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Erik P. Kirk

Washington University School of Medicine in St. Louis

Dominic N. Reeds

Washington University School of Medicine in St. Louis

Brian N. Finck

Washington University School of Medicine in St. Louis

Mitra S. Mayurranjan

Washington University School of Medicine in St. Louis

Bruce W. Patterson

Washington University School of Medicine in St. Louis

See next page for additional authors

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Authors

Erik P. Kirk, Dominic N. Reeds, Brian N. Finck, Mitra S. Mayurranjan, Bruce W. Patterson, and Samuel Klein

Dietary fat and carbohydrates differentially alter insulin sensitivity during caloric restriction

Erik Kirk, Dominic N. Reeds, Brian N. Finck, Mitra S. Mayurranjan, Samuel Klein

Center for Human Nutrition and Division of Geriatrics and Nutritional Science

Washington University School of Medicine, St. Louis, MO

Running title: Acute and Chronic Effects of Calorie Restriction

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Nonstandard abbreviations used: FFA, free fatty acid; FM, fat mass; FFM, fat-free mass; HISI, hepatic insulin sensitivity index; IHTG, intrahepatic triglyceride; Ra, rate of appearance; Rd, rate of disappearance; SAAT, subcutaneous abdominal adipose tissue; TTR, tracer-to-tracee ratio; VAT, visceral adipose tissue;

Corresponding author: Samuel Klein, M.D.
Center for Human Nutrition
Washington University School of Medicine
660 South Euclid Avenue
Campus Box 8031
St Louis, MO 63110
Phone:(314) 362-8708
Fax: (314) 362-8230
E-mail:sklein@wustl.edu

Abstract

Background and Aims: We determined the effects of acute and chronic calorie restriction with either a low-fat, **high-carbohydrate diet** or a **low-carbohydrate diet** on hepatic and skeletal muscle insulin sensitivity. **Methods:** Twenty-two obese subjects (body-mass index, $36.5 \pm 0.8 \text{ kg/m}^2$) were randomized to a **high-carbohydrate** ($>180 \text{ g/d}$) or **low-carbohydrate** ($<60 \text{ g/d}$) energy-deficit diet. A euglycemic–hyperinsulinemic clamp, muscle biopsies, and magnetic resonance spectroscopy were used to determine insulin action, cellular insulin signaling and intrahepatic triglyceride content before, after 48 h, and after ~11 wks (7% weight loss) of **diet therapy**. **Results:** At 48 h, **intrahepatic triglyceride content** decreased more in the **low-carbohydrate** than the **high-carbohydrate diet** group ($29.6 \pm 4.8\%$ vs. $8.9 \pm 1.4\%$; $P < 0.05$), but was similar in both groups after 7% **weight loss** (**low-carbohydrate diet**, $38.0 \pm 4.5\%$ vs. **high-carbohydrate diet**, $44.5 \pm 13.5\%$). Basal glucose production rate decreased more in the low-carbohydrate than the high-carbohydrate diet group at 48 h ($23.4 \pm 2.2\%$ vs. $7.2 \pm 1.4\%$, $P < 0.05$) and after 7% **weight loss** ($20.0 \pm 2.4\%$ vs. $7.9 \pm 1.2\%$, $P < 0.05$). Insulin-mediated glucose uptake did not change at 48 h, but increased similarly in both groups after 7% **weight loss** ($48.4 \pm 14.3\%$, $P < 0.05$). In both groups, insulin-stimulated phosphorylation of Jun N-terminal kinase decreased by $29 \pm 13\%$ and phosphorylation of Akt and **insulin receptor substrate -1** increased by $35 \pm 9\%$ and $36 \pm 9\%$, respectively, after 7% **weight loss** (all $p < 0.05$). **Conclusion:** Moderate **calorie restriction** causes temporal changes in liver and skeletal muscle metabolism; 48 h of **calorie restriction** affects the liver (**intrahepatic triglyceride content**, hepatic insulin sensitivity, and glucose production), whereas moderate weight loss affects muscle (insulin-mediated glucose uptake and insulin signaling).

2 Insulin resistance is the most common metabolic complication associated with obesity,
3 and is associated with an increased risk of developing nonalcoholic fatty liver disease (NAFLD)
4 and type 2 diabetes ^{1, 2}. A reduced calorie diet is a primary therapy for insulin-resistant obese
5 persons, because even moderate diet-induced weight loss (5%-10% of body weight) decreases
6 intrahepatic triglyceride content (IHTG) and improves hepatic and skeletal muscle insulin
7 sensitivity ³⁻⁹. However, the effect of brief calorie restriction (CR) (≤ 3 d) is confusing because
8 short-term therapy with a very-low calorie diet (≤ 800 kcal/d) improves insulin action ^{10, 11},
9 whereas short-term fasting induces insulin resistance ^{12, 13}.

10 The mechanism responsible for the apparent discrepancy between severe and complete
11 CR on insulin action is not clear, but it is possible that differences in total carbohydrate intake
12 could be responsible. Data from studies that used the hyperinsulinemic-euglycemic clamp
13 technique to assess insulin action found that short-term CR with low carbohydrate intake (0-50
14 g/d) is associated with a decline in hepatic and skeletal muscle insulin sensitivity ^{14, 15}, whereas
15 short-term CR with adequate carbohydrate intake (100 g/d) is associated with an increase in
16 both hepatic and skeletal muscle insulin sensitivity ⁴. We previously found that carbohydrate
17 restriction, not total energy restriction, is responsible for initiating the lipolytic response to
18 fasting; providing daily energy requirements by infusing a lipid emulsion (carbohydrate
19 restriction) resulted in the same increase in lipolytic rate that occurred after complete fasting ¹⁶.
20 The summation of these data suggest that short-term CR with a low-carbohydrate (LC) diet
21 could have adverse effects on insulin sensitivity because of increased FFA release into the
22 circulation, which can cause both hepatic ^{17, 18} and skeletal muscle ¹⁹ insulin resistance.

