Intermittent fasting does not affect whole-body glucose, lipid, or protein metabolism

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ABSTRACT

Background: Intermittent fasting (IF) was shown to increase whole-body insulin sensitivity, but it is uncertain whether IF selectively influences intermediary metabolism. Such selectivity might be advantageous when adapting to periods of food abundance and food shortage.

Objective: The objective was to assess effects of IF on intermediary metabolism and energy expenditure.

Design: Glucose, glycerol, and valine fluxes were measured after 2 wk of IF and a standard diet (SD) in 8 lean healthy volunteers in a crossover design, in the basal state and during a 2-step hyperinsulinemic euglycemic clamp, with assessment of energy expenditure and phosphorylation of muscle protein kinase B (AKT), glycogen synthase kinase (GSK), and mammalian target of rapamycin (mTOR). We hypothesized that IF selectively increases peripheral glucose uptake and lowers proteolysis, thereby protecting protein stores.

Results: No differences in body weight were observed between the IF and SD groups. Peripheral glucose uptake and hepatic insulin sensitivity during the clamp did not significantly differ between the IF and SD groups. Likewise, lipolysis and proteolysis were not different between the IF and SD groups. IF decreased resting energy expenditure. IF had no effect on the phosphorylation of AKT but significantly increased the phosphorylation of glycogen synthase kinase (GSK) and mammalian target of rapamycin (mTOR). We hypothesized that IF selectively increases peripheral glucose uptake and lowers proteolysis, thereby protecting protein stores.

Conclusions: IF does not affect whole-body glucose, lipid, or protein metabolism in healthy lean men despite changes in muscle phosphorylation of GSK and mTOR. The decrease in resting energy expenditure after IF indicates the possibility of an increase in weight during IF when caloric intake is not adjusted. This study was registered at www. trialregister.nl as NTR1841. Am J Clin Nutr 2009;90:1244–51.

INTRODUCTION

Intermittent fasting (IF) has been suggested to mimic cycles of feast and famine (food abundance and food shortage) as may have been physiologic in the late Paleolithic era (1). Accordingly, it has been postulated that humans have developed metabolic pathways that oscillate with the cycles of feast-famine and physical activity–rest (2). Such adaptive mechanisms were proposed to be part of a thrifty genotype, necessary for survival during shortage of food, protecting muscle lipid and glycogen stores as well as lean body mass (LBM, ie, body protein) during fasting while replenishing fuel stores during refeeding (2, 3).

Previous human studies of the metabolic effects of IF are scarce and unequivocal (1, 4, 5). However, the increase of whole-body insulin sensitivity after 2 wk of IF in healthy volunteers as shown by Halberg et al (1) is of interest because it may provide a simple tool to improve insulin sensitivity in subjects with insulin resistance.

IF consists of repetitive bouts of short-term fasting. The latter is characterized by integrated alterations in intermediary metabolism that guarantee substrate availability for energy production necessary for survival (6–9). Specifically, fasting (ie, 62 h) induces a reduction in peripheral insulin sensitivity via a decrease in phosphorylation of both protein kinase B (AKT) and AS160 after short-term fasting (8). Hepatic insulin sensitivity may be affected by fasting as well (10), but not all studies have confirmed this (11). To date, it is uncertain whether IF influences hepatic insulin sensitivity (1).

Furthermore, short-term fasting increases proteolysis (12, 13) and decreases protein synthesis (14). The fall in protein synthesis and the rise in proteolysis during short-term fasting occur through decreased activation of AKT and the downstream mammalian target of rapamycin (mTOR) (15). It is currently unknown whether IF affects protein metabolism via changes in activity of mTOR.

In this study, we measured glucose, glycerol, and valine fluxes after 2 wk of IF and after 2 wk of a standard isocaloric diet (SD) both in the basal state and during a 2-step hyperinsulinemic euglycemic clamp (stable-isotope technique). Immunoblots were performed of crucial signaling proteins in the insulin and mTOR signaling pathways in muscle tissue samples obtained in the basal state and during the clamp. We hypothesized that IF selectively increases peripheral insulin sensitivity of glucose uptake via increased phosphorylation of AKT and AS160. Additionally, we...
expected proteolysis to be lower after IF, thereby protecting protein stores.

