-6 (IL-6), and tumor necrosis factor-α (TNFα) levels were measured after an overnight fast and found to not change in either CR or CON over the intervention (Supplemental Figure 5).

**TXNIP expression levels**

TXNIP mRNA levels were measured in the vastus lateralis biopsy sample taken after the 6-hour hyperinsulemic-euglycemic clamp and the morning after an
overnight ten-hour fast. In the biopsy sample taken after the insulin clamp levels of TXNIP significantly declined after the 16-week intervention in the CR group (Figure 4A, P < 0.05). Furthermore, the change in TXNIP expression across all participants (CR and CON) significantly correlated with the change in GIR during the hyperinsulinemic euglycemic clamp (Figure 4B, r = -0.71, R² = 0.50, P = 0.001). We further measured TXNIP protein content in the biopsy sample after the insulin clamp to see if the change in mRNA expression levels resulted in changes in protein content. TXNIP significantly declined after the 16-week intervention in the CR group (Figure 4C, P < 0.05) and the change in TXNIP protein content significantly correlated with the change in GIR during the hyperinsulinemic euglycemic clamp (Figure 4D, r = -0.5017, R² = 0.25, P = 0.034). There was no change in TXNIP mRNA expression levels in the overnight fasted sample (Supplemental Figure 6).

DISCUSSION

The finding in the current study that insulin-induced skeletal muscle TXNIP mRNA and protein expression change after CR provides novel insight into the mechanism by which peripheral insulin sensitivity is enhanced by CR in adults at high risk of developing T2D. Importantly these findings potentially provide an important link between skeletal muscle substrate metabolism and insulin sensitivity. A primary defect in T2D individuals is reduced skeletal muscle non-oxidative glucose disposal under insulin-stimulated conditions (34). In the current study, enhanced non-oxidative glucose disposal, likely occurring in skeletal muscle as glycogen synthesis (35) primarily accounted for higher glucose disposal rates (Table 2). However, we did not observe any changes in many commonly purported determinants of insulin resistance in skeletal muscle. We did not observe any impact of CR on intramuscular DAG, ceramide, or amino acid metabolites (Supplemental Figures 3, & 4, Supplemental Table 1). No effect on skeletal muscle mitochondrial oxidative capacity or mTH₂O₂ emissions was also observed (Figure 2). The most important finding is the significant decrease in skeletal muscle TXNIP transcript and protein expression following the hyperinsulinemic-euglycemic clamp (Figure 4A) and lack of a similar change in TXNIP following the CON period. We further observed that the change in glucose disposal during the hyperinsulinemic-euglycemic clamp over 16-week period in all participants was significantly correlated to the change in TXNIP in response to insulin (Figure 4B & D) supporting a hypothesis that the reduction in TXNIP at least partly explains the increase in insulin induced glucose disposal. The results thus provide new mechanistic insight on how CR enhances insulin-stimulated glucose disposal through a key redox sensitizing protein in skeletal muscle. TXNIP impairs insulin-signaling by inhibiting thioredoxin NADPH-dependent reduction of protein disulfides on phosphatidylinositol 3-phosphatase (PTEN) (36). When stabilized, PTEN has been shown to oppose insulin signaling in skeletal muscle (19,37). Furthermore skeletal muscle TXNIP deletion that protects against high-fat diet induced insulin resistance (19,21), is independent of any apparent changes in mitochondrial function (22). Participants in the current study showed no changes in any of the measured indices of mitochondrial
function, or mtH_2O_2 emissions (Figure 3), but demonstrated changes in insulin sensitivity (Figure 2) supporting an independent role for TXNIP from mitochondrial function.

There is growing interest in understanding how lifestyle interventions such as CR mediate their insulin sensitizing effects in obese individuals at high risk of developing T2D. We previously showed that life-long CR enhanced skeletal muscle antioxidant status and reduced oxidative damage to proteins, suggesting improved cellular redox status (38). More recently we demonstrated that 12-weeks of aerobic exercise training insulin resistant women with polycystic ovary syndrome improved insulin sensitivity enhanced skeletal muscle endogenous antioxidant activity, reduced markers of cellular oxidative stress, and lowered chronically elevated mtH_2O_2 emissions to that of healthy controls. The current study demonstrated that improvement of insulin sensitivity by weight loss in CR is mediated by mechanisms other than by altered mitochondrial function, although we did not measure mitochondrial fatty acid oxidative capacity.

