Available online at www.sciencedirect.com
ScienceDirect
Metabolism Clinical and Experimental 59 (2010) 1633 – 1641
Metabolism
Clinical and Experimental
www.metabolismjournal.com

# Low-carbohydrate diet does not affect intramyocellular lipid concentration or insulin sensitivity in lean, physically fit men when protein intake is elevated

Jackson G. Green[a,b,*], Nathan A. Johnson[b,c], Toos Sachinwalla[d], Christopher W. Cunningham[a], Martin W. Thompson[b], Stephen R. Stannard[e]

[a]*Research Centre for Maori Health and Development, Massey University, 4442, New Zealand*
[b]*Discipline of Exercise and Sport Science, The University of Sydney, NSW 2006, Australia*
[c]*Institute of Obesity, Nutrition and Exercise, The University of Sydney, NSW 2006, Australia*
[d]*Department of Magnetic Resonance, Rayscan Imaging, Liverpool, NSW 2170, Australia*
[e]*Institute of Food, Nutrition and Human Health, Massey University, 4442, New Zealand*

Received 14 September 2009; accepted 19 March 2010

**Abstract**

It has been speculated that dietary carbohydrate restriction is solely responsibly for mobilization of endogenous lipid stores, elevation of plasma free fatty acid (FFA) concentration, and an associated reduction in insulin sensitivity seen in starvation and low-carbohydrate diets. In 6 healthy men, dietary carbohydrate was eliminated but gluconeogenic substrate supply was maintained by 3 days of very low-carbohydrate/high-protein (HPLC) diet. Results were compared with 3-day starvation and 3-day mixed-carbohydrate diet. Intramyocellular lipid (IMCL) concentration was measured by $^{1}$H magnetic resonance spectroscopy, and insulin sensitivity was determined by intravenous glucose tolerance test. Fasting plasma glucose was significantly reduced ([starvation] 3.5 ± 0.3 vs [HPLC] 4.2 ± 0.4 and [mixed] 4.5 ± 0.3 mmol $L^{-1}$, $P < .01$), and IMCL to water ratio (25.6 ± 5.9 vs 13.6 ± 6.1 and 13.6 ± 3.3 × $10^{-3}$, $P < .01$) and fasting FFA (1179 ± 294 vs 387 ± 232 and 378 ± 120 $\mu$mol $L^{-1}$, $P < .05$) were significantly elevated after starvation but were unchanged after HPLC. Minimal model insulin sensitivity was reduced after starvation (5.7 ± 1.5 vs 14.5 ± 4.8 and 16.5 ± 6.8 L $min^{-1}$ $mU^{-1}$, $P < .05$). Plasma glucose, plasma FFAs, IMCLs, and insulin sensitivity are maintained when an HPLC diet is consumed, despite other forms of carbohydrate deprivation producing marked changes in these measures. We conclude that dietary carbohydrate restriction does not cause circulating FFA to become elevated. However, it remains possible that circulating carbohydrate status has an important influence on plasma FFA and therefore insulin sensitivity in healthy people.


## 1. Introduction

The pathologic association between excess adiposity and the development of insulin resistance is well established [1,2]. On the other hand, insulin resistance has also been shown to be a rapidly adaptive, short-term physiologic response in lean, physically fit humans. To[AU1] fully understand the pathophysiology linking obesity and insulin resistance, we must understand how "healthy" insulin resistance can develop in the lean, physically fit individual. In the latter group, "healthy" insulin resistance is coincident with changes in fat partitioning induced by high-fat diets and lipid heparin infusion [3], situations that result in elevated blood lipids, whereas in the former group, it can be improved with thiazolidinediones [4] and nicotinic acid [5], agents that reduce blood lipids. The common denominator in reduced insulin sensitivity in both groups appears to be accumulation of lipid or its precursors within skeletal muscle fiber [6,7] resulting from a mismatch between fatty acid uptake from the circulation and oxidation by muscle [8]. Although lean, endurance-trained individuals are very insulin sensitive but reportedly possess higher muscle lipid content, situations

Institutional approval: The study was approved by the local institutional human ethics committee and conformed to the Declaration of Helsinki.

* Corresponding author. Massey University, Palmerston North, New Zealand. Tel.: +64 6 350 5799x5264; fax: +64 6 350 5799x5781.
*E-mail address:* j.green@massey.ac.nz (J.G. Green).

0026-0495/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.metabol.2010.03.013

where the intramyocellular lipid (IMCL) store is increased also reduce insulin sensitivity within this group [6,9].

In vitro research has also demonstrated a causative relationship between elevated concentrations of lipids in the extracellular environment, increased accumulation of IMCL [10], impairment of insulin signaling [11], and reduced glucose uptake by the muscle fiber [10,12]. However, although insulin resistance may be caused by an excess of lipid at a cellular level, it is not exclusively a function of dietary fat intake.

A reduction in muscle and whole-body insulin sensitivity is also brought about by short-term (3-day) starvation [6,13,14]. Lean, physically fit men experience equivalent reduction in circulating carbohydrate, accumulation of IMCL, and reduction in insulin sensitivity when exposed to 2 different forms of dietary carbohydrate restriction: starvation or very low-carbohydrate/high-fat diet [6]. Thus, despite a vast difference in whole-body energy intake, at a cellular level, starvation and high-fat/low-carbohydrate diet both result in IMCL accumulation and insulin resistance. The common factors between starvation and low-carbohydrate/high-fat diet–induced insulin resistance in healthy individuals are reduced endogenous (circulating) carbohydrate concentration and increased circulating free fatty acid (FFA) concentration.

It has been speculated that, in lean, healthy individuals, dietary carbohydrate restriction independently mediates IMCL accumulation and insulin resistance [15]. However, previous research has not been able to minimize exogenous carbohydrate intake without also reducing circulating carbohydrate concentrations. As such, it is not clear whether the root cause of insulin resistance in lean, healthy individuals is endogenous or exogenous carbohydrate restriction.

High dietary protein intake provides an excess supply of gluconeogenic substrates and, in conjunction with minimal dietary carbohydrate (<5% of energy), causes an up-regulation in hepatic gluconeogenesis [16-20]. By ensuring high dietary protein intake, yet minimizing carbohydrate intake, we can increase the rate of hepatic gluconeogenic substrates and presumably support normal endogenous carbohydrate concentrations despite minimal exogenous supply. By uncoupling exogenous and endogenous carbohydrate supply, it is possible to determine the independent effects of each on IMCL accumulation and insulin sensitivity.