SUBJECTS AND METHODS

Subjects

Eight men were recruited via advertisements in local magazines. Subjects were in self-reported good health, confirmed by medical history and physical examination. Criteria for inclusion were 1) absence of a family history of diabetes; 2) age 18–35 y; 3) white race; 4) body mass index (in kg/m²) of 20–25; 5) normal oral-glucose-tolerance test result according to criteria of the American Diabetes Association (16); 6) normal results from a routine blood examination; 7) no excessive sport activities, ie, <3 times/wk; and 8) no medication use. Additionally, subjects who were not in the habit of eating breakfast every day were excluded. Written informed consent was obtained from all subjects after they received an explanation of the purpose, nature, and potential risks of the study. The study was approved by the Medical Ethical Committee of the Academic Medical Center of the University of Amsterdam.

Experimental protocol

The subjects were studied twice in balanced assignment (crossover design) to 2 wk of IF or 2 wk of an SD. The study days were separated by ≥4 wk to minimize influences of the previous diet. Subjects were fasting from 2000 on the evening before the study days and were allowed to drink water only.

After admission at the Metabolic Unit at 0730, a catheter was inserted into an antecubital vein for infusion of stable-isotope tracers, insulin, and glucose. Another catheter was inserted retrogradely into a contralateral hand vein and kept in a thermoregulated (60°C) clear plastic box for sampling of arterialized venous blood. Saline was infused as 0.9% NaCl at a rate of 50 mL/h to keep the catheters patent. [6,6-2H₂]Glucose, [1,1,2,3,3-2H₅]glycerol, and L-[1-13C]valine were used as tracers (>99% enriched; Cambridge Isotopes, Andover, MA) to study glucose kinetics, lipolysis (total triglyceride hydrolysis), and proteolysis, respectively.

At t = 0 h (0800), blood samples were drawn for determination of background enrichments. Then, a primed continuous infusion of isotopes was started: [6,6-2H₂]glucose (prime: 8.8 μmol/kg; continuous: 0.11 μmol · kg⁻¹ · min⁻¹), [1,1,2,3,3-2H₅]glycerol (prime: 1.6 μmol/kg; continuous: 0.11 μmol · kg⁻¹ · min⁻¹), and L-[1-13C]valine (prime: 13.7 μmol/kg; continuous: 0.15 μmol · kg⁻¹ · min⁻¹) and continued until the end of the study. After a 2-h equilibration period (14 h of fasting), 3 blood samples were drawn for isotope enrichments and 1 sample for glucoregulatory hormones and free fatty acids (FFAs). Thereafter (t = 3.0 h), a 2-step hyperinsulinemic euglycemic clamp was started: step 1 included an infusion of insulin at a rate of 10 mU · m⁻² · min⁻¹ (Actrapid 100 IU/mL; Novo Nordisk Farma BV, Alphen aan den Rijn, Netherlands) to assess hepatic insulin sensitivity. Glucose 20% was started to maintain a plasma glucose concentration of 5 mmol/L. [6,6-2H₂]Glucose was added to the 10% glucose solution to achieve glucose enrichments of 1% to approximate the values for enrichment reached in plasma and thereby minimizing changes in isotopic enrichment due to changes in the infusion rate of exogenous glucose (17).

Plasma glucose concentrations were measured every 5 min at the bedside. After 2 h (t = 5 h), 5 blood samples were drawn at 5-min intervals for the measurement of glucose concentrations and isotopic enrichments. Another blood sample was drawn for measurement of glucoregulatory hormones and FFAs. Hereafter, insulin infusion was increased to a rate of 40 mU · m⁻² · min⁻¹ (step 2) to assess peripheral insulin sensitivity. After another 2 h (t = 7 h), blood sampling was repeated.

Intermittent fasting and standard diet

The 2 wk of IF in the present study were the same as in the study by Halberg et al (1), in which the subjects fasted every second day for 20 h (Figure 1). Fasting started at 2200 and ended at 1800 the following day. In total, the subjects fasted 7 times. During the SD, the subjects were not allowed to skip meals. The day before the study days, subjects were not fasting, but consumed their diet as on nonfasting days.

The caloric intake during both diet periods was equal to avoid energy restriction with secondary effects on metabolism. Distribution of calories between carbohydrates, fat, and protein were kept equal as well. To increase comparability, the volunteers ate mainly bread, fruit, and dairy products (60% of daily energy intake) supplemented with liquid meals (40% of daily energy intake): Nutridrink [Nutricia Advanced Medical Nutrition, Zoetermeer, Netherlands; per serving (200 mL): 300 kcal, 12.0 g protein, 36.8 g carbohydrate, and 11.6 g fat].