23 The current recommended dietary guidelines for treating obesity is to reduce daily
24 energy intake by 500-1000 kcal ²⁰. Although, both low-carbohydrate (LC) and high-
25 carbohydrate (HC), low-fat diets are frequently used to lose weight, it is not known whether the
26 acute and chronic effects of CR on IHTG content and insulin action in liver and muscle differs
27 between diets. Therefore, the purpose of the present study was to evaluate the acute and

28 chronic metabolic effects of a 1000 kcal/d deficit HC ($\geq 180\text{g/d}$) or LC ($\leq 50\text{g/d}$) diet in obese
29 insulin-resistant subjects. A euglycemic-hyperinsulinemic clamp procedure, in conjunction with
30 stable isotope tracer infusion, was performed to assess hepatic and muscle insulin sensitivity,
31 vastus lateralis muscle samples were obtained to determine the concentration of key factors
32 that regulate skeletal muscle insulin sensitivity, and magnetic resonance spectroscopy was
33 used to determine IHTG content after short-term CR (48 h) and moderate (7%) weight loss. We
34 hypothesized that, compared with an energy-deficit HC diet, consuming an energy-deficit LC
35 diet has adverse effects on insulin action.

36

37 **METHODS**

38

39 **Subjects**

40 Twenty-two obese subjects (4 men and 18 women; 43.6 ± 2.5 years old, $\text{BMI} = 36.5 \pm 0.8$
41 kg/m^2) participated in this study. All subjects completed a medical evaluation, which included a
42 history and physical examination, standard blood and urine tests, an electrocardiogram, and a
43 2-h oral glucose-tolerance test (OGTT). All subjects were considered insulin-resistant, defined
44 as homeostasis model assessment of insulin resistance (HOMA-IR) value > 3.0 ²¹. **In addition,**
45 **63% of subjects had impaired glucose tolerance based on a plasma glucose concentration**
46 **between 140 and 199 mg/dL at 2 h after a 75 g oral glucose load**²². Subjects who had diabetes,
47 a history of excessive alcohol consumption, liver disease, or evidence of other serious illnesses
48 or organ dysfunction, and subjects who smoked tobacco products or took medications that are
49 known to alter glucose metabolism were excluded from the study. All subjects were weight
50 stable ($\leq 2\%$ change in body weight) and had been sedentary (< 1 h of exercise per week) for at
51 least 3 months before being enrolled in the study.

52 The study was approved by the Human Studies Committee of Washington University
53 School of Medicine in St. Louis, MO. Written informed consent was obtained from each subject
54 before their participation in this study.

55 **Experimental Design**

56

57 ***Body Composition Assessments***

58 Total body fat mass (FM) and fat-free mass (FFM) were determined by using dual-
59 energy x-ray absorptiometry (DXA, Hologic QDR 4500, Waltham, MA)²³. Total abdominal,
60 subcutaneous abdominal, and intra-abdominal fat volumes were quantified by using magnetic
61 resonance imaging (MRI, Siemens Vision 1.5 Tesla imager). Intrahepatic triglyceride (IHTG)
62 content was determined by using proton magnetic resonance spectroscopy (MRS) with a 1.5T
63 scanner (Magnetom Vision Scanner; Siemens, Erlanger, Germany)²⁴; three 2 x 2 x 2 voxels were
64 analyzed for each subject and the values were averaged for data analyses. These body
65 composition assessments were made at baseline (before diet intervention), after 48 h of CR with
66 either a HC or LC diet, and after subjects lost 7% of their initial body weight and were weight
67 stable for 4 weeks.

68

69 ***Euglycemic-hyperinsulinemic clamp procedure***

70 Subjects were admitted to the inpatient unit of the General Clinical Research Center
71 (GCRC) on two separate occasions. A euglycemic-hyperinsulinemic clamp procedure, in
72 conjunction with stable isotopically labeled tracer infusion, was performed at baseline (before
73 diet intervention), after 48 h of CR with either a HC or LC diet, and after subjects lost 7% of their
74 initial body weight and were weight stable for 4 weeks. Subjects were instructed to abstain from
75 exercise and to maintain their regular diet for at least 3 days and to abstain from caffeine and
76 alcohol for at least 24 h before each admission. Female subjects were studied during the
77 follicular phase of their menstrual cycle.

78 During the first GCRC admission subjects were admitted for 4 days. In the evening on
79 the day of admission, subjects consumed a standard meal, containing 15 kcal/kg FFM and 55%
80 of total energy as carbohydrates, 30% as fat, and 15% as protein at ~1800 h and then fasted
81 (except for water) and rested in bed until completion of the clamp procedure the next day. The
82 following morning, at 0600 h, a catheter was inserted into an antecubital vein of one arm to
83 infuse stable isotopically labeled glucose, insulin and dextrose; another catheter was inserted in
84 a contralateral hand vein, which was placed in a thermostatically controlled (65°C) box to obtain
85 arterialized blood ²⁵. At 0630 h, resting energy expenditure was determined by using a
86 metabolic measuring cart (Delta Trac; SensorMedics, Yorba Linda, CA). At ~0700 h, after a
87 blood sample was obtained to determine the background glucose enrichment, a primed,
88 continuous infusion of [6,6-²H₂]glucose was started and maintained for 7 h. At 210 min after
89 starting the tracer infusion, insulin was infused at a rate of 40 mU·m² body surface area (BSA)
90 ¹·min⁻¹ for 210 min (initiated with a two-step priming dose of 160 mU·m² BSA⁻¹·min⁻¹ for 5 min
91 followed by 80 mU·m² BSA⁻¹·min⁻¹ for 5 min). Dextrose (20%), enriched with [6,6-²H₂]glucose to
92 ~2.5% to minimize changes in plasma glucose enrichment ²⁶, was infused at a variable rate to
93 maintain euglycemia (plasma glucose concentration of 5.6 mM). The infusion rate of [6,6-
94 ²H₂]glucose was decreased by 75% during the clamp procedure to account for the expected
95 decline in hepatic glucose production. Blood samples were taken every 10 min during the last
96 30 min of the basal period and the clamp procedure to determine plasma glucose TTR and
97 concentration and plasma insulin concentration during basal conditions and insulin infusion. A
98 muscle biopsy from the vastus lateralis was taken at 240 min (i.e., 30 min after starting the
99 insulin infusion) to assess specific cellular factors involved in insulin sensitivity. The tissue was
100 immediately frozen in liquid nitrogen and then stored at -80°C until final analyses.