The aim of this study was to compare the effects of moderate-carbohydrate diet vs 2 forms of carbohydrate restriction, very low-carbohydrate/high-protein (HPLC) diet and starvation, on insulin sensitivity and IMCL. We exposed lean, physically fit men to 2- to 3-day dietary treatments that eliminated dietary carbohydrate but supplied differing amounts of gluconeogenic substrates, namely, starvation and HPLC diet, as well as approximately 3 days of standardized moderate-carbohydrate diet. We hypothesized that the carbohydrate restriction diets would induce equivalent reductions in levels of blood glucose with an associated increase in circulating FFA, increase in IMCL accumulation, and reduction in insulin sensitivity when compared with the moderate-carbohydrate diet.

## 2. Materials and methods

### 2.1. Participants

Six healthy, physically fit men (age = 38.8 ± 12.7 years, body mass = 72.9 ± 8.8 kg) volunteered for this study. All participants reported regularly undertaking exercise for more than 1.5 hours daily at least 5 days per week. Participants' physical characteristics are given in Table 1. Participants were informed of the study protocol and risks before providing their written consent. The study was approved by the local institutional human ethics committee and conformed to the Declaration of Helsinki.

### 2.2. Preliminary testing

One week before participation in the dietary intervention, submaximal and maximal oxygen uptake tests were performed on an electronically braked cycle ergometer (Lode ergometer, Groningen, the Netherlands) as previously described [21]. External power output and $V_{O_2}$ attained during the final minute of each submaximal workload and the maximal ramp were used to formulate regression equations from which workloads for the control exercise bout were derived. On a separate occasion, participants presented at the laboratory after a 12-hour overnight fast for measurement of resting metabolic rate (RMR) using respiratory gas analysis lying supine after 30-minute rest. Body density was assessed via hydrodensitometry; and percentage body fat was then calculated using a 2-compartment model, as previously described [22]. Underwater body weight measurements were corrected for measured residual lung volume using the methods of van der Ploeg et al [23].

### 2.3. Experimental protocol

All participants underwent 3 supervised dietary interventions in random order, each separated by at least 7 days. Each diet period was of 67 hours' duration and comprised a water-only starvation diet, an HPLC diet, or a mixed control diet. Dietary intake and physical activity before initiation of each diet were strictly controlled as previously described [6].

Table 1
Participants' physical characteristics

| | |
|---|---|
| Body fat (%) | 13.5 ± 2.1 |
| RMR (mL $O_2$ $min^{-1}$) | 306 ± 35 |
| $V_{O_{2max}}$ (mL $kg^{-1}$ $min^{-1}$) | 75.5 ± 16.0 |

Values are mean ± standard deviation. $V_{O_{2max}}$ indicates maximal oxygen consumption.

Upon initiation of the diet, participants ingested one of the following: a carbohydrate snack, a protein snack, or nothing, according to their allocation to the mixed diet, HPLC diet, or starvation, respectively. This meal provided 1 g carbohydrate per kilogram body weight (100% energy from carbohydrate) in the mixed diet, an isocaloric meal supplying 1% of energy from carbohydrate and 99% from protein in the HPLC diet, or water only in the starvation diet. Except for the starvation diet, participants ingested an evening meal 2 hours later. In the mixed diet, this meal contained 1.5 g carbohydrate per kilogram body weight and supplied 50% of energy from carbohydrate, 35% of energy from fat, and 15% of energy from protein. In the HPLC diet, this was an isocaloric meal supplying 2% of energy from carbohydrate, 35% of energy from fat, and 63% of energy from protein. Beginning the following morning and continuing for the remainder of the dietary treatment (48 hours), participants received diets in both the mixed and HPLC treatments that provided energy to match a daily expenditure of 1.5× RMR to maintain energy balance. Diets were designed to deliver 50% of energy from carbohydrate, 35% of energy from fat, and 15% of energy from protein in the mixed diet and negligible carbohydrate, 35% of energy from fat, and 65% of energy from protein in the HPLC diet. After exercise in the starvation treatment, participants continued a water-only diet until completion of the experimental treatment. Diet composition was quantified via Foodworks (Xyris Software, Melbourne, Australia) using the New Zealand–Standard database. In all dietary interventions, participants were instructed to maintain activities of daily living and avoid all forms of recreational exercise.

### 2.4. Determination of IMCL content

After 65 hours of each diet, vastus lateralis proton magnetic resonances ($^1$H magnetic resonance spectroscopy) were obtained as previously described [6].

Spectral data were postprocessed by magnetic resonance user interface software (jMRUI version 3.0, EU Project). Vastus lateralis IMCL content was determined by the ratio of the methylene (—$[CH_2]_n$) resonance from IMCL at 1.3 ppm and intracellular water [24]. A 10-resonance model was used to determine IMCL concentration as we have detailed previously [6]. Muscle water signal amplitudes were measured from the non–water-suppressed spectrum using Hankel Lanczos squares singular values decomposition. The $^1$H magnetic resonance spectroscopy processing was performed by an experimenter who was blinded to treatment allocation.

### 2.5. Intravenous glucose tolerance test

After determination of IMCL content, participants reported to the laboratory where glucose tolerance was assessed by frequently sampled intravenous glucose tolerance test (IVGTT) without modification by insulin infusion as previously described [6,25]. Intravenous rather than oral glucose tolerance test was chosen to avoid potential confounding effects of diet (including starvation) [26] on gastric emptying and subsequent glucose tolerance [27]. The IVGTTs were undertaken after a 12-hour overnight fast (or 67-hour fast in the starvation intervention). Additional blood samples were collected at 15, 30, 60, and 120 minutes for determination of plasma FFA concentrations.

### 2.6. Blood sampling

Before the IVGTT, 3 mL of venous blood was sampled by syringe, transferred into EDTA, placed on ice, and then centrifuged at 2000*g* for 8 minutes within 30 minutes of collection. Plasma was decanted off and stored at −85°C for later analysis of FFA concentration. An additional 2 mL of venous blood was drawn into a lithium heparin–pelleted syringe. This sample was mixed and rested on ice for approximately 5 minutes, after which 1.3 mL was transferred into blood tubes and centrifuged at 2000*g* for 8 minutes, and the plasma was frozen (−85°C) for later determination of plasma insulin concentration. Residual blood remained on ice in the lithium heparin–pelleted syringe for later determination of plasma glucose concentration. Blood for glucose, insulin, and FFA measurement was sampled according to these methods during the ensuing IVGTT (1 mL for EDTA) according to the sampling schedule outlined above.

