Published in final edited form as:

Metabolism. 2013 April ; 62(4): 595–603. doi:10.1016/j.metabol.2012.10.010.

Acute effects of different diet compositions on skeletal muscle insulin signalling in obese individuals during caloric restriction

Cecilia C.L. Wang^{1,2}, Rebecca L. Adochio^{1,2}, J. Wayne Leitner¹, Ian M. Abeyta², Boris Draznin^{1,2}, and Marc-Andre Cornier²

Cecilia C.L. Wang: Cecilia.Wang@ucdenver.edu; Rebecca L. Adochio: Rebecca.Adochio@integris-health.com; J. Wayne Leitner: Wayne.Leitner@va.gov; Ian M. Abeyta: Ian.Abeyta@ucdenver.edu; Boris Draznin: Boris.Draznin@ucdenver.edu; Marc-Andre Cornier: Marc.Cornier@ucdenver.edu

¹Research Service, Department of Veterans Affairs, Denver, Colorado, USA

²Department of Medicine, University of Colorado Anschutz Medical Campus, Aurora, Colorado, USA

Abstract

Objective—The cellular effects of restricting fat versus carbohydrate during a low-calorie diet are unclear. The aim of this study was to examine acute effects of energy and macronutrient restriction on skeletal muscle insulin signalling in obesity.

Materials/Methods—Eighteen obese individuals without diabetes underwent euglycemic-hyperinsulinemic clamp and skeletal muscle biopsy after: (a) 5 days of eucaloric diet (30% fat, 50% carbohydrate), and (b) 5 days of a 30% calorie-restricted diet, either low fat/high carbohydrate (LF/HC: 20% fat, 60% carbohydrate) or high-fat/low carbohydrate (HF/LC: 50% fat, 30% carbohydrate).

Results—Weight, body composition, and insulin sensitivity were similar between groups after eucaloric diet. Weight loss was similar between groups after hypocaloric diet, 1.3 ± 1.3 kg ($p < 0.0001$ compared with eucaloric). Whole-body insulin sensitivity was unchanged after calorie restriction and similar between groups. However, *ex vivo* skeletal muscle insulin signalling differed depending on macronutrient composition of calorie-restricted diet. Skeletal muscle of the LF/HC group had increased insulin-stimulated tyrosine phosphorylation of IRS-1, decreased insulin-stimulated Ser 307 phosphorylation of IRS-1, and increased IRS-1-associated phosphatidylinositol (PI)3-kinase activity. Conversely, insulin stimulation of tyrosine phosphorylated IRS-1 was absent and serine 307 phosphorylation of IRS-1 was increased on HF/LC, with blunting of IRS-1-associated PI3-kinase activity.

© 2012 Elsevier Inc. All rights reserved.

Corresponding Author: Cecilia C. L. Wang, University of Colorado School of Medicine, Anschutz Medical Campus, MS8106, RC-1 South, Room 7103, 12801 E. 17th Avenue, Aurora, CO 80045.

Disclosure statement

The authors have no conflicts of interest to declare.

Author contributions

MC, BD and RA conceived of the study and participated in its design and coordination. JL carried out the *ex vivo* analyses. MC and RA performed the *in vivo* studies. MC and IA performed the statistical analyses. CW analysed and interpreted the data, drafted the manuscript and revised it critically. MC and BD revised the manuscript critically. All authors read and approved the final manuscript.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Conclusion—Acute caloric restriction with a LF/HC diet alters skeletal muscle insulin signalling in a way that improves insulin sensitivity, while acute caloric restriction with a HF/LC diet induces changes compatible with insulin resistance. In both cases, *ex vivo* changes in skeletal muscle insulin signalling appear prior to changes in whole body insulin sensitivity.

Key terms

hypocaloric diet; carbohydrate vs. fat; insulin sensitivity; intracellular signalling

Background

Chronic reduced-calorie diets have been demonstrated to enhance insulin sensitivity [1]. Lifestyle modification interventions promoting reduced calorie and fat intake, increased physical activity, and weight loss have also been demonstrated to reduce the incidence of type 2 diabetes in individuals with insulin resistance and impaired glucose tolerance [2–4]. Studies published decades ago indicated that a diet higher in carbohydrate and lower in fat enhances insulin sensitivity in patients with diabetes [5]. Over the past 10 years, several randomized controlled trials examining effects of differing macronutrient composition of calorie-restricted diets have been performed in non-diabetic individuals [6–9]. With the exception of one study which showed greater improvement in insulin sensitivity with a carbohydrate-restricted diet compared with a fat-restricted diet [8], all of the studies show an improvement in insulin sensitivity without detectable differences among diets. Surprisingly, two studies show a reversion of insulin sensitivity back to baseline even with continued weight loss [6,9], and one study demonstrated similar degree of improvement in insulin sensitivity between diets despite greater amount of weight loss on the carbohydrate-restricted diet [9].

Despite the numerous studies of calorie-restricted diets, the acute effects of restricting fat versus carbohydrate on insulin sensitivity and action are not clear. Kolterman, et al. demonstrated previously that short-term 5-day high carbohydrate (75%) feeding results in enhanced insulin sensitivity as evidenced by increased ability of insulin to promote removal of glucose from plasma [10]. In the current study, we examined acute effects of energy and macronutrient restriction on insulin signalling at the level of the whole body and at the tissue level, in skeletal muscle. We previously demonstrated that 5 days of either high fat or high carbohydrate overfeeding in lean individuals resulted in differential responses in skeletal muscle insulin signalling despite unchanged whole body insulin sensitivity [11]. We thus hypothesized that the macronutrient composition of a hypocaloric diet may differentially affect skeletal muscle insulin signalling acutely, reflecting diverse adaptive changes on a cellular level. Furthermore, we hypothesized that acute hypocaloric feeding would alter skeletal muscle insulin signalling prior to observable changes in whole body insulin sensitivity. The present study was designed to examine these hypotheses.