101

102

103

104 ***Diet intervention***

105 After completing the first insulin clamp procedure, subjects were randomized to
106 treatment with either a low-calorie HC diet or an LC diet. The energy content of the HC and LC
107 diets were designed to provide a 1000 kcal daily energy deficit, based on an estimated daily
108 energy requirement (calculated as 1.3 times measured resting energy expenditure); the average
109 total daily energy intake was ~1100 kcal. The HC diet provided ≥ 180 g carbohydrates (CHO)
110 per day and ~65% of total daily energy intake as CHO, 20% as fat, and 15% as protein; the LC
111 diet provided ≤ 60 g CHO per day and ~10% of daily energy intake as CHO, 75% as fat, and
112 15% as protein.

113 Subjects remained in the GCRC until the second insulin clamp procedure and body
114 composition assessment were completed. All food was provided by the GCRC metabolic
115 kitchen and subjects' food intake was monitored. On the first day of the diet intervention (i.e.,
116 the day of the first clamp procedure), the calorie and CHO contents of the diet were adjusted to
117 account for the glucose calories infused during the clamp procedure. On the third morning in
118 the GCRC, the insulin clamp procedure was repeated after 48 h of consuming either a low-
119 calorie HC or low-calorie LC diet. After completing the second insulin clamp procedure, the
120 calorie and CHO contents of the diet were again adjusted to account for the glucose calories
121 infused during the clamp procedure. The following morning (day 4 in the GCRC), IHTG content
122 and body composition were evaluated and subjects were then discharged from the GCRC.

123 All subjects received detailed dietary instructions by a registered dietician and were
124 instructed to follow the HC and LC diet until they lost 7% of their total body weight. Subjects
125 received weekly individual or group behavior therapy and diet education with a registered
126 dietician and experienced behavior counselor to enhance dietary compliance. Once subjects
127 achieved a 7% body weight loss (on average after 6 ± 1 wks), total calorie intake was adjusted to
128 maintain a constant body weight and prevent further weight loss. After being weight stable at

129 their new body weight for at least 4 weeks, subjects were readmitted to the GCRC and the
130 insulin clamp procedure and body composition analyses were repeated.

131

132 **Sample Analyses**

133 *Plasma substrate and hormone concentrations.* Plasma glucose concentration was
134 determined by using an automated glucose analyzer (YSI 2300 STAT Plus, Yellow Spring
135 Instrument Co., Yellow Springs, OH). Plasma insulin and leptin concentrations were measured
136 by using radioimmunoassay and enzyme-linked immunosorbent assay kits were used to
137 measure plasma adiponectin concentrations (Linco Research, St Louis, MO). The relative
138 changes in plasma 3-hydroxybutyrate concentrations at 48 h and ~11 wks of CR compared with
139 baseline values were determined by using a gas chromatography-mass spectrometry platform,
140 as described previously ²⁷

141 *Plasma glucose isotopic enrichment.* Plasma glucose tracer to tracee ratio (TTR) was
142 determined by using gas chromatography-mass spectrometry (Agilent Technologies/HP 6890
143 Series GC System – 5973 Mass Selective Detector, Hewlett-Packard, Palo Alto, CA), after
144 preparing the heptafluorobutyryl derivative of glucose and selectively monitoring ions at m/z 519
145 and 521 ²⁸.

146 *Muscle Akt/PKB, IRS-1, and JNK 1 phosphorylation were determined by using Western*
147 *blotting analyses (Muscle Akt/PKB, and JNK 1 phosphorylation) and immunoprecipitation (IRS-1*
148 *phosphorylation).* Muscle samples were homogenized in lysis buffer (50 mM Tris, 150 mM
149 NaCl, and 1% NP40), containing a cocktail of protease and phosphatase (NaF and NaVO₄)
150 inhibitors ²⁹. Protein content was quantified and then 60 µg protein was electrophoresed by
151 SDS-PAGE and transferred to nitrocellulose membranes. Blots were probed with polyclonal
152 antibodies directed against total Akt/protein kinase B (PKB) (Amersham Biosciences,
153 Pittsburgh, PA), Akt/ PKB phosphorylated at serine 473 (Amersham Biosciences, Pittsburgh,
154 PA), total c-Jun N-terminal kinase (JNK; EMD Biosciences, San Diego, CA), and JNK

155 phosphorylated at threonine 183 (EMD Biosciences, San Diego, CA). To evaluate IRS-1
156 tyrosine phosphorylation, IRS-1 was immunoprecipitated from 500 µg of protein using a
157 polyclonal antibody against IRS-1 (gift of Mike Mueckler) prior to SDS-PAGE and
158 immunoblotting with an antibody directed against **phosphotyrosine** (Cell Signaling, Danvers,
159 MA) or IRS-1 (gift of Mike Mueckler). The intensity of bands obtained by Western blotting
160 analyses was quantified by digitizing the autoradiographic images and using Image Processing
161 and Analysis in Java Program (ImageJ, National Institutes of Health, Version 1.36b). The
162 intensity of the phosphorylated forms of the proteins were corrected for total content of that
163 protein and normalized to the baseline value (i.e., before intervention); therefore, values are
164 expressed as percentage change from baseline.

165

166 **Calculations**

167 Total (endogenous and exogenous) glucose rate of appearance (Ra) in plasma during
168 basal conditions and the clamp procedure were calculated by dividing the glucose tracer
169 infusion rate by the average plasma glucose TTR between 180 and 210 minutes (basal) and
170 390 and 420 min (clamp)³⁰. Basal, endogenous glucose Ra was calculated by subtracting the
171 glucose tracer infusion rate from total glucose Ra. It was assumed that glucose Rd was equal
172 to total glucose Ra.

173 The homeostasis model assessment of insulin resistance (HOMA-IR) was determined by
174 dividing the product of plasma glucose concentration (in mM) and plasma insulin concentration
175 (in mU/L) by 22.5²¹. Hepatic insulin sensitivity index was assessed as the reciprocal of the
176 Hepatic Insulin Resistance Index, which is calculated as the product of the basal hepatic
177 glucose production rate (in µmol·kg FFM⁻¹·min⁻¹) and fasting plasma insulin concentration (in
178 mU/L)^{31, 32}. Skeletal muscle insulin sensitivity was determined by evaluating the ability of
179 insulin to stimulate skeletal muscle glucose uptake, assessed as the relative increase in whole-
180 body glucose Rd during insulin infusion compared with baseline values.