### 2.7. Analytical procedures and calculations

Plasma glucose concentration was measured by autoanalyzer (EML 105; Radiometer, Copenhagen, Denmark). The plasma concentration of FFAs was determined using a Wako NEFA C Test kit (WAKO Chemical, Richmond, VA) scaled for use in a microplate (Bio-Rad, Hercules, CA). All measurements were made in duplicate, and the mean is reported. Glucose tolerance was determined by the rate of decline in plasma glucose concentration between 10 and 40 minutes of IVGTT ($K_g$) as outlined by Galvin et al [28] where $K_g$ is the slope of the least squares linear regression of ln(glucose concentration) vs time between 10 and 40 minutes of the IVGTT. A measure of glucose tolerance was also made by calculation of the incremental area under the curve (iAUC) for plasma glucose above the basal glucose concentration for glucose vs time [29]. Insulin sensitivity index ($S_i$) and glucose effectiveness ($S_g$) were determined via the minimal model analysis of the plasma glucose and insulin response to the IVGTT [25] using MINMOD Millenium (version 6.02, MinMod; University of Southern California, Los Angeles, CA). For these calculations, plasma insulin concentrations were converted from units of picomoles per liter to microunits per liter (1 $\mu$U $L^{-1}$ = 6 pmol $L^{-1}$), and glucose concentrations were converted from units of millimoles per liter to milligrams per deciliter (1 mmol $L^{-1}$ = 18 mg $dL^{-1}$).

Table 2
Forty-eight–hour dietary analysis of group daily macronutrient intake

| | As a percentage energy intake | | | Relative to body mass (kg) | | |
|---|---|---|---|---|---|---|
| | Mixed | HPLC | Starvation | Mixed | HPLC | Starvation |
| Carbohydrate (%) | 49.5 ± 0.7 | 2.0 ± 0.4* | 0 ± 0*,† | 5.2 ± 0.7 | 0.2 ± 0.1* | 0 ± 0*,† |
| Fat (%) | 34.3 ± 1.2 | 34.6 ± 1.4 | 0 ± 0*,† | 1.6 ± 0.2 | 1.6 ± 0.2 | 0 ± 0*,† |
| Protein (%) | 15.8 ± 0.9 | 63.4 ± 1.1* | 0 ± 0*,† | 1.7 ± 0.3 | 6.7 ± 1.0* | 0 ± 0*,† |
| Energy (kJ $d^{-1}$) | 12299 ± 843 | 12314 ± 842 | 0 ± 0*,† | 168.9 ± 23.7 | 169.1 ± 23.5 | 0 ± 0*,† |

Group daily macronutrient intake as percentage energy intake and relative to body mass in kilograms. Values are means ± SD; n = 7 participants.
* Significantly different vs mixed diet ($P < .001$).
† Significantly different vs HPLC diet ($P < .001$).

Indices of estimated whole-body insulin sensitivity were also obtained via (*a*) the ratio of the iAUC for plasma glucose to insulin (SIAUC) [30], (*b*) the method proposed by Galvin et al [28]:

$$SI_{Galvin} = K_g/(\text{iAUC for insulin between 0 and 40 minutes of IVGTT})$$

and (*c*) the index of insulin sensitivity (ISI) described by Matsuda and DeFronzo [31], assuming its application to the IVGTT:

$$ISI = 10000/\left([\text{glucose}]_r \times [\text{insulin}]_r\right) \times \left([\text{glucose}]_{IVGTT} \times [\text{insulin}]_{IVGTT}\right)$$

where $[\text{glucose}]_r$ is the resting glucose concentration in milligrams per deciliter, $[\text{insulin}]_r$ is the resting insulin concentration in milliunits per liter, $[\text{glucose}]_{IVGTT}$ is the mean concentration of glucose during IVGTT in milligrams per deciliter, and $[\text{insulin}]_{IVGTT}$ is the mean concentration of insulin during the IVGTT in milliunits per liter.

### 2.8. Analysis

Differences in nutritional intake, all basal measures (plasma glucose, insulin, FFA, and IMCL to water ratio), and differences in indices of glucose tolerance, insulin sensitivity, and $S_g$ between conditions were compared by 1-way repeated-measures analysis of variance. Plasma glucose, insulin, and FFA concentrations during IVGTT were compared by 2-way repeated-measures analysis of variance for investigation of treatment and time (diet-time) interactions. Pearson correlation coefficients (2-tailed) were used to express the relationship between IMCL to water ratio and $S_i$. Statistical significance was accepted at $P < .05$. All values are expressed as mean ± standard deviation.

## 3. Results

### 3.1. Dietary intake

Carbohydrate intake was significantly lower in the HPLC diet than in the mixed diet ($P < .001$), the diets providing 15 ± 4 and 389 ± 22 g $d^{-1}$, respectively. Dietary protein intake was significantly higher in the HPLC vs the mixed diet ($P < .001$, Table 2), yet daily fat and energy intakes were not different between the HPLC and mixed diets. Despite our efforts to abolish its intake, dietary carbohydrate consumption in the HPLC was significantly greater than during starvation ($P < .001$, Table 2), though by only 0.2 (±0.1) g $kg^{-1}$ $d^{-1}$.

### 3.2. IMCL content

The ratio of vastus lateralis IMCL to water ratio was significantly higher in the starvation condition (25.6 ± 5.9 × $10^{-3}$) than in the mixed (13.6 ± 6.1 × $10^{-3}$, $P < .01$) or HPLC (13.6 ± 3.3 × $10^{-3}$, $P < .01$) conditions. The ratio of IMCL to water was not different between the mixed and HPLC conditions ($P > .99$, Fig. 1). Intramyocellular lipid content demonstrated a significant within-subject correlation between the mixed and starvation conditions

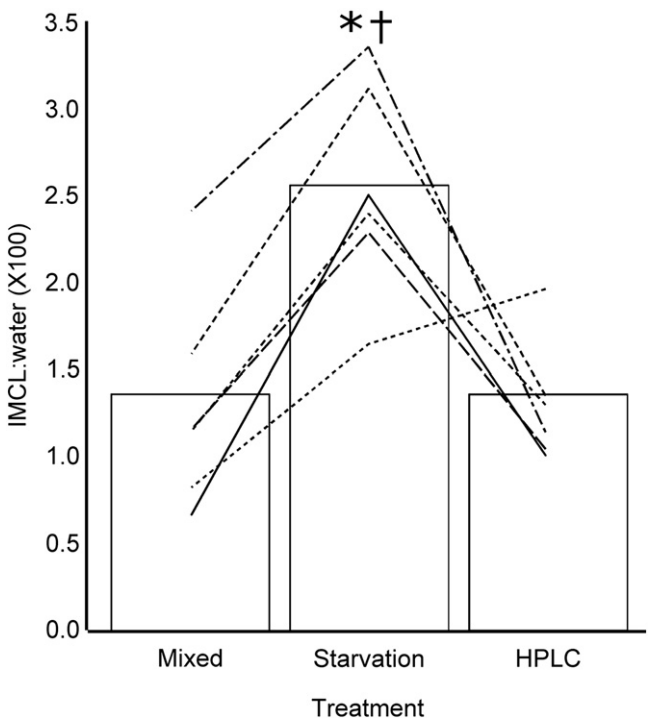


Fig. 1. Effect of 67 hours of mixed diet, HPLC diet, or starvation on IMCL to water ratio. n = 6 subjects. *Significantly different from mixed diet ($P < .01$). †Significantly different from HPLC diet ($P < .01$). Dashed lines indicate individual subject data.