Materials and Methods

Subjects and study design (Figure 1)

The study was approved by the Colorado Multiple Institutional Review Board, and all subjects gave informed consent prior to any study procedures being performed. Inclusion criteria included the following: body mass index (BMI) between 30 and 40 kg/m², 20–45 years of age, free of significant medical illness as determined by history, physical examination and screening laboratory tests, and weight stable (+/– 5 pounds) by self-report over the past year. Subjects were excluded if they had: diabetes as determined by history and/or oral glucose tolerance test (OGTT), coronary artery disease, hyperlipidemia, uncontrolled hypertension, untreated hyper- or hypothyroidism, gastrointestinal disorders

affecting food intake, cancer, current smoking, post-menopausal status, use of any medications thought to affect lipid metabolism, body weight, energy expenditure or appetite, presence of alcohol or other substance abuse, presence of anorexia or bulimia as determined by history and the Eating Attitudes Test, or serious psychiatric disease. After initial screening with clinical history, physical examination and laboratory assessments, subjects underwent assessments of resting metabolic rate (RMR) by indirect calorimetry (Sensormedics, Yorba Linda, CA), and body composition by dual X-ray absorptiometry (DPX whole-body scanner, Lunar Radiation Corp., Madison, WI). Subjects were queried regarding preferred foods from a pre-set list. For the 5-day eucaloric diet phase, subjects presented to the Clinical Translational Research Center (CTRC) to pick up meals and snacks chosen from their preference list in amounts to ensure energy and macronutrient balance. Estimates of daily energy needs were made using lean body mass (as determined by dual X-ray absorptiometry) in the following equation: $RMR = (\text{fat free mass} \cdot 23.9) + 372$. The estimates were confirmed using RMR as assessed by indirect calorimetry, multiplied by an activity factor of 1.3. The eucaloric diet consisted of 30% fat, 50% carbohydrate, and 20% protein. Subjects returned to the CTRC once more during the 5 day period to pick up meals and snacks for the remainder of the 5-day period. On the evening of the 5th day, subjects were admitted to the CTRC and fasted for 12 hours. They then underwent a euglycemic-hyperinsulinemic clamp and skeletal muscle biopsy the following morning. Following these procedures, subjects were randomized to receive a low fat/high carbohydrate (LF/HC) or a high fat/low carbohydrate (HF/LC) hypocaloric diet, provided at an energy level of 30% below maintenance energy needs, and received meals and snacks to bring home. They returned to the CTRC once again during the 5-day hypocaloric diet phase to pick up additional meals and snacks for the remainder of the 5-day period. Insulin action studies and skeletal muscle biopsies were performed after 5 days of assigned hypocaloric diet as with the eucaloric phase. The hypocaloric diets consisted of either LF/HC (20% fat, 60% carbohydrate, 20% protein) or HF/LC (50% fat, 30% carbohydrate, 20% protein). The macronutrient composition of each hypocaloric diet was chosen to provide similar amounts of protein but marked differences in carbohydrate and fat intake. The diets contained a 1:1:1 ratio of monounsaturated, polyunsaturated and saturated fats for palatability. Food for all meals and snacks during both phases of the study was provided by the CTRC kitchen. Subjects presented to the CTRC at the beginning of each diet period and once again during each diet period to pick up food and to be weighed. Dietary compliance was assessed by asking subjects to bring back their coolers containing food wrappers and containers, and any uneaten food. Subjects were asked to maintain their usual level of activity and not consume any alcohol- or calorie-containing beverages throughout each diet phase.

Materials

Antibodies to insulin receptor substrate (IRS)-1, phospho-serine (Ser 307) IRS-1, p85 α and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were purchased from Millipore (Lake Placid, NY). The PY20 antibody was from Santa Cruz Biotechnology (Santa Cruz, CA), as were protein A/G agarose beads. The p110 antibody was purchased from BD Transduction Laboratories (San Jose, CA). Secondary horseradish-peroxidase-conjugated antibody, protein A Sepharose, ECL Western Blotting Analysis System, and ImageQuant TL software were from GE Healthcare Bio Sciences, Piscataway, NJ. Radioactivity in the form of $\gamma^{32}\text{P}$ -ATP was purchased from Perkin-Elmer (Boston, MA). Analytical grade resins, polyvinylidene difluoride membranes, and reagents for polyacrylamide gel electrophoresis were from Bio-Rad Laboratories (Hercules, CA).

Study Days

Subjects were admitted to the inpatient CTRC the night before each of the two study days for a 12-hour overnight fast. The next morning, a euglycemic-hyperinsulinemic clamp and a

skeletal muscle biopsy were performed as previously described [11]. Briefly, an antecubital venous catheter was placed in one arm for infusions, and another catheter was placed retrograde in a dorsal hand vein of the contralateral arm for sampling, using the heated hand technique to obtain arterialized venous blood. After baseline blood samples were obtained, a primed (19 $\mu\text{mol/kg}$), constant (0.22 $\mu\text{mol/kg/min}$) infusion of [6,6- $^2\text{H}_2$] glucose was used to determine glucose disposal rate. A primed, continuous insulin infusion at a rate of 40 mU/ m^2/min was then initiated and continued from time 120 to 240 minutes. Blood samples were taken every 5 minutes during the insulin infusion for bedside glucose analysis, and a 20% dextrose solution enriched with [6,6- $^2\text{H}_2$] was infused and adjusted to maintain euglycemia at a blood glucose level of approximately 90 mg/dL. Rates of glucose appearance (R_a) and disappearance (R_d) were calculated using the modified Steele equation [12,13]. Women were studied during the follicular phase of their menstrual cycle, between days 1 and 7 of a typical 28-day cycle.

Skeletal muscle biopsy of the vastuslateralis muscle was performed toward the beginning of the euglycemic hyperinsulinic clamp once the insulin prime was complete (at 15 minutes), to assess insulin-stimulated PI3-kinase activity. For biopsies, 1% lidocaine was used to infiltrate subcutaneous tissue overlying the vastuslateralis muscle. A scalpel was used to make a small incision to the level of the fascia, and a Bergstrom side cut needle used along with suction to remove approximately 0.25 g skeletal muscle tissue. Tissue samples were frozen immediately in liquid nitrogen and stored at -80°C until used.