Early reports indicated that CR enhanced peripheral insulin sensitivity in obese and T2D individuals in association with declines in skeletal muscle lipid content (39). However, subsequent investigations of exercise training (40,41) and acute lipid infusions in rodents (42) have identified that lipid content, while predictive of T2D risk (7,35), are unlikely to be directly involved in reducing insulin action on skeletal muscle. Instead much work in the field has identified bioactive lipid species including ceramide (43), DAGs (33) and elevated amino acid metabolites (44) as causes of skeletal muscle insulin resistance. We measured each of those known causes in the current study and found that CR did not change their levels in skeletal muscle but did improve peripheral insulin sensitivity. Our current results are therefore in contrast to others (17), who found significant decreases in both skeletal muscle DAG and ceramide levels after 16-weeks of CR. We analyzed not only total ceramide and DAG, but also the subcellular localization of DAG species that are likely to impact insulin sensitivity (Supplemental Figures 3 & 4). Of interest, anserine, an amino acid metabolite involved in scavenging endogeneous carbonyl’s (45) increased after CR. This may potentially contribute to the reduction of carbonylated proteins, nucleic acids, and aminophospholipids with a potential impact on protein function. It is reasonable to state that while on CR both insulin sensitivity and many metabolites may change but following CR and then a weight maintaining only insulin sensitivity improves without much change in metabolites supporting a notion that improvement of insulin sensitivity following CR-related weight loss has a mechanism unrelated to the changes in metabolites.

Insulin resistance has also been linked to metabolic inflexibility originally described across the leg in insulin resistant individuals (13). Recent exercise training (46–48) and diet plus exercise training (49) programs have shown improvements in metabolic inflexibility, changes in lipid content of hepatic and skeletal muscle and insulin sensitivity. The link between metabolic inflexibility and
insulin sensitivity is proposed to be through mitochondria (50), however, in the current study metabolic inflexibility improved (Table 2) with no changes in any indices of mitochondrial function. The improvement in metabolic inflexibility was due to higher whole body lipid oxidation in the overnight fasted state (Table 2), a finding that is supported by elevated β-hydroxybutyrate concentrations after CR in the fasted state (Table 1). Lack of change in plasma FFA concentrations after an overnight fast on CR is consistent with a previous CR study in obese people (12) and may represent increased clearance of FFA despite increased mobilization. It remains to be resolved how metabolic flexibility, substrate partitioning and insulin sensitivity are mechanistically linked. The current study did however, demonstrate that CR enhances insulin-stimulated glucose disposal during hyperinsulinemic conditions mainly by non-oxidative glucose disposal in the peripheral tissues that is likely to occur primarily in skeletal muscle (51).

Our two-stage pancreatic clamp technique also allowed us to evaluate responsiveness of EGP to insulin (Figure 2). In those with obesity and overt T2D, CR restores elevated levels of EGP to those of normal controls in concert with substantial declines in hepatic lipid content (52). The current results in obese individuals, who are not overt diabetics demonstrated that the effect of CR was primarily on peripheral glucose disposal with a minimal effect on EGP and future investigations should be focused on if hepatic lipid content is affected by CR in insulin resistant pre-diabetic individuals.

In summary, 16-weeks of CR and related weight loss in obese participants at a high risk of developing T2D improved whole body insulin sensitivity with the main impact on peripheral glucose disposal. This improvement cannot be attributed to alterations in skeletal muscle mitochondrial oxidative capacity, mtH$_2$O$_2$ emissions or intramuscular content of ceramide, DAG, or amino acid metabolites. However, the CR induced improvement in insulin sensitivity occurred in concert with increased post absorptive whole body lipid oxidation. Moreover we found that CR reduced levels of skeletal muscle TXNIP expression following hyperinsulinemia. Furthermore, the changes in TXNIP expression correlated with changes in glucose disposal rate during hyperinsulinemic state over the 16-week period. Together these results support a likely role of TXNIP in CR induced improvement in insulin sensitivity.
Acknowledgements
M.L.J contributed to conceptual design, data collection, data analysis, and data interpretation and wrote the manuscript. K.K. contributed to data collection, data analysis, manuscript writing and editing. I.R.L. contributed to conceptual design, data collection, data interpretation, manuscript writing, and editing. B.A.I contributed to conceptual design, data collection, data interpretation, manuscript writing, and editing. M.M.R. and A.R.K contributed to data collection, data analysis, and manuscript editing. G.I.S performed tissue DAG and ceramide analyses, manuscript writing and editing. K.S.N. contributed to conceptual design, supervised the execution of the study, data analysis, data interpretation, and manuscript writing and editing. K.S.N. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