($r$ = 0.82, $P$ < .05), but there were no significant correlations between the HPLC condition and either the mixed or starvation condition.

### 3.3. Basal plasma metabolite and insulin concentrations

Basal plasma glucose concentrations were lower after starvation than after either the mixed ($P$ < .01) or HPLC ($P$ < .05) diet. There was no difference in basal glucose concentration between the mixed and HPLC diets ($P$ = .63, Table 3). The basal plasma insulin concentration was significantly higher after the mixed diet than after either starvation ($P$ < .01) or the HPLC diet ($P$ < .05), but there was no significant difference in basal plasma insulin between starvation and the HPLC diet (Table 3). The basal plasma FFA concentration was significantly higher after starvation than after either the mixed or HPLC diet ($P$ < .01 for both), and there was no difference between basal plasma FFA between the mixed and HPLC diets (Table 3).

### 3.4. Plasma metabolite and insulin responses to IVGTT

In all 3 treatments, the plasma glucose concentration increased rapidly after the intravenous glucose infusion at the initiation of the IVGTT, reaching a peak between 3 and 14 minutes after initiation. Glucose concentration subsequently declined and was not different between treatments and not different from baseline by the last sample of the IVGTT at 180 minutes (Fig. 2A). There was a significant treatment-time interaction effect ($P$ < .05), with starvation resulting in greater plasma glucose concentrations compared with mixed from 30 to 140 minutes and compared with HPLC from 60 to 140 minutes (Fig. 2A). There was also a significant within-subject main effect of treatment, with the overall glucose concentration throughout the IVGTT being significantly lower after the mixed diet (7.6 ± 2.9 mmol $L^{-1}$) than after either the HPLC diet (8.1 ± 3.4 mmol $L^{-1}$, $P$ < .05) or starvation (8.2 ± 2.5 mmol $L^{-1}$, $P$ < .01).

There were 7 data points in which the plasma insulin concentration was below the detectable limit of the assay (6 pmol $L^{-1}$), and a value of 5 pmol $L^{-1}$ was assumed for these points. Plasma insulin concentration increased rapidly after the intravenous glucose infusion at the initiation of the IVGTT, reaching a peak between 3 and 8 minutes after initiation. Insulin concentration subsequently declined and was not different from baseline by 140 minutes and not different between treatments by 40 minutes after initiation of the IVGTT (Fig. 2B). There was a significant treatment-time interaction effect ($P$ < .001), with mixed diet resulting in greater plasma insulin concentrations compared with starvation at baseline and from 30 to 40 minutes and compared with HPLC diet at baseline and from 19 to 40 minutes. Starvation also resulted in significantly greater plasma insulin concentration than HPLC diet at 3 and 19 minutes (Fig. 2B).

In all treatments, plasma FFA concentrations declined after glucose infusion, reaching a minimum at 60 minutes,

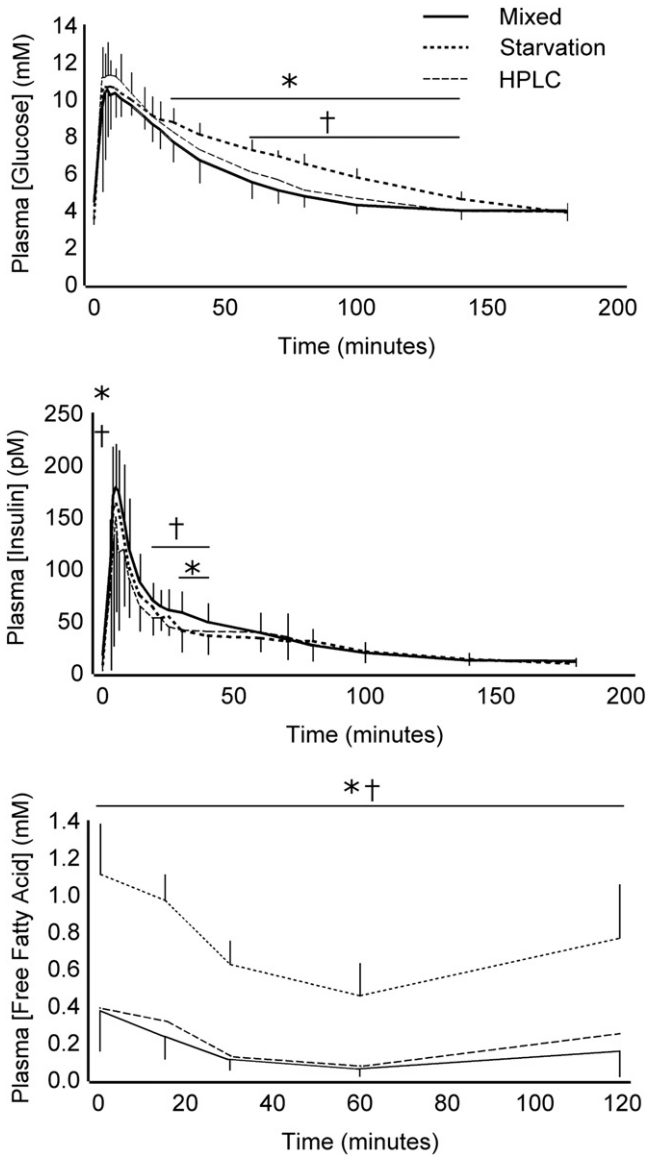


Fig. 2. Effect of 67 hours of mixed diet, HPLC diet, or starvation on plasma glucose, insulin, and FFA concentrations during an IVGTT. n = 6 subjects. *Significantly different, starvation vs mixed diet ($P$ < .05). †Significantly different, starvation vs HPLC diet ($P$ < .05). Error bars indicate 1 standard deviation.