Clinical Laboratory Measurements

Plasma glucose levels from blood obtained every 5 minutes throughout the euglycemic-hyperinsulinemic clamp studies were assessed using a bedside YSI glucose analyzer (YSI Inc, Yellow Springs, OH). Baseline and steady-state analyses included blood sampling for insulin, glucose, free fatty acids, glycerol, triglycerides, leptin, adiponectin, tumor necrosis factor (TNF)- α , and interleukin (IL)-6. Insulin was determined by radioimmunoassay (Diagnostic Systems Laboratory, Webster, TX). Serum glucose levels were determined by enzymatic assay (Olympus America, Inc., Center Valley, PA), as were free fatty acids (FFA) (WaKo Chemicals, Richmond, VA) and glycerol (R-Biopharm Inc., Marshall, MI). Leptin and adiponectin levels were determined by radioimmunoassay (Linco Research, Inc., St. Charles, MO). TNF- α and IL-6 were determined by enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN). Glucose isotopic enrichment was measured by gas chromatography/mass spectrometry (Metabolic Solutions, Inc., Nashua, NH).

Western blot analysis of skeletal muscle lysates

Preparation of lysates from biopsy samples was performed in the setting of liquid nitrogen to maintain integrity of proteins. After determination of protein concentration, equal aliquots of skeletal muscle lysates were immunoprecipitated for IRS-1 for analysis of tyrosine phosphorylation, serine 307 phosphorylation, total IRS-1 and p110. Western blotting for total p85 α expression was performed on non-immunoprecipitated lysates, which were also immunoblotted with anti-GAPDH antibody as a loading control. All samples were kept at -20°C until analyzed. Antibodies were diluted to a concentration of 1:1000 in 1% milk in Tris buffered saline Tween 20 (TBST) before being used for immunoblotting. Equal aliquots of proteins were loaded in each lane.

IRS-1-associated phosphatidylinositol (PI) 3-kinase activity

As above, preparation of lysates from skeletal muscle biopsy samples was performed in the setting of liquid nitrogen to maintain integrity of proteins. After determination of protein concentration, aliquots of lysates were immunoprecipitated for IRS-1 with 1:100 dilution of specific antibody. IRS-1-associated PI3-kinase activity was determined in

immunoprecipitate by thin-layer chromatography as previously described [14]. Briefly, samples were resuspended in PI3-kinase buffer (25 mmol/L 3-(n-morpholino) propane sulfonic acid, 1 mmol/L ethylene glycol tetraacetic acid (EGTA), and 5 mmol/L magnesium chloride in distilled water) and PI3-kinase reaction mixture, which included sonicated L- α -phosphatidylinositol. The reaction was allowed to proceed for 30 minutes after adding gamma- 32 phosphate-ATP. After stopping the reaction, the organic layer was extracted twice with chloroform, combined, and dried. Samples were resuspended in a 2:1 chloroform:methanol solution just prior to spotting onto thin-layer chromatography (TLC) plates that had been activated then dried. Plates were placed in developing solution consisting of 65% 1-propanol and 1% glacial acetic acid, then left overnight. The next morning, plates were air-dried and exposed to film. Densitometry of the TLC spots was performed using a BioRad Fluor-S MultiImager, and quantified using QuantOne software (Bio-Rad, Hercules, CA).

Glucose rates of appearance and disappearance

Glucose rates of appearance (Ra) and disappearance (Rd) were calculated using a modified Steele equation for steady-state conditions as described by Finegood and co-authors [12,13] and as we have published previously [15]. Basal Ra and Rd were calculated during the last 20 minutes of the baseline infusions (between 90 and 110 minutes) using the following equations:

$$Ra = \frac{F - pV \cdot [(C_2 + C_1)/2] \cdot (E_2 E_1)/(t_2 - t_1)}{(E_2 + E_1)/2}$$

$$Rd = Ra - pV \cdot (C_2 - C_1)/(t_2 - t_1)$$

Insulin-suppressed Ra and insulin-stimulated Rd were calculated during the last 20 minutes of each insulin clamp. To account for the tracer in the “spiked” dextrose solution, equations were used for measurements made during the clamp as described in a previous publication by our group [15].

Statistical Analysis

Except where noted, data are presented as mean $\pm$ SE. Statistical analyses were performed using SigmaStat software (Jandel Scientific, San Rafael, CA). The effects of study diets were analyzed using repeated measures analysis of variance (ANOVA). Between-group analyses were performed using unpaired *t*-tests, while differences between time points were analysed using paired *t*-tests. P-values of <0.05 were considered statistically significant. No gender differences were found, so data from men and women were combined.

Results

Subject Characteristics

Eighteen obese subjects (8 men, 10 women) participated in this study. The mean age was 32.4 ± 7.2 years, weight 103.3 ± 3.1 kg, and body mass index (BMI) 34.7 ± 0.6 kg/m². Other characteristics were similar between groups (Table 1). There was a significant, albeit modest decrease in body weight and fat mass after five days of hypocaloric feeding ($p < 0.001$ compared with eucaloric), but this was not significantly different between hypocaloric diet groups (Table 2). After 5 days of hypocaloric diet, fasting levels of insulin and leptin decreased, while free fatty acids increased on both diets. The decrease in leptin

was driven by the effects on HF/LC diet. Adiponectin, TNF- α , and IL-6 did not change significantly after the hypocaloric diet (data not shown).

Insulin sensitivity

Steady state glucose (83.3 ± 4.6 mg/dL) and insulin (113.7 ± 33.7 μ IU/mL) concentrations during the clamp studies were similar between groups and after each of the diet phases. Whole body insulin sensitivity as determined by euglycemic-hyperinsulinemic clamp was also similar between groups at the 2 time points (Table 3): neither group demonstrated significant changes in M-value, glucose rate of appearance (Ra) or glucose rate of disappearance (Rd).

Insulin signalling

Skeletal muscle biopsies were obtained in the insulin-stimulated condition at 2 time points: following the 5-day eucaloric diet phase and the 5-day hypocaloric diet phase. Tyrosine phosphorylation of IRS-1 increased significantly in response to insulin *in vivo* in skeletal muscle from subjects on the LF/HC hypocaloric diet, but not on the HF/LC hypocaloric diet (Figure 2A, B). Conversely, phosphorylation at the serine-307 position of IRS-1 in response to insulin was significantly increased on the HF/LC hypocaloric diet while it decreased on the LF/HC hypocaloric diet (Figure 2A, C). Total IRS-1 protein was unchanged in both diet groups between the eucaloric diet phase (Day 0) and hypocaloric diet phase (Day 5) (Figure 2A).