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Statistical Analysis

A two-way analysis of variance with repeated measures was used to compare between and within group differences in the changes in outcome measures from baseline to 48 hours and from baseline to 7% weight loss. Tukey's post-hoc procedure was used to locate differences, if a significant main effect was found. **The relationship between the percent change in intra-abdominal fat volume and the percent change in IHTG content and HISI were assessed by using linear regression analysis.** A P-value of ≤ 0.05 was considered statistically significant. Data are expressed as means \pm SEM. All data were analyzed using SAS (8.2, Cary, NC).

RESULTS

Study subject characteristics

Baseline metabolic variables and body composition measurements were not different between subjects randomized to the HC and LC diet groups (Table 1). Fifty percent of subjects in the HC diet group and 58% of subjects in the LC diet group had nonalcoholic fatty liver disease, defined as IHTG content $>5.6\%$ ³³.

Dietary compliance

Changes in plasma 3-hydroxybutyrate concentrations during CR suggest that study subjects in both the HC and LC diet groups were compliant with their dietary assignment. In subjects randomized to CR with an HC diet, plasma β -hydroxybutyrate increased ~ 2 -fold at 48 h of CR ($P=0.02$) and returned to baseline values at 11 wks of CR. In subjects randomized to CR with a LC diet, plasma 3-hydroxybutyrate increased ~ 10 -fold at 48 h of CR ($P<0.0001$) and remained 10-fold greater than baseline at 11 wks of CR ($P=0.002$).

207 **Body weight and body composition**

208 Short-term CR caused a similar decrease in body weight at 48 h with either diet (Table
209 2) (mean weight loss for both groups combined= $2.0 \pm 0.2\%$, $P < 0.0001$). Long-term weight loss
210 after completing the diet intervention was also similar in both groups (Table 2) (mean weight
211 loss for both groups combined at ~11 wks of dieting= $7.5\% \pm 0.4\%$ $P < 0.0001$). The time to
212 achieve 7% weight loss was not different between the AC diet group (6.2 ± 1.0 wks) and the LC
213 diet group (5.9 ± 1.0 weeks).

214 Changes in body FM and FFM at ~11 wks of dieting and 7% weight loss were not
215 different between the HC and LC groups (the average decreases in FM, FFM, and intra-
216 abdominal fat volume in all subjects were $11.3 \pm 0.9\%$, $3.8 \pm 0.6\%$, and $12.0 \pm 2.8\%$,
217 respectively; all $P < 0.001$) (Figure 1). Calorie restriction with either the HC or LC diet caused a
218 progressive decrease in IHTG content. The relative decrease in IHTG was ~3 times greater in
219 the LC group than in the HC group at 48 h of CR, but was not different between groups after
220 ~11 wks of CR (~7% weight loss) (Figure 1). **There was not a significant relationship between**
221 **percent change in intra-abdominal fat volume and the percent change in IHTG ($R^2=0.001$,**
222 **$P > 0.05$).**

223

224 **Plasma adipokine and hepatic enzymes concentrations.**

225 Plasma leptin concentration decreased similarly in both groups after 48 h ($10.8 \pm 3.6\%$)
226 decrease from baseline in combined groups, $P < 0.01$) and ~11 wks ($19.4 \pm 6.8\%$) decrease from
227 baseline in combined groups, $P < 0.01$) of CR. Plasma adiponectin concentrations decreased in
228 both groups after 48 h ($8.8 \pm 3.5\%$ decrease from baseline in combined groups, $P < 0.05$) and
229 tended to increase after ~11 wks ($12.1 \pm 7.2\%$ increase from baseline in combined groups,
230 $P > 0.05$) of CR.

231 Plasma ALT and AST concentrations did not change after 48 h and ~11 wks of CR in
232 either the HC or LC diet groups. In the combined groups, plasma ALT concentrations were 29.2
233 ± 2.4 , 31.1 ± 3.4 and 33.4 ± 5.2 IU/L and plasma AST concentrations were 25.5 ± 2.0 , 28.0 \pm
234 2.9, and 26.2 ± 2.8 IU/L at baseline, 48 h and 11 wks of CR, respectively.

235

236 ***In vivo measures of insulin sensitivity and glucose homeostasis***

237 *Plasma glucose, c-peptide, and insulin concentrations.* Calorie restriction caused a
238 decline in plasma glucose, c-peptide and insulin concentrations both after 48 h and ~11 wks
239 (~7% weight loss) of dieting in the HC and LC groups (Table 2). There was a trend toward a
240 greater decrease in both plasma glucose, c-peptide and insulin concentrations in the LC group
241 than the HC group after both short-term and long-term dieting. However, only the decrease in
242 plasma glucose concentration after 48 h of CR and the decrease in plasma insulin concentration
243 after 7% weight loss were significantly different between groups.

244 *Homeostasis model assessment of insulin resistance.* HOMA-IR improved in both
245 groups after 48 h of CR and did not change further after ~11 wks of dieting (~7% weight loss)
246 (Table 2). However, the decrease in HOMA-IR was greater in the LC than the AC diet group
247 both after 48 h of CR and 7% weight loss (Table 2).

248 *Hepatic Insulin Sensitivity Index.* Hepatic insulin sensitivity increased after 48 h of CR in
249 both the AC and LC groups, but did not improve further after 11 wks of CR (7% weight loss)
250 (Figure 3, top panel). However, the improvement in hepatic insulin sensitivity was greater in the
251 LC than the AC group, after both 48 h CR and 7% weight loss (Figure 3A). There was not a
252 significant correlation between percent changes in IHTG content and HISI value ($R^2=0.083$,
253 $P>0.05$).

254 *Basal glucose kinetics.* Basal glucose Ra decreased after 48 h of CR in both the AC and
255 LC groups, but was not different between groups and did not change further with more

256 prolonged CR and 7% weight loss. **Glucose Ra in the combined groups were 13.8 ± 0.4 ,**
257 **12.0 ± 0.4 , and 12.2 ± 0.3 $\mu\text{mol/kg FFM/min}$ at baseline, and at 48 h and 11 wks of CR,**
258 **respectively ($p < 0.001$ for each CR value compared with baseline value).** The decline in basal
259 glucose Ra was greater in the LC than the AC group after both short-term (48 h) and long-term
260 (~11 wks, 7% weight loss) CR (Figure 2).