Table 3
Basal plasma substrate and insulin concentrations after 67 hours of dietary intervention

| | Mixed | HPLC | Starvation |
|---|---|---|---|
| Plasma glucose (mmol $L^{-1}$) | 4.5 ± 0.3 | 4.2 ± 0.4 | 3.5 ± 0.3*,‡ |
| Plasma insulin (pmol $L^{-1}$) | 19.8 ± 8.3 | 12.7 ± 3.8† | 8.5 ± 5.1* |
| Plasma FFAs ($\mu$mol $L^{-1}$) | 378 ± 120 | 387 ± 232 | 1179 ± 294*,§ |

Values are means ± SD; n = 6 participants.
* Significantly different vs mixed diet ($P$ < .01).
† Significantly different vs mixed diet ($P$ < .05).
‡ Significantly different vs HPLC diet ($P$ < .01).
§ Significantly different vs HPLC diet ($P$ < .05).

after which time they began to rise again (Fig. 2C). After starvation, there were significantly greater ($P < .001$) plasma FFA concentrations throughout the IVGTT compared with the mixed or HPLC diets. There was also a significant treatment-time interaction ($P < .01$) during the IVGTT, with plasma FFA concentrations declining more rapidly after starvation than after the mixed or HPLC diet (Fig. 2C).

### 3.5. Minimal model analysis

Insulin sensitivity index determined by the minimal model was significantly lower after starvation compared with after mixed diet ($P < .01$) or HPLC diet ($P < .05$). There was no difference in $S_i$ between the mixed and HPLC diets (Table 4). Glucose effectiveness was significantly greater after the HPLC diet than after either the mixed diet or starvation ($P < .01$ for both, Table 4).

### 3.6. Glucose tolerance

Glucose tolerance as estimated by $K_g$ was significantly lower after starvation than after the HPLC diet ($P < .05$). The $K_g$ after the mixed diet was not significantly different from the other treatments. Glucose AUC was significantly higher after starvation than the other treatments ($P < .01$ for both), and there was no difference in AUC between the mixed and HPLC diets (Table 4).

### 3.7. Other estimates of insulin sensitivity

The SIAUC was significantly impaired after starvation ($P < .01$) and HPLC diet ($P < .05$) compared with after the mixed diet and was significantly more impaired after starvation than after HPLC diet ($P < .05$). The $SI_{Galvin}$ was significantly greater after the HPLC diet than after the other interventions ($P < .01$ for both, Table 4). There were no significant differences in ISI between treatments.

Table 4
Effect of 67 hours of mixed diet, HPLC diet, or starvation on indices of glucose tolerance and insulin sensitivity during an IVGTT

| | Mixed | HPLC | Starvation |
|---|---|---|---|
| Minimal model | | | |
| $S_i$ (L $min^{-1}$ $mU^{-1}$) | 16.5 ± 6.8 | 14.5 ± 4.8 | 5.7 ± 1.5*,§ |
| $S_g \times 1000$ ($min^{-1}$) | 4.4 ± 2.2 | 13.2 ± 2.2* | 6.4 ± 2.3‡ |
| Glucose tolerance | | | |
| $K_g \times 10^{-2}$ (mmol $L^{-1}$ $min^{-1}$) | 1.1 ± 0.4 | 1.4 ± 0.5 | 0.8 ± 0.2§ |
| Glucose iAUC (mmol $L^{-1}$ min) | 268 ± 91 | 339 ± 72 | 447 ± 50*,‡ |
| Insulin sensitivity indices | | | |
| SIAUC $\times 10^{-1}$ ($mmol^{-1}$ $\mu U^{-1}$) | 3.4 ± 1.2 | 5.3 ± 1.6† | 7.6 ± 1.8*,‡ |
| $SI_{Galvin}$ ($uU^{-1}$ $min^{-2}$) | 2.9 ± 1.4 | 4.2 ± 0.7* | 2.0 ± 0.4‡ |
| ISI | 17.4 ± 3.0 | 24.4 ± 8.6 | 26.9 ± 10.0 |

Values are means ± SD; n = 6 participants. $S_i$ indicates minimal model insulin sensitivity index; $S_g$, minimal model glucose effectiveness index; $K_g$, rate of decline of ln(plasma glucose) between 10 and 40 minutes of IVGTT [28], with higher values indicating better glucose tolerance; iAUC, incremental area under curve, with higher values reflecting worse glucose tolerance; SIAUC, insulin sensitivity index = iAUC for glucose/insulin, with higher values reflecting lower insulin sensitivity; $SI_{Galvin}$, insulin sensitivity determined via the method proposed by Galvin et al (1992) [28], with higher values indicating higher insulin sensitivity; ISI, insulin sensitivity index [31], with higher values indicating lower insulin sensitivity.

* Significantly different vs mixed diet ($P < .01$).
† Significantly different vs mixed diet ($P < .05$).
‡ Significantly different vs HPLC diet ($P < .01$).
§ Significantly different vs HPLC diet ($P < .05$).

## 4. Discussion

The primary finding of this study is that, in lean, healthy men, carbohydrate restriction in the form of 3 days of HPLC diet does not cause any change in circulating FFA concentration, whole-body insulin sensitivity, or vastus lateralis intramuscular lipid concentration compared with 3 days of mixed, carbohydrate-containing diet. These results contrast with similar protocols that use other forms of dietary carbohydrate restriction (starvation or very high-fat/low-carbohydrate diet) and have produced marked reductions in insulin sensitivity and increases in IMCL concentrations [3,6,32-34].

The almost identical fasting plasma glucose and FFA concentrations after the HPLC and mixed diets, and the marked differences after starvation (Table 3) suggest that there was a continuing supply of glucose entering the blood throughout the HPLC condition. This could be accounted for either by a failure to sufficiently reduce dietary carbohydrate intake in the HPLC diet or by an up-regulation of gluconeogenesis in the face of minimal carbohydrate intake and abundant supply of gluconeogenic substrates. The latter is more likely, as basal insulin concentrations were significantly lower in HPLC than in the mixed condition. Furthermore, we have previously used diets containing approximately 2% of energy from carbohydrate to reduce fasting plasma glucose, increase fasting FFA, and induce insulin resistance and IMCL accumulation almost identically to starvation [6].