The authors are greatly indebted to the skillful assistance of Katherine Klaus, Daniel Jakaitis, Jill Schimke, Dawn Morse, Roberta Soderberg, Deborah Sheldon, Lynne Johnson, and Melissa Aakre in the Division of Endocrinology and Metabolism Mayo Clinic College of Medicine Rochester, Minnesota.

Funding
Funding for this work was provided by, R01 DK41973 (KSN), U24DK100469, DK50456, T32 DK007198 (M.L.J), KL2 TR000136-07 (MLJ), KL2 RR024151 (BAI), R01 DK-49230 (GIS), R24 DK-090963 (GIS), T32 DK007352 (MMR and ARK) and UL1 TR000135. Additional support was provided by the Mayo Foundation and the Murdock-Dole Professorship (to K.S.N.). Meal replacements for the caloric restriction group were donated by Newlifestylediet (San Ramon, CA).

Duality of Interest
No potential conflicts of interest relevant to this article were reported.
References:


10. Butler PC, Rizza RA. Contribution to postprandial hyperglycemia and effect on initial splanchnic glucose clearance of hepatic glucose cycling in glucose-intolerant


Table 1: Characteristics of the Subjects

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Caloric Restriction</th>
<th>Control</th>
<th>P (group)</th>
<th>P (time)</th>
<th>P (interaction)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline (N=11)</td>
<td>Post (N=11)</td>
<td>Baseline (N=9)</td>
<td>Post (N=9)</td>
<td></td>
</tr>
<tr>
<td><strong>Demographic and body-composition characteristics</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age – yr.</td>
<td>55.3±1.8</td>
<td>52.7±1.6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Height - cm</td>
<td>169.6±2.4</td>
<td>177.8±3.9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight – kg</td>
<td>101.8±4.9</td>
<td>91.3±4.6****</td>
<td>109±7.4</td>
<td>110.3±7.7</td>
<td>0.1401 &lt; 0.001  &lt; 0.001</td>
</tr>
<tr>
<td>Weight % change</td>
<td>-10.1±1.2</td>
<td>+0.8±0.6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI</td>
<td>35.2±1.3</td>
<td>31.8±1.1****</td>
<td>34.4±1.4</td>
<td>34.6±1.5</td>
<td>0.6103 &lt; 0.001  &lt; 0.001</td>
</tr>
<tr>
<td>glucose – mg/dL</td>
<td>106.6±2.5</td>
<td>102.3±2.2</td>
<td>105.0±3.3</td>
<td>105.3±4.1</td>
<td>0.8716 0.1161 0.0767</td>
</tr>
<tr>
<td>insulin - µIU/mL</td>
<td>12.7±1.8</td>
<td>6.7±0.9****</td>
<td>11.3±2.5</td>
<td>11.2±2.3</td>
<td>0.5616 &lt; 0.002  &lt; 0.002</td>
</tr>
<tr>
<td>Free Fatty Acids - mM</td>
<td>0.38±0.04</td>
<td>0.45±0.05</td>
<td>0.38±0.06</td>
<td>0.39±0.03</td>
<td>0.5620 0.1876 0.3880</td>
</tr>
<tr>
<td>β-Hydroxybutyrate – mM</td>
<td>0.23±0.01</td>
<td>0.28±0.01***</td>
<td>0.24±0.01</td>
<td>0.25±0.01</td>
<td>0.3826 0.0012 0.0387</td>
</tr>
<tr>
<td>Body fat - %</td>
<td>45.9±1.6</td>
<td>42.4±1.5****</td>
<td>43.9±2.0</td>
<td>44.3±1.8</td>
<td>0.9918 &lt; 0.001  &lt; 0.001</td>
</tr>
<tr>
<td>Lean mass – kg</td>
<td>52.5±3.2</td>
<td>50.3±2.9**</td>
<td>59.5±5.3</td>
<td>59.1±5.0</td>
<td>0.1854 0.0034 0.0347</td>
</tr>
<tr>
<td>Fat mass – kg</td>
<td>39.6±4.6</td>
<td>36.8±2.2****</td>
<td>45.9±3.2</td>
<td>46.2±3.2</td>
<td>0.1246 &lt; 0.001  &lt; 0.001</td>