261 *Insulin-mediated glucose uptake.* Plasma insulin concentrations during the clamp
262 procedure were not different between the HC and LC groups at any time point during the study.
263 However, plasma insulin concentrations after 48 h (84.3 ± 3.4 $\mu\text{U/mL}$) and 11 wks
264 ($84.5 \pm .2$ $\mu\text{U/mL}$) of CR were ~10% lower than values at baseline (95.4 ± 3.3 $\mu\text{U/mL}$; $p < 0.0001$).
265 **Glucose Rd values during insulin infusion was similar in both groups: 30.0 ± 2.6 , 25.0 ± 1.4 , and**
266 **31.1 ± 2.5 $\mu\text{mol/kg FFM/min}$ at baseline, and at 48 h and 11 wks of CR for the combined groups,**
267 **respectively.** The relative increase in glucose Rd during insulin infusion was not greater at 48 h
268 of CR than at baseline before CR in either diet group. However, the relative increase in glucose
269 Rd during insulin infusion was greater after 7% weight loss than at baseline in both diet groups.
270 Both short-term (48 h) and long-term (11 wks, 7% weight loss) CR caused similar changes in
271 insulin-mediated increases in glucose Rd in the AC and LC groups, so the data from both
272 groups are combined (Figure 3, bottom panel).

273

274 ***Cellular insulin signaling in skeletal muscle***

275 At 48 h of CR, skeletal muscle phosphorylation of Tyr183 JNK, Tyr IRS1, and Ser473
276 Akt/PKB content assessed after insulin stimulation (30 min of insulin infusion) was not
277 significantly different than baseline (before CR) in either diet group (Figure 4). However, at 11
278 weeks of CR (7% weight loss), insulin-stimulated skeletal muscle phosphoTyr IRS1, and
279 phosphoSer473 Akt/PKB content increased, whereas phosphoTyr183 JNK content decreased,
280 compared with baseline in both diet groups. Changes in phosphorylation status of Tyr183 JNK,

281 Tyr IRS1, and Ser473 Akt/PKB were similar in the HC and LC groups, so the data from both
282 groups are combined in Figure 4.

283

284 **DISCUSSION**

285 An energy-deficit diet is the cornerstone of therapy for obesity. However, the most
286 appropriate macronutrient composition of diet therapy needed to improve metabolic health
287 remains controversial. In the present study, we carefully evaluated the longitudinal metabolic
288 effects of short-term (48 h; 2% weight loss) and longer-term (11 wks; 7% weight loss) calorie
289 restriction (1000 kcal/d energy deficit) with either a high- or low- carbohydrate diet in obese,
290 insulin-resistant but non-diabetic adults. Our data demonstrate that short-term CR caused a
291 rapid decrease in IHTG content, increase in hepatic insulin sensitivity, and decrease in
292 endogenous glucose production rate, whereas longer-term CR and moderate 7% weight loss
293 improved skeletal muscle insulin sensitivity, in conjunction with an increase in cellular insulin
294 signaling. In addition, short-term CR with a low-carbohydrate diet caused a greater change in
295 liver fat content and metabolic function than short-term CR with a high-carbohydrate diet. These
296 data underscore the complexity of the metabolic effects of CR with diets that differ in
297 macronutrient composition, and demonstrate temporal differences among organ systems in the
298 adaptive response to CR itself and subsequent weight loss.

299 Our results refute our original hypothesis that a LC diet will cause insulin resistance
300 because of increased adipose tissue lipolytic rates and excessive FFA release into the
301 bloodstream. In fact, we found that LC intake rapidly caused a greater reduction in IHTG content,
302 improvement in hepatic insulin sensitivity, and decrease in endogenous glucose production rate
303 than consumption of an isocaloric low-fat diet. The mechanism responsible for the early
304 beneficial effects on liver metabolism is not known, but is probably related to the greater
305 decrease in plasma insulin concentrations in subjects consuming the low-carbohydrate diet.
306 The decline in circulating insulin likely decreased IHTG because of enhanced lipolysis of IHTG

307 and hepatic fatty acid oxidation¹⁴, and decreased hepatic glucose production because of hepatic
308 glycogen depletion¹⁷ and decreased glycogenolysis^{4, 34}. These metabolic alterations are similar
309 to the physiologic adaptations that occur during the early response to starvation, which are also
310 triggered by a reduction in carbohydrate intake¹⁶. However, in contrast with data obtained from
311 studies evaluating the metabolic effects of brief fasting¹²⁻¹⁴, we did not detect a significant
312 decline in skeletal muscle insulin sensitivity after 48 h of CR with a low-carbohydrate diet.

313 Weight loss, but not short-term CR, was necessary to increase skeletal muscle insulin-
314 mediated glucose disposal. The improvement in muscle insulin sensitivity we observed *in vivo*
315 is explained by enhanced cellular insulin signaling (increased insulin stimulated IRS-1 tyrosine
316 and Akt/PKB serine phosphorylation) detected after 7% weight loss but not after 48 h of CR.
317 These results are consistent with data from a study conducted in subjects with type 2 diabetes
318 that found insulin-stimulated Akt/PKB did not change after 2 days of CR³⁵. In addition, our data
319 suggest that the mechanism responsible for the increase in insulin signaling involves down-
320 regulation of JNK, which inhibits IRS-1 serine phosphorylation and the proximal component of
321 the insulin signaling cascade³⁶. Therefore, these findings demonstrate that the increase in
322 JNK associated with obesity and type 2 diabetes is responsive to nutritional manipulation and
323 can be normalized by weight loss.