To the authors' knowledge, no study has measured the rate of gluconeogenesis or gluconeogenic enzyme activity in humans who have consumed a high-protein/very low-carbohydrate (<5% of energy from carbohydrate) diet. However, where carbohydrate is not eliminated, increased protein intake up-regulates gluconeogenesis. Humans who routinely consume greater amounts of protein have increased overnight-fasted hepatic glucose output and an increased proportion of hepatic glucose output from gluconeogenesis (~65% vs ~45%) compared with controls matched for age, sex, body mass index, and energy intake [35,36]. Infusion of a physiologic dose of gluconeogenic amino acids after an overnight fast doubles the rate of gluconeogenesis [37,38]. If somatostatin is also infused to prevent changes in insulin and glucagon concentrations, amino acid infusion results in increased gluconeogenesis and hyperglycemia [37]. Similarly, when carbohydrate intake was eliminated by 3 to 4 weeks of starvation in obese humans, infusion of alanine caused massive up-regulation of gluconeogenesis and hyperglycemia [16,17].

In the present study, the large amounts of protein consumed would be expected, at least initially, to increase both gluconeogenesis and hepatic glucose output. However, the reduced fasting insulin concentration in HPLC compared with mixed diet suggests that as liver glycogen stores were depleted, glycogenolysis and total hepatic glucose output declined despite increased gluconeogenesis. This is consistent with data showing that gluconeogenesis accounts for only 47% of hepatic glucose output after 14 hours of fasting but 93% after 42 hours of starvation [39]. Thus, in the HPLC condition, we succeeded in eliminating dietary carbohydrate yet maintaining an endogenous carbohydrate supply, albeit at a reduced rate.

It has been proposed that one physiologic stimulus for IMCL accumulation and an associated insulin resistance in the skeletal muscle of lean, healthy men is an absence of dietary carbohydrate [6]. According to this proposal, dietary carbohydrate restriction, reduced blood glucose, and subsequent suppression of insulin and increased circulating FFAs encourage elevations in IMCL, which in turn protects blood glucose concentrations by blunting glucose disposal by skeletal muscle [6]. In the current study, dietary carbohydrate restriction and replacement with protein did not cause a reduction in blood glucose; so the alterations in circulating FFA and IMCL were also absent. The present results suggest therefore that circulating carbohydrate status rather than exogenous carbohydrate intake is an important factor influencing circulating FFA concentration and therefore IMCL accumulation and changes in insulin sensitivity.

It is important to note that carbohydrate restriction is not the only possible cause of elevated FFA. Circulating FFA concentration also increases in response to high dietary fat intake [40,41] even when carbohydrate intake is very high [42]. This effect is likely to be responsible for some of the elevation in circulating FFA observed during a very low-carbohydrate/high-fat diet [40,41]. However, this increase in circulating FFAs from an exogenous source is clearly the result of different physiologic processes to the increase in circulating FFAs from an endogenous source, as seen in starvation conditions [6,32,33] and under investigation in the current study.

The possibility exists that the physiologic stimulus for elevated circulating FFA in starvation conditions is dietary protein or energy restriction per se rather than circulating carbohydrate status. However, in an evolutionary context, the most parsimonious explanation remains that endogenous FFA production is increased in response to reduced circulating glucose. To conclusively demonstrate that circulating carbohydrate status is the stimulus for elevated circulating FFAs in low-energy conditions, it would be necessary to show elevated circulating FFAs in response to a normal-fat, normal-protein, and low-carbohydrate diet and normal circulating FFAs in response to an energy-matched low-fat, normal-protein, and moderate-carbohydrate diet.

The well-established link between an increase in circulating FFA concentration, accumulation of IMCL and its intermediates, and insulin resistance within a homogenous population [43] is further supported by our data that show a good correlation between IMCL to water ratio and $S_i$ (Pearson coefficient $r = -0.68$, $P < .01$). In the present study, it is also important to consider the potential for other effects of a high protein intake on insulin sensitivity. Where dietary carbohydrate intake is not eliminated, high-protein diet [36] and intravenous infusion of amino acids [38] both decrease insulin sensitivity. Conversely, high protein intake triggers an increase in myocellular insulin-like growth factor 1 concentration [44,45], which in turn could increase muscular insulin sensitivity [46] or mask any decrease that would otherwise have occurred. However, as the relationship between IMCL and insulin sensitivity is maintained in the present study, it is unlikely that other effects of dietary protein are quantitatively important.

Another striking feature of the results of current study was the very high glucose effectiveness indicated by high $S_g$ values in the HPLC condition. The $S_g$ is an estimate of the ability of circulating glucose to trigger its own removal from the blood. The high glucose effectiveness in the HPLC condition is also reflected in the high values for $SI_{Galvin}$, as this estimate does not include measures of insulin concentration and so will be inflated by high glucose effectiveness (Table 4). To conclusively determine the tissues responsible for the high $S_g$ values would require tracer techniques that were not used in the current study. Notwithstanding, it seems likely that the liver was the main site of altered non–insulin-stimulated glucose disposal during the IVGTT in the HPLC condition. As discussed above, hepatic glucose output is upregulated by high-protein feeding. In rats, when protein is administered without simultaneous carbohydrate feeding, the increased rate of hepatic glucose output depletes liver glycogen despite increased gluconeogenesis [47,48]. It is teleologically prudent that the liver should replenish its glycogen stores before skeletal muscle, as glycogen in skeletal muscle is difficult to recycle for use by glucose obligate tissues [49]; and in any case, it seems likely that skeletal muscle glycogen stores were not depleted in the HPLC condition, as plasma glucose concentration did not change. Furthermore, a single protein meal increases splanchnic blood flow [50,51], which could potentiate the influence of the liver on whole-body glucose effectiveness. We speculate that, in the HPLC condition, the IVGTT glucose infusion caused the liver to halt glucose output and begin net glucose uptake more rapidly than in the mixed condition and before an insulin response was able to trigger glucose uptake by skeletal muscle.

The primary measure of insulin sensitivity and glucose effectiveness used in this study was the minimal model developed by Bergman et al [52]. The minimal model has good correlation with the hyperinsulinemic-euglycemic clamp [53] and low coefficient of variation [54]. Furthermore, the IVGTT will not be affected by alterations in glucose absorption by the gut, as might be expected after 72 hours of starvation or HPLC diet. The minimal model has

been shown to systematically overestimate $S_g$, with the degree of overestimation increasing with increasing insulin response during the first 20 minutes of the IVGTT. As such, caution should be used when comparing $S_g$ results between groups with differing insulin response [55]. However, in the present study, there were no between-treatment differences in insulin response during the first 20 minutes of the IVGTT (Fig. 2); so the overestimation should remain consistent, and comparison between treatments is valid.