</tr>
<tr>
<td>Visceral fat – cm²</td>
<td>14.6±2.2</td>
<td>11.4±1.3*</td>
<td>12.1±1.6</td>
<td>13.9±1.9</td>
<td>0.9891 0.4132 0.0081</td>
</tr>
<tr>
<td>Subcutaneous fat – cm²</td>
<td>45.1±2.6</td>
<td>36.9±2.6****</td>
<td>43.2±3.9</td>
<td>44.2±4.1</td>
<td>0.5722 0.0078 0.0015</td>
</tr>
<tr>
<td>REE – kcals/day</td>
<td>1,668±93</td>
<td>1,615±92</td>
<td>1,865±128</td>
<td>1,867±109</td>
<td>0.1356 0.4925 0.4745</td>
</tr>
<tr>
<td>VO₂peak – l/min</td>
<td>1.9±0.1</td>
<td>2.2±0.2</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Measurements were made prior to randomization (baseline) and again after 16-weeks of CR or CON in the fasting state. Means ± SEM are given, and a 2-way (group, time) repeated measures ANOVA was used to compare outcomes across groups. Precise P
values are given for the ANOVA. When a significant interaction was found a Sidak post-hoc test was performed. * P < 0.05, ** P < 0.01 *** P 0.001, **** P < 0.0001
<table>
<thead>
<tr>
<th>Table 2: Metabolic Flexibility</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td><strong>Respiratory exchange ratio</strong></td>
</tr>
<tr>
<td>Basal</td>
</tr>
<tr>
<td>Clamp</td>
</tr>
<tr>
<td>Delta</td>
</tr>
<tr>
<td><strong>CHO oxidation (µmol<em>kgFFM</em>min)</strong></td>
</tr>
<tr>
<td>Basal</td>
</tr>
<tr>
<td>Clamp</td>
</tr>
<tr>
<td><strong>Nonoxidative glucose disposal (µmol<em>kgFFM</em>min)</strong></td>
</tr>
<tr>
<td>Basal</td>
</tr>
<tr>
<td>Clamp</td>
</tr>
<tr>
<td><strong>Lipid oxidation (µmol<em>kgFFM</em>min)</strong></td>
</tr>
<tr>
<td>Basal</td>
</tr>
<tr>
<td>Clamp</td>
</tr>
</tbody>
</table>

Measurements were made prior to randomization (baseline) and again after 16-weeks of CR or CON. Means ± SEM are given, and a 2-way (group, time) repeated measures ANOVA was used to compare outcomes across groups. Precise P values are given for the ANOVA. When a significant interaction was found a Sidak post-hoc test was performed. * P < 0.05, ** P < 0.01 *** P 0.001, **** P < 0.0001
**Figures:**

**Figure 1. Experimental Design.** Before and after 16-weeks of CR or CON, two outpatient visits and one inpatient visit were scheduled. One outpatient visit consisted of magnetic resonance imaging (NMR), while the second outpatient visit was for measurements of resting energy expenditure (REE), DEXA scan, and maximal oxygen uptake (VO₂peak) test on a bicycle ergometer. An inpatient visit at baseline and after 16-weeks of the intervention was conducted after 5-days of a weight maintaining diet provided by the CRU metabolic kitchen. The inpatient visit consisted of a two-stage (low and high dose insulin) hyperinsulemic-euglycemic pancreatic clamp over 6-hours, followed by a skeletal muscle biopsy. Blood samples were obtained every 10 min to adjust the glucose infusion rate to maintain euglycemia at approximately 90 mg/dL. After the clamp and biopsy was completed, standardized meals were provided to keep participants weight stable. The following morning in the post absorptive state a second fasted skeletal muscle biopsy was performed. *Blood draw.