324 Nonalcoholic fatty liver disease is associated with insulin resistance^{37, 38} and is an
325 important risk factor for diabetes³⁹. We previously found a linear inverse correlation between
326 IHTG content and insulin sensitivity in both liver and skeletal muscle³⁸. In the present study,
327 dietary manipulation of IHTG content allowed us to dissociate the interrelationships among
328 IHTG and insulin sensitivity in liver and skeletal muscle. After 48 h of CR, IHTG content
329 decreased by ~20%, which was associated with a decrease in basal glucose production rate
330 and an increase in hepatic insulin sensitivity, whereas skeletal muscle insulin sensitivity did not
331 change. Continued CR until subjects lost 7% of initial body weight caused a further decrease in
332 IHTG content, without a further decrease in basal glucose production or improvement in hepatic

333 insulin sensitivity. However, 7% weight loss up-regulated skeletal muscle insulin signaling and
334 increased muscle insulin sensitivity. These data support the notion of a causal link between
335 steatosis and hepatic insulin resistance. The mechanism responsible for the link between IHTG
336 content and hepatic insulin sensitivity is unknown, but could be related to an accumulation of
337 intracellular fatty acid metabolites, which can antagonize the effects of insulin signaling on
338 **endogenous** glucose production⁴⁰

339 Our data provide new insights into the potential mechanism responsible for the marked
340 improvement in glycemic control observed within days after Roux-en-y gastric bypass (RYGP)
341 surgery in obese patients with type 2 diabetes⁴¹. For example, in one study, 90% of patients
342 were able to discontinue all diabetes medications and maintain normal glycemia at discharge
343 from the hospital 6 days after RYGP surgery, before much weight loss occurred⁴². These
344 observations have led to the hypothesis that diversion of ingested nutrients from the upper
345 gastrointestinal tract has beneficial effects on glucose homeostasis, possibly because of an
346 altered incretin response to meals⁴³. However, our results suggest that the rapid decrease in
347 liver fat and improvement in hepatic insulin sensitivity that occur after brief CR can completely
348 explain the early improvement in glucose homeostasis observed after bariatric surgery. Food
349 intake is limited after RYGP surgery, and patients usually consume less than 250 kcal/d for
350 several days after the operation⁴¹. Therefore, the marked postoperative reduction in **calorie**
351 intake, itself, likely has profound effects on hepatic fat content and metabolism⁴⁰. Moreover, the
352 decrease in calorie intake makes it is unlikely that diversion of ingested nutrients from the upper
353 gastrointestinal tract has an important effect on glucose metabolism.

354 In summary, the data from this study demonstrate that the effect of moderate calorie
355 restriction in obese subjects with either a low-fat or low-carbohydrate diet on metabolic function
356 is a continuum, with differential effects on specific organ systems. Brief (48 h) CR and minimal
357 weight loss (~2% of initial body weight) primarily affects the liver, manifested by a decrease in
358 IHTG content, an increase in hepatic insulin sensitivity, and a decrease in endogenous glucose

359 production, whereas longer (~11 wks) CR and moderate weight loss (~7% of initial body weight)
360 primarily affects skeletal muscle, manifested by an increase in muscle insulin-mediated glucose
361 uptake and enhanced cellular insulin signaling. These findings help explain the rapid
362 improvement in glucose homeostasis observed after low-calorie diet therapy and bariatric
363 surgery.

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500 study subjects for their participation.

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503 **Table 1.** Baseline body composition and metabolic characteristics of the study subjects

	High-carbohydrate diet group (n=11)	Low-carbohydrate diet group (n=11)	All subjects (n=22)
Age (yrs)	45.4 ± 4.0	41.8 ± 3.1	43.6 ± 2.5
Body weight (kg)	101.0 ± 4.1	101.9 ± 4.0	101.5 ± 2.8
BMI (kg/m ²)	36.9 ± 1.2	36.1 ± 1.0	36.5 ± 0.8
Fat-free mass (kg)	57.2 ± 3.1	57.9 ± 3.2	57.6 ± 2.2
Fat mass (kg)	41.7 ± 2.4	42.1 ± 1.7	41.9 ± 1.4
Fat mass (% body weight)	42.3 ± 1.9	42.3 ± 1.4	42.3 ± 1.1
Total abdominal fat volume (cm ³)	5625 ± 233	5753 ± 321	5686 ± 191
Subcutaneous abdominal fat volume (cm ³)	4010 ± 243	4208 ± 385	4105 ± 219
Intra-abdominal fat volume (cm ³)	1556 ± 234	1544 ± 221	1550 ± 158
Intrahepatic triglyceride content (%)	11.2 ± 2.9	12.4 ± 2.9	11.8 ± 2.0
Plasma glucose (mg/dL)	96.8 ± 2.7	101.5 ± 4.5	99.1 ± 2.6
Plasma insulin (μU/mL)	15.5 ± 2.8	18.7 ± 2.4	17.1 ± 1.8
Plasma triglyceride (mg/dL)	138.9 ± 17	147.7 ± 21.0	143.5 ± 13
HDL-cholesterol (mg/dL)	45.2 ± 2.7	44.1 ± 3.7	44.6 ± 2.2
LDL-cholesterol (mg/dL)	93.3 ± 5.5	96.7 ± 7.3	95.0 ± 4.5

504

505 Values are means ± SEM

506

507 **Table 2.** Percent change from baseline in body weight and metabolic variables after 48 h and
 508 11 wks (7% weight loss) of calorie restriction (CR) in subjects consuming a high carbohydrate
 509 (HC) or low-carbohydrate (LC) diet.

	Percent change after 48 h CR		Percent change after ~11 weeks CR	
	HC	LC	HC	LC
Body weight	-1.6 ± 0.2**	-2.2 ± 0.2**	-7.3 ± 0.6**	-7.6 ± 0.5**
Plasma glucose	-2.6 ± 2.3	-9.8 ± 2.4* [#]	-6.2 ± 1.6*	-8.9 ± 3.0*
Plasma insulin	-22.0 ± 5.1*	-33.9 ± 6.4**	-22.0 ± 5.7*	-38.4 ± 5.2** [#]
C-Peptide	-14.4 ± 3.5*	-26.3 ± 4.5**	-12.0 ± 3.1*	-25.3 ± 3.6**
Free fatty acids	13.9 ± 6.2*	32.1 ± 8.0*	-1.5 ± 9.9	-1.5 ± 7.5
HOMA-IR	-23.8 ± 5.9*	-40.3 ± 6.1** [#]	-27.1 ± 5.1**	-44.0 ± 4.7** [#]

510 Values are means ± SEM.

511 Value significantly different from baseline value: * p<0.05, ** P<0.001.

512 Value significantly different from value in HC group, [#] P<0.05.

513 HOMA-IR: Homeostasis model assessment of insulin resistance

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517 **Figure legends**

518

519 **Figure 1.** Changes in body composition and intrahepatic triglyceride (IHTG) content after 48 h
520 (2% weight loss) and ~11 weeks (7% weight loss) of calorie restriction in obese subjects
521 consuming either a high-carbohydrate or low-carbohydrate 1000 kcal/d deficit diet. Values are
522 means \pm SEM. Value significantly different from baseline value; *P<0.05, **P<0.001; #Value
523 significantly different from corresponding high-carbohydrate diet group, P<0.05. FM=Fat Mass,
524 FFM= Fat-free Mass

525

526 **Figure 2.** Relative changes in basal glucose Rate of appearance (Ra) in plasma after 48 h of
527 calorie restriction and 7% weight loss. Values are means \pm SEM. *Value significantly different
528 from baseline value; P<0.001. # Value significantly different from value in AC group; P<0.001.