## 5. Conclusion

Normal levels of plasma glucose, plasma FFAs, IMCLs, and insulin sensitivity are maintained when an HPLC diet is consumed, despite other forms of carbohydrate deprivation producing marked changes in these measures. We conclude that dietary carbohydrate restriction does not per se cause circulating FFA to become elevated. However, it remains probable that circulating carbohydrate status has an important influence on circulating FFA concentrations and therefore insulin sensitivity and healthy IMCL accretion in lean, healthy people.

## Acknowledgment

The authors wish to thank David Walton for his assistance with spectroscopy and Dr Patricia Ruell for her assistance with biochemical analysis. This research was supported by the Peter Snell Doctoral Scholarship in Public Health and Exercise Science.

## References

[1] Arner P. Insulin resistance in type 2 diabetes: role of fatty acids. Diabetes Metab Res Rev 2002;18:S5-9.
[2] Henriksen EJ. Exercise effects of muscle insulin signaling and action: invited review: effects of acute exercise and exercise training on insulin resistance. J Appl Physiol 2002;93:788-96.
[3] Bachmann OP, Dahl DB, Brechtel K, Machann J, Haap M, Maier T, et al. Effects of intravenous and dietary lipid challenge on intramyocellular lipid content and the relation with insulin sensitivity in humans. Diabetes 2001;50:2579-84.
[4] Nolan JJ, Ludvik B, Beerdsen P, Joyce M, Olefsky J. Improvement in glucose tolerance and insulin resistance in obese subjects treated with troglitazone. N Engl J Med 1994;331:1188-93.
[5] Vaag A, Skött P, Damsbo P, Gall MA, Richter EA, Beck-Nielsen H. Effect of the antilipolytic nicotinic acid analogue acipimox on whole-body and skeletal muscle glucose metabolism in patients with non–insulin-dependent diabetes mellitus. J Clin Invest 1991;88:1282.
[6] Johnson NA, Stannard SR, Rowlands DS, Chapman PG, Thompson CH, O'Connor H, et al. Effect of short-term starvation versus high-fat diet on intramyocellular triglyceride accumulation and insulin resistance in physically fit men. Experimental Physiology 2006;91:693-703.
[7] Krssak M, Falk Petersen K, Dresner A, DiPietro L, Vogel SM, Rothman DL, et al. Intramyocellular lipid concentrations are correlated with insulin sensitivity in humans: a 1H NMR spectroscopy study. Diabetologia 1999;42:113-6.
[8] van Loon L, Goodpaster B. Increased intramuscular lipid storage in the insulin-resistant and endurance-trained state. Pflugers Arch 2006;451:606-16.
[9] Johnson NA, Stannard SR, Rowlands DS, Chapman PG, Thompson CH, Sachinwalla T, et al. Short-term suppression of plasma free fatty acids fails to improve insulin sensitivity when intramyocellular lipid is elevated. Diabet Med 2006;23:1061-8.
[10] Thompson AL, Lim-Fraser MYC, Kraegen EW, Cooney GJ. Effects of individual fatty acids on glucose uptake and glycogen synthesis in soleus muscle in vitro. Am J Physiol Endocrinol Metab 2000;279:E577-84.
[11] Schmitz-Peiffer C, Craig DL, Biden TJ. Ceramide generation is sufficient to account for the inhibition of the insulin-stimulated PKB pathway in C2C12 skeletal muscle cells pretreated with palmitate. J Biol Chem 1999;274:24202-10.
[12] Shillabeer G, Chamoun C, Hatch G, Lau DCW. Exogenous triacylglycerol inhibits insulin-stimulated glucose transport in L6 muscle cells in vitro. Biochem Biophys Res Commun 1995;207:768-74.
[13] Mansell PI, Macdonald IA. The effect of starvation on insulin-induced glucose disposal and thermogenesis in humans. Metabolism 1990;39:502-10.
[14] Webber J, Taylor J, Greathead H, Dawson J, Buttery PJ, Macdonald IA. Effects of fasting on fatty acid kinetics and on the cardiovascular, thermogenic and metabolic responses to the glucose clamp. Clin Sci 1994;87:697-706.
[15] Stannard SR, Johnson NA. Energy well spent fighting the diabetes epidemic. Diabetes Metab Res Rev 2006;22:11-9.
[16] Felig P, Marliss E, Owen OE, Cahill GF. Role of substrate in the regulation of hepatic gluconeogenesis in fasting man. Adv Enzyme Regul 1969;7:41-6.
[17] Felig P, Marliss E, Pozefsky T, Cahill GFJ. Amino acid metabolism in the regulation of gluconeogenesis in man. Am J Clin Nutr 1970;23:986-92.
[18] Kettelhut IC, Foss MC, Migliorini RH. Glucose homeostasis in a carnivorous animal (cat) and in rats fed a high-protein diet. Am J Physiol Regul Integr Comp Physiol 1980;239:R437-44.
[19] Rossetti L, Rothman DL, DeFronzo RA, Shulman GI. Effect of dietary protein on in vivo insulin action and liver glycogen repletion. Am J Physiol Endocrinol Metab 1989;257:E212-9.
[20] Cowey CB, Knox D, Walton MJ, Adron JW. The regulation of gluconeogenesis by diet and insulin in rainbow trout (*Salmo gairdneri*). Br J Nutr 1977;38:463-70.
[21] Stannard SR, Thompson MW. The effect of participation in Ramadan on substrate selection during submaximal cycling exercise. J Sci Med Sport 2008;11:510-7.
[22] Brozek J, Grande F, Anderson JT, Keys A. Densitometric analysis of body composition: revision of some quantitative assumptions. Ann N Y Acad Sci 1963;110(Body Composition Part I):113-40.
[23] van der Ploeg GE, Gunn SM, Withers RT, Modra AC, Crockett AJ. Comparison of two hydrodensitometric methods for estimating percent body fat. J Appl Physiol 2000;88:1175-80.
[24] Szczepaniak LS, Babcock EE, Schick F, Dobbins RL, Garg A, Burns DK, et al. Measurement of intracellular triglyceride stores by H spectroscopy: validation in vivo. Am J Physiol Endocrinol Metab 1999;276:E977-89.
[25] Pacini G, Bergman RN. MINMOD: a computer program to calculate insulin sensitivity and pancreatic responsivity from the frequently sampled intravenous glucose tolerance test. Comput Methods Programs Biomed 1986;23:113-22.
[26] Corvilain B, Abramowicz M, Fery F, Schoutens A, Verlinden M, Balasse E, et al. Effect of short-term starvation on gastric emptying in humans: relationship to oral glucose tolerance. Am J Physiol Gastrointest Liver Physiol 1995;269:G512-7.
[27] Horowitz M, Edelbroek MAL, Wishart JM, Straathof JW. Relationship between oral glucose tolerance and gastric emptying in normal healthy subjects. Diabetologia 1993;36:857-62.
[28] Galvin P, Ward G, Walters J, Pestell R, Koschmann M, Vaag A, et al. A simple method for quantitation of insulin sensitivity and insulin