Consistent with these changes in IRS-1 phosphorylation, IRS-1-associated PI3-kinase activity in skeletal muscle from subjects receiving 5 days of the LF/HC hypocaloric diet increased significantly in response to insulin stimulation *in vivo*, while it was blunted in skeletal muscle from subjects receiving 5 days of the HF/LC hypocaloric diet (Figure 3). Insulin-stimulated IRS-1-associated PI3-kinase activity after the hypocaloric diets were significantly different between groups ($p < 0.05$).

In concert with these changes in PI3-kinase activity, the following changes in subunits of PI3-kinase were observed in skeletal muscle from subjects on the LF/HC hypocaloric diet: protein expression of insulin-stimulated IRS-1-associated p110 catalytic subunit of PI3-kinase was significantly increased (Figure 2A,D; Figure 4), and total p85 α subunit protein expression was significantly decreased. In contrast, skeletal muscle from subjects on the HF/LC hypocaloric diet demonstrated decreased insulin-stimulated IRS-1-associated p110 and increased total p85 α expression (Figure 2A, D; Figure 4).

Discussion

Although consuming a reduced-calorie diet is known to result in weight loss and improvements in health [16–18], the effects of varying macronutrient composition in a calorie-restricted diet on insulin action at the tissue level in obese humans are less clear. The salient findings from this study are that: (1) acute changes in insulin signalling are observed in skeletal muscle of obese subjects after 5 days of a hypocaloric diet despite the absence of significant changes in whole-body insulin sensitivity, and (2) these changes in insulin signalling differ depending on macronutrient composition of the hypocaloric diet.

At a whole body level, insulin resistance is present when higher than normal concentrations of insulin are necessary to maintain euglycemia. On a cellular level, metabolic insulin resistance often displays reduced strength of signalling via the insulin receptor substrate (IRS)-phosphatidylinositol (PI) 3-kinase pathway. In almost all cases of insulin resistance, there is a decline in PI3-kinase activity [19,20]. Two complementary mechanisms have

emerged as potential explanations for the reduced strength of the IRS-1-PI3-kinase signalling pathway. First, PI3-kinase activity is reduced by serine phosphorylation of IRS proteins because of a diminished ability of IRS proteins to attract PI3-kinase in this situation [21,22]. There is a subsequent decline in tyrosine phosphorylation of IRS-1 and IRS-1-associated PI3-kinase activity. Although mechanisms leading to serine phosphorylation of IRS proteins have been explored, the nutritional effect on this process in humans is not completely understood. Second, disruption in the balance of various PI3-kinase subunits may play a role in the development of insulin resistance [23]. PI3-kinase consists of a regulatory subunit, p85, and a catalytic subunit, p110. Normally, p85 monomers exist in excess relative to p110. Since the p85 subunit of PI3-kinase binds directly to IRS-1, free p85 monomer and p85-p110 heterodimers compete for the binding to IRS-1 [24]. However, only the p85-p110 heterodimer is responsible for PI3 kinase activity. Therefore, increases or decreases in p85 expression lead to decreased or increased PI3-kinase activity in an inverse manner [14,25].

We found that 5 days of a calorie-restricted diet was too early to detect significant changes in whole-body insulin sensitivity in our cohort. Interestingly, though, we observed changes at the skeletal muscle level. Skeletal muscle insulin signalling on the LF/HC diet demonstrated changes consistent with increased insulin sensitivity, with increased insulin-stimulated tyrosine phosphorylation of IRS-1 and PI3-kinase activity [19,26]. In contrast, changes in skeletal muscle insulin signalling on the HF/LC diet were consistent with insulin resistance: lack of insulin stimulation of tyrosine phosphorylation of IRS-1 and PI3-kinase activity, increased serine phosphorylation of IRS-1 and increased expression of p85 α subunit [14,25,27]. Previously, Kolterman, et al. also demonstrated acute improvement in insulin sensitivity after 5 days on a very high carbohydrate diet (75% of calories)[10]. In addition, they found decreased insulin binding to adipocytes in this situation. This apparent paradox was hypothesized to be due to enhanced insulin signalling distal to the insulin receptor.

A potential explanation for the observation of enhanced insulin signalling on the LF/HC diet is that this represents an adaptive response at the tissue level to handle the relatively higher carbohydrate load on this diet. On the other hand, the higher fat load encountered on the HF/LC non-ketogenic diet resulted in blunting of insulin signalling. This could occur: (1) in response to the higher absolute saturated fat content of the diet with resulting activation of IKK-beta and NF-kappaB [28], or (2) as an adaptive response to the lower carbohydrate load. A reduction in carbohydrate oxidation and an increase in fat oxidation may be observed with carbohydrate restriction and may take up to 7 days to appear. These adaptive changes are hypothesized to occur in order to reduce the reliance on glucose as a substrate for energy production, increase reliance on lipid sources, allow for more effective use of dietary fat [29], and prevent hypoglycaemia when less carbohydrate is available [30–32]. The elevated fasting FFA observed on the hypocaloric HF/LC diet was likely from greater mobilization of fat stores during this acute period of low carbohydrate intake, and may be an additional explanation for the differences observed between groups: elevated FFA would be expected to induce greater insulin resistance, which was supported in the skeletal muscle biopsy findings. Boden, et al. have demonstrated that acute elevation of free fatty acids by lipid and heparin infusion in rats results in hepatic insulin resistance as determined by insulin suppression of endogenous glucose production and glucose uptake via activation of the NF-kB pathway [33,34]. Furthermore, lowering of fasting free fatty acids using acipimox over night improves insulin resistance acutely in humans [35].

Few studies have been published examining skeletal muscle insulin signalling in response to calorie restriction in humans. Among these, Kim, et al, demonstrated increased phosphorylation of IRS-1 and PI3-kinase activity following 3 months of calorie restriction in

obese individuals [36]. Even less has been reported regarding acute effects of caloric restriction on insulin signalling at a cellular level. One such study by Kirk and colleagues randomized obese individuals to a high carbohydrate (180 g carbohydrate/day; 20% fat, 65% CHO, 15% protein vs. low carbohydrate (50 g carbohydrate/day; 75% fat, 10% CHO, 15% protein) energy-reduced diet (1000 kcal energy restriction/day) [37]. A euglycemic-hyperinsulinemic clamp and muscle biopsy were performed 48 hours after starting the diet. Subjects experienced a significant decrease in weight acutely after beginning the diet. Similar to our observations, insulin-mediated glucose uptake was unchanged acutely, but they did not see changes in insulin signalling at the tissue level at 48 hours. The authors hypothesized that acute calorie restriction results in improved hepatic insulin sensitivity while chronic calorie restriction results in improved whole-body glucose uptake and muscle insulin signalling. Differences between their study and our observations may be explained by the length of the dietary interventions: 5 days may result in greater adaptation than 48 hours of a hypocaloric diet. Other differences may be related to degree of carbohydrate- and calorie-restriction.