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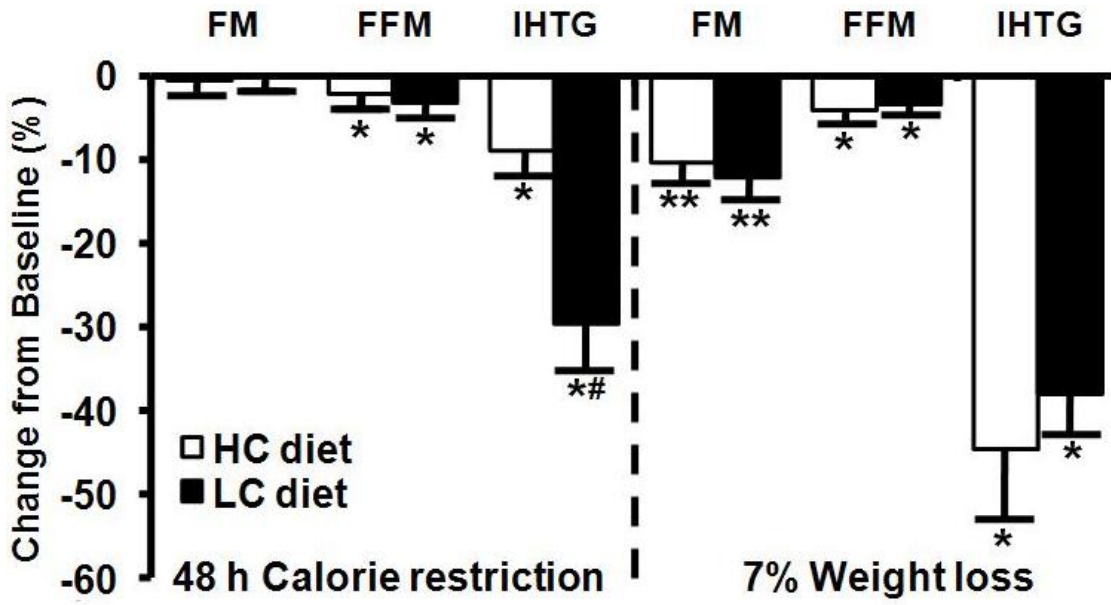
530 **Figure 3.** Hepatic insulin sensitivity index (HISI) (top panel) in subjects consuming either a high-
531 carbohydrate or low-carbohydrate diet and changes in insulin mediated glucose uptake, an
532 index of skeletal muscle insulin sensitivity, in both groups combined (bottom panel) after 48 h
533 and ~11 wks (7% weight loss) of calorie restriction. Value significantly different from baseline
534 value: * P<0.05, ** P<0.001. Value significantly different from value in HC group, # P<0.05.

535

536 **Figure 4.** Changes in phosphoTyr183 JNK, phosphoTyr IRS, and phosphoSer473 Akt/PKB
537 protein levels in vastus lateralis muscle biopsies obtained after 30 min of insulin infusion during
538 a euglycemic-hyperinsulinemic clamp procedure after 48 h and ~11 wks (7% weight loss) of
539 calorie restriction. Values are corrected for total JNK, IRS1, and Akt/PKB protein content and
540 normalized (=0) to values from baseline samples (day 0). Values are means \pm SEM. Value
541 significantly different from corresponding baseline value, *p<0.05.