release from an intravenous glucose tolerance test. Diabet Med 1992;9:921-8.
[29] Sparti A, Decombaz J. Effect of diet on glucose tolerance 36 hours after glycogen-depleting exercise. Eur J Clin Nutr 1992;46:377-85.
[30] Horowitz JF, Kaufman AE, Fox AK, Harber MP. Energy deficit without reducing dietary carbohydrate alters resting carbohydrate oxidation and fatty acid availability. J Appl Physiol 2005;98:1612-8.
[31] Matsuda M, DeFronzo RA. Insulin sensitivity indices obtained from oral glucose tolerance testing: comparison with the euglycemic insulin clamp. Diabetes Care 1999;22:1462-70.
[32] Wietek BM, Machann J, Mader I, Thamer C, Haring HU, Claussen CD, et al. Muscle type dependent increase in intramyocellular lipids during prolonged fasting of human subjects: a proton MRS study. Horm Metab Res 2004;36:639-44.
[33] Stannard SR, Thompson MW, Fairbairn K, Huard B, Sachinwalla T, Thompson CH. Fasting for 72 h increase intramyocellular lipid content in nondiabetic, physically fit men. Am J Physiol Endocrinol Metab 2002;283:E1185-91.
[34] Schrauwen-Hinderling VB, Kooi ME, Hesselink MKC, Moonen-Kornips E, Schaart G, Mustard KJ, et al. Intramyocellular lipid content and molecular adaptations in response to a 1-week high-fat diet. Obesity research 2005;13:2088.
[35] Linn T, Geyer R, Prassek S, Laube H. Effect of dietary protein intake on insulin secretion and glucose metabolism in insulin-dependent diabetes mellitus. J Clin Endocrinol Metab 1996;81:3938-43.
[36] Linn T, Santosa B, Grönemeyer D, Aygen S, Scholz N, Busch M, et al. Effect of long-term dietary protein intake on glucose metabolism in humans. Diabetologia 2000;43:1257-65.
[37] Krebs M, Brehm A, Krssak M, Anderwald C, Bernroider E, Nowotny P, et al. Direct and indirect effects of amino acids on hepatic glucose metabolism in humans. Diabetologia 2003;46:917-25.
[38] Tremblay F, Krebs M, Dombrowski L, Brehm A, Bernroider E, Roth E, et al. Overactivation of S6 kinase 1 as a cause of human insulin resistance during increased amino acid availability. Diabetes 2005;54: 2674-84.
[39] Landau BR, Wahren J, Chandramouli V, Schumann WC, Ekberg K, Kalhan SC. Contributions of gluconeogenesis to glucose production in the fasted state. J Clin Invest 1996;98:378-85.
[40] Schenk S, Cook JN, Kaufman AE, Horowitz JF. Postexercise insulin sensitivity is not impaired after an overnight lipid infusion. Am J Physiol Endocrinol Metab 2005;288:E519-25.
[41] Schenk S, Horowitz JF. Acute exercise increases triglyceride synthesis in skeletal muscle and prevents fatty acid–induced insulin resistance. J Clin Invest 2007;117:1690-8.
[42] Zehnder M, Christ E, Ith M, Acheson K, Pouteau E, Kreis R, et al. Intramyocellular lipid stores increase markedly in athletes after 1.5 days lipid supplementation and are utilized during exercise in proportion to their content. European Journal of Applied Physiology 2006;98:341-54.
[43] He J, Goodpaster BH, Kelley DE. Effects of weight loss and physical activity on muscle lipid content and droplet size. Obesity Res 2004;12: 761-9.
[44] Sanchez-Gomez M, Malmlöf K, Mejia W, Bermudez A, Ochoa MT, Carrasco-Rodriguez S, et al. Insulin-like growth factor–I, but not growth hormone, is dependent on a high protein intake to increase nitrogen balance in the rat. Br J Nutr 1999;81:145-52.
[45] Caverzasio J, Shigematsu T, Rizzoli R, Bonjour JP. Potential role of IGF-1 in parathyroid hormone-related renal growth induced by high protein diet in uninephrectomized rats. Kidney Int 1995;48:33-8.
[46] Moses AC, Young SC, Morrow LA, O'Brien M, Clemmons DR. Recombinant human insulin-like growth factor I increases insulin sensitivity and improves glycemic control in type II diabetes. Diabetes 1996;45:91-100.
[47] Moundras C, Remesy C, Demigne C. Dietary protein paradox: decrease of amino acid availability induced by high-protein diets. Am J Physiol Gastrointest Liver Physiol 1993;264:G1057-65.
[48] Gannon MC, Nuttall FQ. Physiological doses of oral casein affect hepatic glycogen metabolism in normal food-deprived rats. J Nutr 1995;125:1159-66.
[49] Wagenmakers AJ. Muscle amino acid metabolism at rest and during exercise: role in human physiology and metabolism. Exerc Sport Sci Rev 1998;26:287.
[50] Brandt JL, Castleman L, Ruskin HD, Greenwald J, Kelly JJ, Jones A. The effect of oral protein and glucose feeding on splanchnic blood flow and oxygen utilization in normal and cirrhotic subjects. J Clin Invest 1955;34(7 Pt 1):1017.
[51] Wahren J, Felig P, Hagenfeldt L. Effect of protein ingestion on splanchnic and leg metabolism in normal man and in patients with diabetes mellitus. J Clin Invest 1976;57:987.
[52] Bergman RN, Ider YZ, Bowden CR, Cobelli C. Quantitative estimation of insulin sensitivity. Am J Physiol Endocrinol Metab 1979;236: E667-77.
[53] Bergman RN, Prager R, Volund A, Olefsky JM. Equivalence of the insulin sensitivity index in man derived by the minimal model method and the euglycemic glucose clamp. J Clin Invest 1987;79:790.
[54] Ferrari P, Alleman Y, Shaw S, Riesen W, Weidmann P. Reproducibility of insulin sensitivity measured by the minimal model method. Diabetologia 1991;34:527-30.
[55] Cobelli C, Bettini F, Caumo A, Quon MJ. Overestimation of minimal model glucose effectiveness in presence of insulin response is due to undermodeling. Am J Physiol Endocrinol Metab 1998;275: E1031-6.