**Figure 2. Insulin Sensitivity.** The glucose infusion rate (GIR) required to maintain euglycemia in 10 minute intervals during the 6 hour insulin infusion in CR (A) and CON (B) and corresponding glucose concentrations. The area under the curve (AUC) and rate of glucose disposal (Rd) over the last hour insulin clamp for CR and CON (C & D). Endogenous glucose production (EGP) measured in the basal fasting state (E). EGP percent suppression from overnight fasted to low-dose insulin (F). Means ± SEM are given, and a 2-way (group, time) repeated measures ANOVA was used to compare outcomes across groups. Precise P values are given for the ANOVA. When a significant interaction was found a Sidak post-hoc test was performed. * P < 0.05, ** P < 0.01 *** P 0.001, **** P < 0.0001

**Figure 3. Mitochondrial Function.** Mitochondrial oxygen consumption rates (JO₂) were measured with carbohydrate-based mitochondrial substrates for CR (A) and CON (B) and then normalized for mitochondrial protein (C & D). In-vivo oxidative capacity measured by magnetic resonance spectroscopy before and after the 16-week period (E). Mitochondrial coupling was assessed from the respiratory control ratio (RCR) (F). mthH₂O₂ emissions were evaluated in isolated mitochondria under state 2 conditions (G). Means ± SEM are given, and a 2-way (group, time) repeated measures ANOVA was used to compare outcomes across groups. Precise P values are given for the ANOVA. When a significant interaction was found a Sidak post-hoc test was performed. * P < 0.05, ** P < 0.01 *** P 0.001, **** P < 0.0001

**Figure 4. Skeletal muscle TXNIP expression.** Skeletal muscle TXNIP expression was evaluated using qPCR and normalized to beta-2-microglobulin in muscle biopsy samples after the insulin clamp (A). The delta change of TXNIP mRNA was correlated using a Pearson’s correlation to delta change in AUC for the 6-hour insulin clamp for all participants (B). Skeletal muscle protein content
was evaluated using western blot and normalized to vinculin (C). The delta change of TXNIP protein was correlated using a Pearson’s correlation to the delta change in the AUC for the 6-hour insulin clamp for all participants (D). Means ± SEM are given, and a 2-way (group, time) repeated measures ANOVA was used to compare outcomes across groups. Precise P values are given for the ANOVA. When a significant interaction was found a Sidak post-hoc test was performed. * P < 0.05, ** P < 0.01 *** P 0.001, **** P < 0.0001
**Study Design**

- Screen: 20 Obese Adults
  - DXA, VO_{peak}, NMR
  - 5-Day standardized weight maintaining diet

**Inpatient Study**

- 16-weeks CR (N=11)
  - Weekly Monitoring
  - DXA, NMR
- 16-weeks CON (N=9)
  - 5-Day standardized weight maintaining diet

**Inpatient Study Details**

- **Overnight Fast**
  - **0400**
    - Primed-continuous infusion D_{2}-glucose
    - Continuous - 4 mg•kgFFM^{−1}•h^{−1}
  - **0630**
    - 6-h Pancreatic Clamp
  - **0700**
    - High-dose insulin
    - Insulin - 2.3 mU•kgFFM^{−1}•min^{−1}
  - **1000**
    - Clamp Ends & Muscle Biopsy
    - *Blood Draws*
  - **1230**
    - Muscle Biopsy
  - **1300**
    - Overnight Fast
    - **0700**

**Note:**
- Insulin - 0.62 mU•kgFFM^{−1}•min^{−1}
- Somatostatin - 0.093 mcg•kgFFM^{−1}•min^{−1}
- Glucagon - 0.001 mcg•kgFFM^{−1}•min^{−1}
- Growth Hormone - 0.0047 mcg•kgFFM^{−1}•min^{−1}
- 40% Dextrose w/2% D_{2} enrichment
A. GIR (µmol/kgFFM⁻¹·min⁻¹)