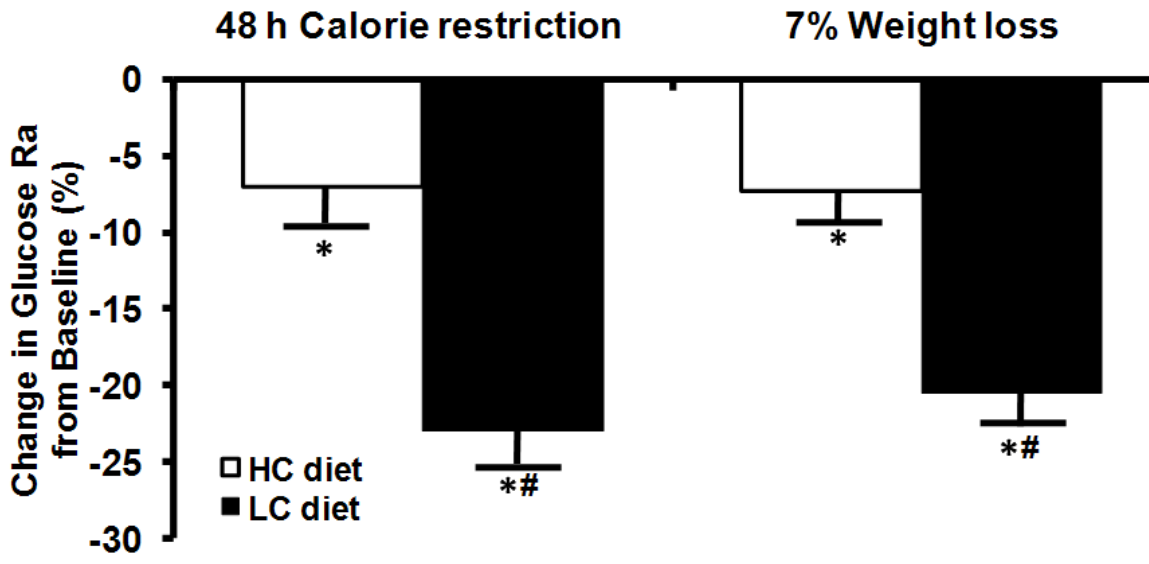
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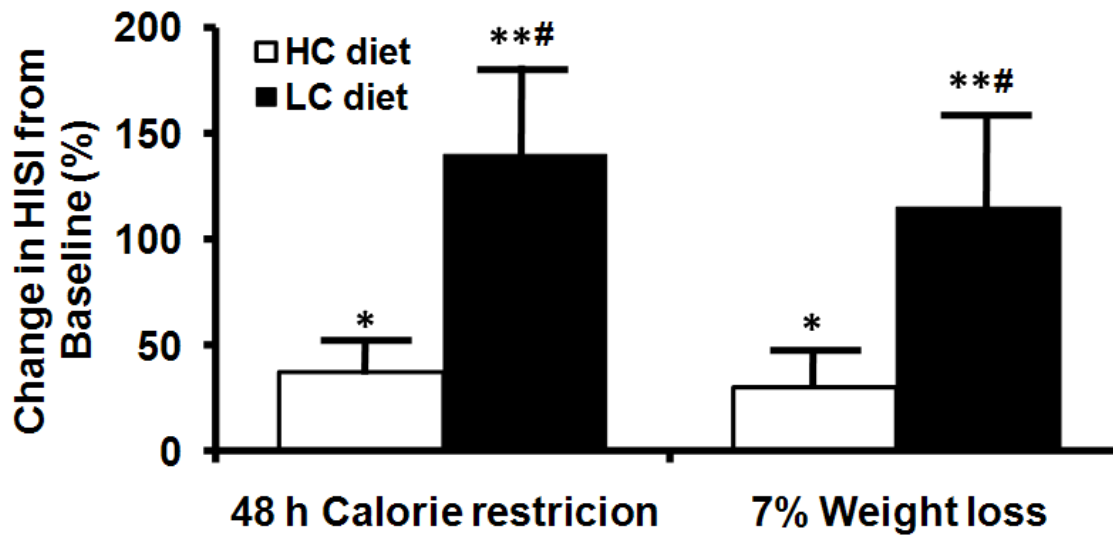
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547 **Figure 1**
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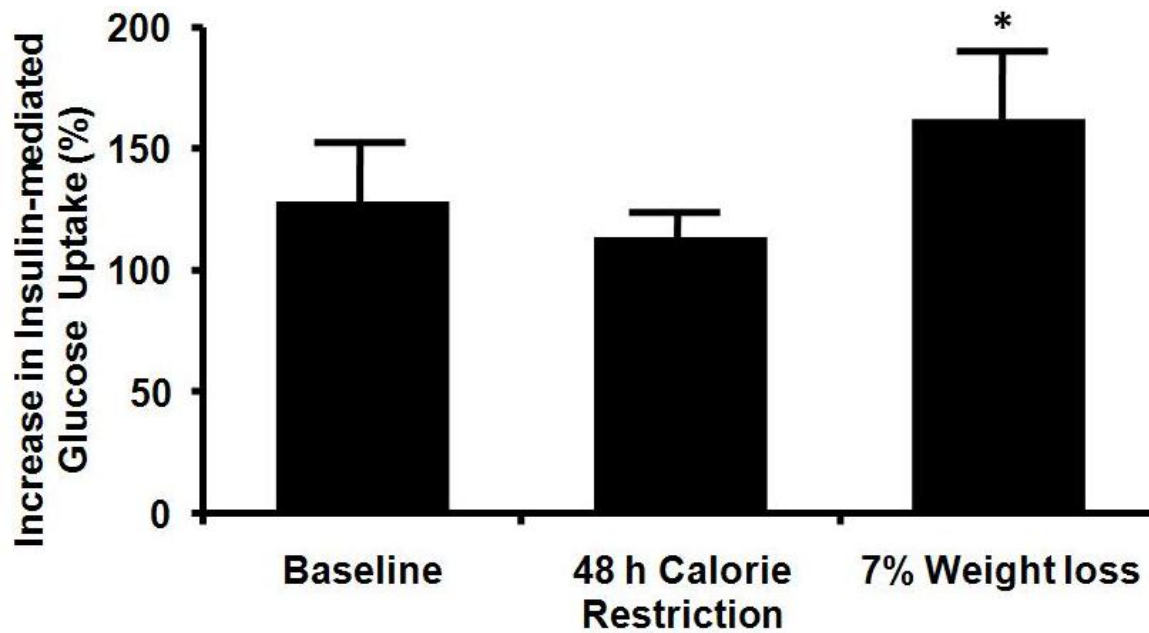


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566 Figure 3.

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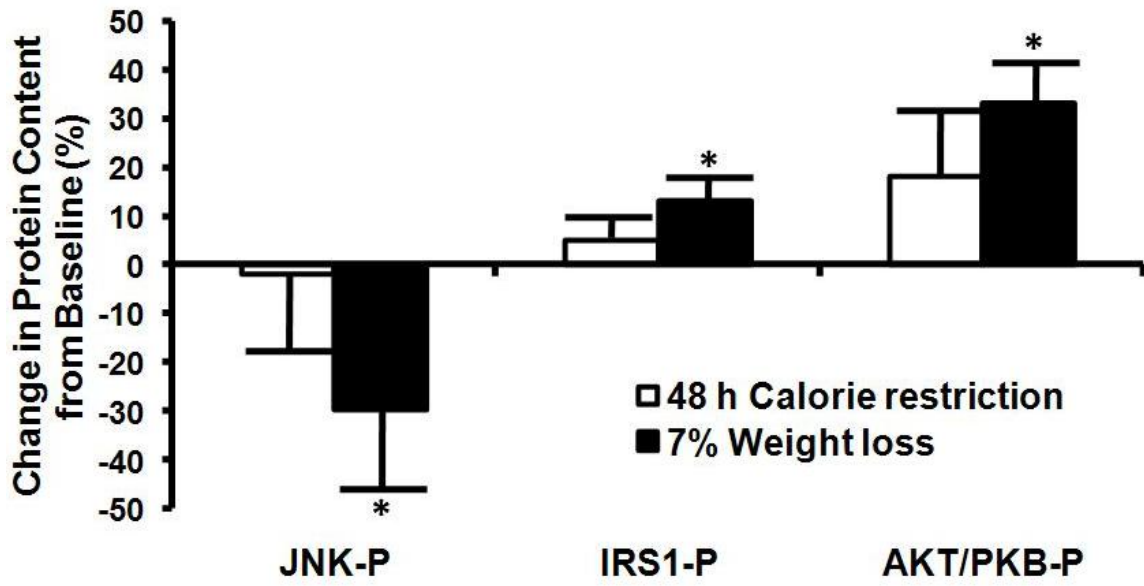
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576 Figure 4

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