The acute changes in insulin signalling we observed may at least partially explain differences in effectiveness of hypocaloric diets in insulin resistant versus insulin sensitive obese subjects. We have previously found that macronutrient composition of a hypocaloric diet has an impact on the amount of weight lost, depending on baseline insulin sensitivity [38]. Obese women who were insulin sensitive at baseline lost more weight on a LF/HC diet than on a HF/LC diet, while the inverse was true for obese women who were insulin resistant at baseline. The current study suggests that acute changes in insulin target tissues such as skeletal muscle could play a role in this difference.

The significant decrease in fasting leptin in the whole group after 5 days of hypocaloric diet was entirely driven by changes on the HF/LC group. This suggests that the leptin resistance manifested by the whole group at baseline was improved acutely by HF/LC and not by LF/HC. Leptin is known to decrease appetite and stimulate energy expenditure. It is secreted by adipocytes in proportion to amount of body fat [39]. Rodents given access to a high fat diet develop hyperleptinemia within a few weeks. Mechanisms for this are unclear, but may include activation of inflammatory pathways and endoplasmic reticulum stress. A majority of obese patients manifest elevated leptin concentrations. This, along with diminished anorectic response to leptin and diminished body weight suppression in response to exogenous leptin, indicates leptin resistance [40]. Leptin resistance occurs not only in peripheral tissues but also in the central nervous system, which triggers an adaptive increase in food intake and weight gain [41]. The interplay between leptin and insulin signalling is complex and not well-understood, but there are emerging data to suggest that leptin action to lower glucose and improve insulin sensitivity can occur independent of insulin, while leptin has stimulatory effects on the PI 3-kinase pathway that could at least partially explain the decrease in PI 3-kinase activity on the HF/LC diet in conjunction with the lower leptin levels. However, further studies to examine this directly are needed.

The strengths of this study are the following: (1) the effects of high fat vs. high carbohydrate in the diet can be understood in a hypocaloric setting; this has been previously studied extensively in the iso- or eucaloric setting; (2) a better understanding of events at the cellular level can be obtained since insulin sensitive target tissue was obtained at different time points of the diet; and (3) because this was a study in human subjects, the results are directly translatable to understanding acute effects of macronutrient composition during a hypocaloric diet. In terms of translational potential, this study highlights cellular differences occurring in response to acute changes in macronutrient composition. The recently-published trials examining weight loss diets with differing macronutrient compositions have not revealed a differential chronic effect of these diets on weight loss when extended for

longer than 6–12 months. This may lead to a premature conclusion that macronutrient composition of weight loss diets is of no consequence. Our findings suggest that there may be differences on a cellular level which may or may not have long term consequences, but should not be ignored until more information is obtained.

There are a few limitations that should be discussed. The number of subjects in this study was small, due in part to the lengthy and involved study procedures. The small number of subjects potentially limits the power to detect changes in whole-body insulin sensitivity due to high inter-individual variability, and also limits the ability to make valid statistical inferences. Furthermore, we used diets with more modest degrees of carbohydrate and fat restriction in order to make the diet palatable. The macronutrient compositions, though, were similar to other published studies examining effects of diet compositions in fat-restricted and in non-ketogenic carbohydrate-restricted diets [6,37]. Last, this study was designed to examine the *acute* effects of calorie restriction and macronutrient composition; the chosen time point of 5 days was consistent with historical studies detecting acute changes after high carbohydrate diets [10]. A longer-term study is needed to determine whether the acute changes in skeletal muscle insulin signalling represent adaptive changes at the tissue level that translate to whole body differences in insulin sensitivity at a later stage.

In conclusion, our findings suggest the presence of an early effect of caloric restriction on insulin signalling at the level of skeletal muscle which differs by relative contribution of fat versus carbohydrate in the diet. A hypocaloric diet lower in fat and higher in carbohydrate results in skeletal muscle insulin signalling changes consistent with increased insulin sensitivity, while a hypocaloric diet higher in fat and lower in carbohydrate content results in skeletal muscle insulin signalling changes consistent with the development of insulin resistance. These changes in skeletal muscle insulin signalling precede detectable changes in whole body insulin sensitivity, and may represent an acute adaptive response at the cellular level.

Acknowledgments

We gratefully acknowledge the participation of the study subjects in this clinical protocol. We also acknowledge the diligence of the staff of the Clinical Translational Research Center.

Funding

This work was supported by **NIH/NCRR Colorado CTSI Grant Number UL1 RR025780**, NIH/NIDDK Clinical Nutrition Research Unit Grant Number DK48520, NIH/NIDDK Grant Number R56 DK077041 (to M. Cornier), and the Department of Veterans Affairs in the form of a Career Development Award-2 (to C. Wang) and a Merit Review Award (to B. Draznin).