- Caloric Restriction Baseline
- Caloric Restriction Post

B. GIR (µmol/kgFFM⁻¹·min⁻¹)

- Control Baseline
- Control Post

C. AUC (µmol/kgFFM/min)

- Baseline
- Post

D. Rd (µmol/kgFFM/min)

- High Insulin

E. EGP (µmol/kgFFM/min)

F. EGP % Suppression

**** Indicates statistical significance.
<table>
<thead>
<tr>
<th></th>
<th>CR Baseline</th>
<th>CR Post</th>
<th>CON Baseline</th>
<th>CON Post</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histidine</td>
<td>8.09±0.58#</td>
<td>8.59±0.71#</td>
<td>6.17±0.36</td>
<td>6.51±0.51</td>
</tr>
<tr>
<td>1-Methylhistidine</td>
<td>2.65±0.67</td>
<td>1.29±0.66</td>
<td>1.38±0.48</td>
<td>1.68±0.67</td>
</tr>
<tr>
<td>Phosphoethanolamine</td>
<td>1.01±0.10</td>
<td>1.54±0.28</td>
<td>0.87±0.09</td>
<td>1.14±0.19</td>
</tr>
<tr>
<td>Carnosine</td>
<td>114.11±8.93</td>
<td>133.12±19.43</td>
<td>111.20±19.73</td>
<td>92.33±11.65</td>
</tr>
<tr>
<td>Anserine</td>
<td>1.65±0.24#</td>
<td>2.06±0.35#</td>
<td>1.04±0.15</td>
<td>1.05±0.14</td>
</tr>
<tr>
<td>Ethanolamine</td>
<td>1.40±0.07</td>
<td>1.55±0.18</td>
<td>1.53±0.15</td>
<td>1.50±0.13</td>
</tr>
<tr>
<td>Aspartic Acid</td>
<td>3.15±0.40</td>
<td>4.53±1.06^</td>
<td>3.12±0.44</td>
<td>4.96±0.96^</td>
</tr>
<tr>
<td>Carnosine</td>
<td>0.18±0.02</td>
<td>0.19±0.04</td>
<td>0.21±0.03</td>
<td>0.21±0.02</td>
</tr>
<tr>
<td>β-Alanine</td>
<td>3.49±0.31</td>
<td>4.57±0.59</td>
<td>2.90±0.50</td>
<td>3.32±0.41</td>
</tr>
<tr>
<td>γ-Amino-N-butyric-acid</td>
<td>0.07±0.01</td>
<td>0.14±0.07</td>
<td>0.05±0.01</td>
<td>0.12±0.05</td>
</tr>
<tr>
<td>α-Aminoacidipic-acid</td>
<td>1.20±0.20</td>
<td>1.22±0.22</td>
<td>0.75±0.16</td>
<td>1.12±0.16</td>
</tr>
<tr>
<td>β-Aminoisobutyric-acid</td>
<td>0.06±0.01</td>
<td>0.11±0.03^</td>
<td>0.07±0.02</td>
<td>0.15±0.05^</td>
</tr>
<tr>
<td>Cystathionine 1</td>
<td>0.20±0.05</td>
<td>0.08±0.03*</td>
<td>0.09±0.03</td>
<td>0.07±0.02</td>
</tr>
<tr>
<td>allo-Isoleucine</td>
<td>0.05±0.01</td>
<td>0.02±0.01^</td>
<td>0.04±0.01</td>
<td>0.03±0.01^</td>
</tr>
<tr>
<td>Arginine</td>
<td>18.23±1.54</td>
<td>15.79±2.43</td>
<td>12.89±1.54</td>
<td>15.42±1.58</td>
</tr>
<tr>
<td>Taurine</td>
<td>236.46±12.38</td>
<td>309.58±21.35^</td>
<td>266.62±21.75</td>
<td>283.52±17.70^</td>
</tr>
<tr>
<td>Serine</td>
<td>10.56±0.90</td>
<td>15.68±2.13^</td>
<td>9.63±0.89</td>
<td>13.91±1.64^</td>
</tr>
<tr>
<td>Glutamine</td>
<td>562.43±37.53</td>
<td>507.89±34.95</td>
<td>483.29±23.18</td>
<td>485.16±32.24</td>
</tr>
<tr>
<td>Glycine</td>
<td>39.23±4.27</td>
<td>52.91±5.99^</td>
<td>37.57±2.51</td>
<td>44.05±4.01^</td>
</tr>
<tr>
<td>Citrulline</td>
<td>7.57±1.87</td>
<td>5.43±1.81</td>
<td>8.48±1.40</td>
<td>7.97±1.41</td>
</tr>
<tr>
<td>Glutamic Acid</td>
<td>83.34±6.61</td>
<td>78.99±9.16</td>
<td>65.81±3.51</td>
<td>80.31±3.82</td>
</tr>
<tr>
<td>Threonine</td>
<td>13.38±0.94</td>
<td>17.57±2.61^</td>
<td>14.78±0.77</td>
<td>19.58±1.70^</td>
</tr>
<tr>
<td>Alanine</td>
<td>87.97±5.48</td>
<td>93.87±6.86</td>
<td>97.17±6.33</td>
<td>107.78±7.26</td>
</tr>
<tr>
<td>Proline</td>
<td>19.06±1.68</td>
<td>21.17±2.42^</td>
<td>20.89±1.96</td>
<td>25.30±2.06^</td>
</tr>
<tr>
<td>Ornithine</td>
<td>5.94±0.40</td>
<td>6.15±0.75</td>
<td>5.40±0.64</td>
<td>6.53±1.17</td>
</tr>
<tr>
<td>Lysine</td>
<td>44.75±3.76</td>
<td>39.55±5.73</td>
<td>30.05±2.75</td>
<td>37.55±4.48</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>2.94±0.34</td>
<td>3.10±0.41</td>
<td>2.61±0.23</td>
<td>3.39±0.38</td>
</tr>
<tr>
<td>Valine</td>
<td>8.98±0.72</td>
<td>9.31±0.84</td>
<td>8.54±0.58</td>
<td>10.27±0.92</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>2.14±0.24</td>
<td>2.12±0.30</td>
<td>1.84±0.18</td>
<td>2.57±0.39</td>
</tr>
<tr>
<td>Leucine</td>
<td>5.78±0.53</td>
<td>6.32±0.90^</td>
<td>5.40±0.41</td>
<td>7.18±0.82^</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>2.99±0.31</td>
<td>3.35±0.40</td>
<td>2.80±0.18</td>
<td>3.50±0.32</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>0.68±0.06</td>
<td>0.75±0.08</td>
<td>0.66±0.05</td>
<td>0.80±0.07</td>
</tr>
</tbody>
</table>