List of Abbreviations

BMI	body mass index
CTRC	Clinical Translational Research Center
EGTA	ethylene glycol tetraacetic acid
FFA	free fatty acids
HF/LC	high fat/low carbohydrate hypocaloric diet
IL-6	interleukin-6
IRS	insulin receptor substrate
LF/HC	low fat/high carbohydrate hypocaloric diet

mTOR	mammalian target of rapamycin
OGTT	oral glucose tolerance test
PI	phosphatidylinositol
Ra	rate of appearance
Rd	rate of disappearance
RMR	resting metabolic rate
TLC	thin-layer chromatography
TNF	tumor necrosis factor

References

- DeFronzo RA, Ferrannini E. Insulin resistance. A multifaceted syndrome responsible for NIDDM, obesity, hypertension, dyslipidemia, and atherosclerotic cardiovascular disease. *Diabetes Care*. 1991; 14(3):173–94. [PubMed: 2044434]
- Tuomilehto J, Lindstrom J, Eriksson JG, et al. Prevention of type 2 diabetes mellitus by changes in lifestyle among subjects with impaired glucose tolerance. *N Engl J Med*. 2001; 344(18):1343–50. [PubMed: 11333990]
- Pan XR, Li GW, Hu YH, et al. Effects of diet and exercise in preventing NIDDM in people with impaired glucose tolerance. The Da Qing IGT and Diabetes Study. *Diabetes Care*. 1997; 20(4):537–44. [PubMed: 9096977]
- Knowler WC, Barrett-Connor E, Fowler SE, et al. Reduction in the incidence of type 2 diabetes with lifestyle intervention or metformin. *N Engl J Med*. 2002; 346(6):393–403. [PubMed: 11832527]
- Brunzell JD, Lerner RL, Hazzard WR, et al. Improved glucose tolerance with high carbohydrate feeding in mild diabetes. *N Engl J Med*. 1971; 284(10):521–4. [PubMed: 5100724]
- Sacks FM, Bray GA, Carey VJ, et al. Comparison of weight-loss diets with different compositions of fat, protein, and carbohydrates. *N Engl J Med*. 2009; 360(9):859–73. [PubMed: 19246357]
- Shai I, Schwarzfuchs D, Henkin Y, et al. Weight loss with a low-carbohydrate, Mediterranean, or low-fat diet. *N Engl J Med*. 2008; 359(3):229–41. [PubMed: 18635428]
- Samaha FF, Iqbal N, Seshadri P, et al. A low-carbohydrate as compared with a low-fat diet in severe obesity. *N Engl J Med*. 2003; 348(21):2074–81. [PubMed: 12761364]
- Foster GD, Wyatt HR, Hill JO, et al. A randomized trial of a low-carbohydrate diet for obesity. *N Engl J Med*. 2003; 348(21):2082–90. [PubMed: 12761365]
- Kolterman OG, Greenfield M, Reaven GM, et al. Effect of a high carbohydrate diet on insulin binding to adipocytes and on insulin action in vivo in man. *Diabetes*. 1979; 28(8):731–6. [PubMed: 446930]
- Adochio RL, Leitner JW, Gray K, et al. Early responses of insulin signaling to high-carbohydrate and high-fat overfeeding. *Nutr Metab (Lond)*. 2009; 6:37. [PubMed: 19781106]
- Steele R. Influences of glucose loading and of injected insulin on hepatic glucose output. *Ann N Y Acad Sci*. 1959; 82:420–30. [PubMed: 13833973]
- Finegood DT, Bergman RN, Vranic M. Estimation of endogenous glucose production during hyperinsulinemic-euglycemic glucose clamps. Comparison of unlabeled and labeled exogenous glucose infusates. *Diabetes*. 1987; 36(8):914–24. [PubMed: 3297886]
- Cornier MA, Bessesen DH, Gurevich I, et al. Nutritional upregulation of p85 alpha expression is an early molecular manifestation of insulin resistance. *Diabetologia*. 2006; 49(4):748–54. [PubMed: 16491394]
- Bergman BC, Cornier MA, Horton TJ, et al. Effects of fasting on insulin action and glucose kinetics in lean and obese men and women. *Am J Physiol Endocrinol Metab*. 2007; 293(4):E1103–E1111. [PubMed: 17684102]

16. Wood PD, Stefanick ML, Williams PT, et al. The effects on plasma lipoproteins of a prudent weight-reducing diet, with or without exercise, in overweight men and women. *N Engl J Med*. 1991; 325(7):461–6. [PubMed: 1852180]
17. Wing RR, Blair EH, Bononi P, et al. Caloric restriction per se is a significant factor in improvements in glycemic control and insulin sensitivity during weight loss in obese NIDDM patients. *Diabetes Care*. 1994; 17(1):30–6. [PubMed: 8112186]
18. Low CC, Grossman EB, Gumbiner B. Potentiation of effects of weight loss by monounsaturated fatty acids in obese NIDDM patients. *Diabetes*. 1996; 45(5):569–75. [PubMed: 8621005]
19. Pessin JE, Saltiel AR. Signaling pathways in insulin action: molecular targets of insulin resistance. *J Clin Invest*. 2000; 106(2):165–9. [PubMed: 10903329]
20. White MF. Insulin signaling in health and disease. *Science*. 2003; 302(5651):1710–1. [PubMed: 14657487]
21. Aguirre V, Werner ED, Giraud J, et al. Phosphorylation of Ser 307 in insulin receptor substrate-1 blocks interactions with the insulin receptor and inhibits insulin action. *J Biol Chem*. 2002; 277(2):1531–7. [PubMed: 11606564]
22. Le RD, Zick Y. Recent advances in our understanding of insulin action and insulin resistance. *Diabetes Care*. 2001; 24(3):588–97. [PubMed: 11289486]
23. Ueki K, Fruman DA, Brachmann SM, et al. Molecular balance between the regulatory and catalytic subunits of phosphoinositide 3-kinase regulates cell signaling and survival. *Mol Cell Biol*. 2002; 22(3):965–77. [PubMed: 11784871]
24. Ueki K, Algenstaedt P, Mauvais-Jarvis F, et al. Positive and negative regulation of phosphoinositide 3-kinase-dependent signaling pathways by three different gene products of the p85 alpha regulatory subunit. *Mol Cell Biol*. 2000; 20(21):8035–46. [PubMed: 11027274]
25. Ueki K, Fruman DA, Yballe CM, et al. Positive and negative roles of p85 alpha and p85 beta regulatory subunits of phosphoinositide 3-kinase in insulin signaling. *J Biol Chem*. 2003; 278(48):48453–66. [PubMed: 14504291]
26. Reusch JE. Current concepts in insulin resistance, type 2 diabetes mellitus, and the metabolic syndrome. *Am J Cardiol*. 2002; 90(5A):19G–26G. [PubMed: 12088773]
27. Barbour LA, Mizanoor RS, Gurevich I, et al. Increased P85 alpha is a potent negative regulator of skeletal muscle insulin signaling and induces in vivo insulin resistance associated with growth hormone excess. *J Biol Chem*. 2005; 280(45):37489–94. [PubMed: 16166093]
28. Cai D, Yuan M, Frantz DF, et al. Local and systemic insulin resistance resulting from hepatic activation of IKK-beta and NF-kappaB. *Nat Med*. 2005; 11(2):183–90. [PubMed: 15685173]
29. Volek JS, Phinney SD, Forsythe CE, et al. Carbohydrate restriction has a more favorable impact on the metabolic syndrome than a low fat diet. *Lipids*. 2009; 44(4):297–309. [PubMed: 19082851]
30. Harber MP, Schenk S, Barkan AL, et al. Alterations in carbohydrate metabolism in response to short-term dietary carbohydrate restriction. *Am J Physiol Endocrinol Metab*. 2005; 289(2):E306–E312. [PubMed: 15797987]
31. Bergouignan A, Gozansky WS, Barry DW, et al. Increasing dietary fat elicits similar changes in fat oxidation and markers of muscle oxidative capacity in lean and obese humans. *PLoS One*. 2012; 7(1):e30164. [PubMed: 22253914]
32. Summermatter S, Mainieri D, Russell AP, et al. Thrifty metabolism that favors fat storage after caloric restriction: a role for skeletal muscle phosphatidylinositol-3-kinase activity and AMP-activated protein kinase. *FASEB J*. 2008; 22(3):774–85. [PubMed: 17928359]
33. Yang G, Li L, Fang C, et al. Effects of free fatty acids on plasma resistin and insulin resistance in awake rats. *Metabolism*. 2005; 54(9):1142–1146. [PubMed: 16125524]
34. Boden G, She P, Mozzoli M, et al. Free fatty acids produce insulin resistance and activate the proinflammatory nuclear factor-kappaB pathway in rat liver. *Diabetes*. 2005; 54(12):3458–3465. [PubMed: 16306362]
35. Santomauro AT, Boden G, Silva ME, et al. Overnight lowering of free fatty acids with Acipimox improves insulin resistance and glucose tolerance in obese diabetic and nondiabetic subjects. *Diabetes*. 1999; 48(9):1836–1841. [PubMed: 10480616]

36. Kim YB, Kotani K, Ciaraldi TP, et al. Insulin-stimulated protein kinase C lambda/zeta activity is reduced in skeletal muscle of humans with obesity and type 2 diabetes: reversal with weight reduction. *Diabetes*. 2003; 52(8):1935–42. [PubMed: 12882908]
37. Kirk E, Reeds DN, Finck BN, et al. Dietary fat and carbohydrates differentially alter insulin sensitivity during caloric restriction. *Gastroenterology*. 2009; 136(5):1552–60. [PubMed: 19208352]
38. Cornier MA, Donahoo WT, Pereira R, et al. Insulin sensitivity determines the effectiveness of dietary macronutrient composition on weight loss in obese women. *Obes Res*. 2005; 13(4):703–9. [PubMed: 15897479]
39. Coppari R, Bjorbaek C. Leptin revisited: its mechanism of action and potential for treating diabetes. *Nat Rev Drug Disc*. 2012; 11:692–708.
40. Konner AC, Bruning JC. Selective insulin and leptin resistance in metabolic disorders. *Cell Metab*. 2012; 16:144–152. [PubMed: 22883229]
41. Konner AC, Klockener T, Bruning JC. Control of energy homeostasis by insulin and leptin: targeting the arcuate nucleus and beyond. *Physiol Behav*. 2009; 97:632–638. [PubMed: 19351541]

Figure 1. Study timeline

After giving written informed consent, subjects were screened to exclude significant medical illnesses including diabetes. Subjects underwent RMR and DEXA, then a 5-day eucaloric diet phase followed immediately by the first hyperinsulinemic-euglycemic clamp and skeletal muscle biopsy. They were then randomized to receive either the LC/HF hypocaloric diet or HC/LF hypocaloric diet for 5 days, followed immediately by the second hyperinsulinemic-euglycemic clamp and skeletal muscle biopsy. RMR, resting metabolic rate by indirect calorimetry; DEXA, dual X-ray absorptiometry; CHO, carbohydrate; Bx, skeletal muscle biopsy; clamp, hyperinsulinemic-euglycemic clamp study; LF/HC, low fat/high carbohydrate; HF/LC, high fat/low carbohydrate; Pro, protein.

Fig 2A

Fig 2B

Fig 2C

Fig 2D

Figure 2. Serine-phosphorylation, tyrosine-phosphorylation, p110 and total IRS-1 expression in skeletal muscle lysates after 5 days of eucaloric (Day 0) vs hypocaloric (Day 5) diet

Skeletal muscle lysates were immunoprecipitated for IRS-1 then immunoblotted for phosphorylated tyrosine, phosphorylated serine-307, PI 3-kinase subunit p110, or total IRS-1. (A) Representative bands from Western blotting for tyrosine phosphorylated IRS-1, serine-307-phosphorylated IRS-1, p110 or total IRS-1 in skeletal muscle at day 0 (following 5 days of eucaloric diet) vs. day 5 (following 5 days of respective hypocaloric diet). (B) Tyrosine phosphorylation of IRS-1 in skeletal muscle increased following the LF/HC diet, but was not stimulated on HF/LC diet. (C) Serine-307 phosphorylation of IRS-1 decreased on LF/HC diet, while it increased on HF/LC diet. (D) IRS-1-associated PI 3-kinase catalytic subunit p110 protein expression was increased on LF/HC, while the HF/LC diet resulted in decreased IRS-1-associated p110 expression. * $p < 0.05$, eucaloric vs. respective hypocaloric diet. † $p < 0.05$, LF/HC vs. HF/LC. OD: optical density. N=18.

Figure 3. Phosphatidylinositol (PI) 3-kinase activity in skeletal muscle lysates after 5 days of eucaloric (Day 0) vs. hypocaloric (Day 5) diet
Skeletal muscle lysates immunoprecipitated for IRS-1 underwent assessment of IRS-1-associated PI3-kinase activity. IRS-1-associated PI3-kinase activity increased on LF/HC hypocaloric diet but decreased on the HF/LC hypocaloric diet. Representative spots from PI3-kinase assays are displayed above respective columns in bar graphs. * $p < 0.05$, eucaloric vs. respective hypocaloric diet. $^{\dagger}p < 0.05$, LF/HC vs. HF/LC. OD: optical density. N=18.