Measurements were made prior to randomization (baseline) and again after 16-weeks of CR or CON. Means ± SEM are given, and a 2-way (group, time) repeated measures ANOVA was used to compare outcomes across groups. # significant group difference. ^ significant effect of time. * when a significant interaction was found a Sidak post-hoc test was performed. P < 0.05
Supplemental Figure 1. Bodyweight before study days

Bodyweights before inpatient

- CR Baseline
- CR Post
- CON Baseline
- CON Post
Supplemental Figure 2. Hormone levels during insulin clamp

Hormone levels during hyperinsulemic-euglycemic pancreatic clamp. Means ± SEM are given.
Supplemental Figure 3. Ceramide Content

Skeletal muscle ceramide content measured by LC-MS/MS in CR (A) and CON (B) participants. Means ± SEM are given, and a 2-way (group, time) repeated measures ANOVA was used to compare outcomes across groups. Precise P values are given for the ANOVA. When a significant interaction was found a Sidak post-hoc test was performed. * P < 0.05, ** P < 0.01 *** P 0.001, **** P < 0.0001
Supplemental Figure 4. DAG content

Skeletal muscle DAG contents measured by LC-MS/MS in a cytosolic fraction CR (A & B) and membrane fraction (C & D). Means ± SEM are given, and a 2-way (group, time) repeated measures ANOVA was used to compare outcomes across groups. Precise P values are given for the ANOVA. When a significant interaction was found a Sidak post-hoc test was performed. * P < 0.05, ** P < 0.01 *** P < 0.001, **** P < 0.0001
C-Reactive protein, IL-6, and TNFα before and after the 16-week for the CR (A,C & E) and CON (B, D & F) groups. Means ± SEM are given, and a 2-way (group, time) repeated measures ANOVA was used to compare outcomes across groups.
Skeletal muscle TXNIP expression was evaluated using qPCR and normalized to beta-2-microglobulin in muscle biopsy samples after and overnight fast. Means ± SEM are given, and a 2-way (group, time) repeated measures ANOVA was used to compare outcomes across groups.