Figure 4. Skeletal muscle PI3-kinase p85 alpha subunit expression after 5 days of eucaloric (Day 0) vs. hypocaloric (Day 5) diet

Skeletal muscle lysates were immunoblotted directly for total p85α expression. This was decreased on LF/HC and increased on HF/LC diet. Representative bands from Western blotting are displayed above respective columns in bar graphs. These samples were also immunoblotted for GAPDH as a loading control. * $p < 0.05$, eucaloric vs. respective hypocaloric diet. † $p < 0.05$, LF/HC vs. HF/LC. OD: optical density. N=18.

Table 1

Subject characteristics after 5 days of eucaloric diet.

Characteristic	All	LF/HC	HF/LC
N	18 (8 M, 10 W)	10 (5 M, 5 W)	8 (3 M, 5 W)
Age $\pm$ SD (y)	32.4 $\pm$ 7.2	33.4 $\pm$ 6.4	31.3 $\pm$ 8.3
Weight (kg)	103.3 $\pm$ 3.1	104.1 $\pm$ 4.8	102.3 $\pm$ 3.8
BMI (kg/m ²)	34.7 $\pm$ 0.6	35.5 $\pm$ 0.8	33.7 $\pm$ 0.9
Fat mass (kg)	41.8 $\pm$ 1.6	42.0 $\pm$ 2.4	41.5 $\pm$ 2.1
<i>Fasting Data</i>			
Glucose (mg/dL)	89.6 $\pm$ 2.1	88.9 $\pm$ 2.7	90.5 $\pm$ 3.6
Insulin (μ IU/mL)	20.8 $\pm$ 1.9	21.4 $\pm$ 2.9	20.1 $\pm$ 2.3
Triglycerides (mg/dL)	126.6 $\pm$ 11.2	124.2 $\pm$ 15.9	129.5 $\pm$ 16.6
Free Fatty Acids (μ Eq/L)	506.5 $\pm$ 18.5	481.4 $\pm$ 21.1	542.3 $\pm$ 29.8
Adiponectin (μ g/mL)	6.7 $\pm$ 0.7	6.3 $\pm$ 1.0	7.2 $\pm$ 0.8
Leptin (ng/mL)	26.2 $\pm$ 3.0	26.5 $\pm$ 4.4	25.9 $\pm$ 4.2
TNF-alpha (pg/mL)	1.30 $\pm$ 0.1	1.16 $\pm$ 0.2	1.45 $\pm$ 0.2
IL-6 (pg/mL)	1.35 $\pm$ 0.2	1.34 $\pm$ 0.1	1.36 $\pm$ 0.3

All data except age are expressed as mean $\pm$ SE

LF/HC, group randomized to low fat, high carbohydrate hypocaloric diet; HF/LC, group randomized to high fat, low carbohydrate hypocaloric diet

Characteristics after 5 days on one of two hypocaloric diets, at the time of the second hyperinsulinemic-euglycemic clamp and skeletal muscle biopsy.

Table 2

Characteristic	All		LF/HC		HF/LC	
	Eucaloric	Hypocaloric	Eucaloric	Hypocaloric	Eucaloric	Hypocaloric
Weight (kg)	103.3 ± 3.1	102.0 ± 3.1 *	104.1 ± 4.8	103.0 ± 4.8 *	102.3 ± 3.8	100.8 ± 3.8 *
BMI (kg/m ²)	34.7 ± 0.6	34.3 ± 0.7	35.5 ± 0.8	35.1 ± 0.9	33.7 ± 0.9	33.2 ± 1.0
Fat mass (kg)	41.8 ± 1.6	40.5 ± 1.7 *	42.0 ± 2.4	40.8 ± 2.6 *	41.5 ± 2.1	40.0 ± 2.2 *
<i>Fasting Data:</i>						
Glucose (mg/dL)	89.6 ± 2.1	87.8 ± 1.7	88.9 ± 2.7	87.0 ± 1.9	90.5 ± 3.6	89.0 ± 3.0
Insulin (μIU/mL)	20.8 ± 1.9	17.3 ± 1.5 *	21.4 ± 2.9	17.9 ± 1.9	20.1 ± 2.3	16.5 ± 2.5
Triglycerides (mg/dL)	126.6 ± 11.2	117.6 ± 11.2	124.2 ± 15.9	123.8 ± 17.8	129.5 ± 16.6	109.8 ± 12.8
Free Fatty Acids (μEq/L)	506.5 ± 18.5	626.7 ± 27.9 *	481.4 ± 21.1	574.6 ± 32.8 *	542.3 ± 29.8	691.9 ± 38.0 *
Leptin (ng/mL)	26.2 ± 3.0	21.6 ± 2.9 *	26.5 ± 4.4	24.1 ± 4.7	25.9 ± 4.2	18.8 ± 3.2 *

Mean ± SE

* p<0.05 compared with eucaloric diet phase

LF/HC, low fat high carbohydrate hypocaloric diet. HF/LC, high fat low carbohydrate hypocaloric diet

Table 3

Hyperinsulinemic euglycemic clamp data following each diet phase.

Characteristic	All		LF/HC		HF/LC	
	Eucaloric	Hypocaloric	Eucaloric	Hypocaloric	Eucaloric	Hypocaloric
N	18 (8 M, 10 W)		10 (5 M, 5 W)		8 (3 M, 5 W)	
M-value (mg/kg _{ffm} /min)	3.36 ± 0.4	3.80 ± 0.4	3.61 ± 0.6	4.12 ± 0.4	3.05 ± 0.05	3.38 ± 0.06
Glucose Rd (mg/min)	354.9 ± 23.8	370.1 ± 27.3	357.9 ± 33.5	410.0 ± 34.1	351.6 ± 36.3	324.5 ± 40.5
Glucose Ra (mg/min)	185.0 ± 8.6	183.3 ± 5.5	185.8 ± 12.4	189.4 ± 8.6	184.1 ± 12.6	175.5 ± 5.8

Mean ± SE

* p<0.05 compared with eucaloric phase

LF/HC, low fat high carbohydrate hypocaloric diet; HF/LC, high fat low carbohydrate hypocaloric diet.
ffm, fat free mass. Rd, rate of disposal. Ra, rate of appearance