Furthermore, the intake of macronutrients was equal and isocaloric during both diet periods. To succeed in finding the correct caloric need for the volunteers, resting energy expenditure (REE) was measured with indirect calorimetry at inclusion. Then total energy requirements were calculated by a dietitian based on REE, dietary history, and activity score, which resulted in an average of advised caloric intake of 130–140% of REE. Our aim was to prevent weight loss, and the diets were adjusted in cases of a 1-kg weight change. Therefore, the volunteers visited the Metabolic Unit for weight control on a validated scale (SECA 701 column scale; SECA, Hamburg, Germany) on day one of each diet and then 2 times/wk.

Body composition, indirect calorimetry, and muscle biopsies

Body composition, oxygen consumption, and carbon dioxide production were measured as described earlier (18). REE,
TABLE 1
Subject characteristics at inclusion

<table>
<thead>
<tr>
<th>Values</th>
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<tbody>
<tr>
<td>Age (y)</td>
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<tr>
<td>Weight (kg)</td>
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<tr>
<td>Height (cm)</td>
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<tr>
<td>BMI (kg/m²)</td>
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<tr>
<td>Lean body mass (%)</td>
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<tr>
<td>Fat mass (%)</td>
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<tr>
<td>FPG (mmol/L)</td>
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<tr>
<td>OGTT (mmol/L)</td>
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<tr>
<td>Energy requirement (kcal/d)</td>
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<tr>
<td>Protein (g/d)</td>
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<tr>
<td>Fat (g/d)</td>
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<tr>
<td>Carbohydrate (g/d)</td>
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</tbody>
</table>

All values are medians (minimum–maximum). FPG, fasting plasma glucose; OGTT, oral-glucose-tolerance test (plasma glucose concentrations of 75 g glucose 2 h after ingestion).

Table 2
Results of indirect calorimetry in the intermittent fasting (IF) and standard diet (SD) groups

<table>
<thead>
<tr>
<th></th>
<th>Basal state</th>
<th>Hyperinsulinemic euglycemic clamp</th>
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<tbody>
<tr>
<td></td>
<td>IF (n = 8)</td>
<td>SD (n = 8)</td>
</tr>
<tr>
<td>REE (kcal/d)</td>
<td>1698 (1280–1754)</td>
<td>1716 (1478–1901)</td>
</tr>
<tr>
<td>CHO (µmol · kg⁻¹ · min⁻¹)</td>
<td>4.4 (1.5–9.2)</td>
<td>6.5 (2.3–15.9)</td>
</tr>
<tr>
<td>FAO (µmol · kg⁻¹ · min⁻¹)</td>
<td>1.5 (1.0–1.8)</td>
<td>1.4 (0.8–2.2)</td>
</tr>
<tr>
<td>RQ</td>
<td>0.76 (0.72–0.83)</td>
<td>0.79 (0.68–0.87)</td>
</tr>
</tbody>
</table>

All values are medians (minimum–maximum). REE, resting energy expenditure; CHO, carbohydrate oxidation; FAO, fatty acid oxidation; RQ, respiratory quotient.

Glucose, lipid, and valine measurements

Plasma glucose concentrations were measured with the glucose oxidase method using a Biosen C-line plus glucose analyzer (EKF Diagnostics, Barleben/Magdeburg, Germany). Plasma FFA concentrations were measured with an enzymatic colorimetric method (NEFA-C test kit; Wako Chemicals GmbH, Neuss, Germany) with an intraassay variation of 1%, interassay variation of 4–6%, and detection limit of 0.02 mmol/L.

Glucoregulatory and thyroid hormones

Insulin was determined on an Immulite 2000 system (Diagnostic Products Corporation, Los Angeles, CA) with a chemiluminescent immunometric assay, intrassay variation of 4–6%, and a detection limit of 15 pmol/L. Glucagon was determined with the Linco Radioimmunoassay (St Charles, MO) with an intraassay variation of 9–10%, an interassay variation of 5–7%, and a detection limit of 15 ng/L. Cortisol, norepinephrine, adrenaline, and adrenocorticotropin were measured as described earlier.

Thyroid-stimulating hormone was measured by time-resolved fluoroimmunoassay (Delfia hTSH Ultra; Wallac Oy, Turku, Finland), and thyroxine, triiodothyronine, and reverse thyroxine were measured by an in-house radioimmunoassay method.

Immunoblotting

Muscle tissue was prepared and separated by electrophoresis on a polyacrylamide gel, and immunoblots were visualized by enhanced chemiluminescence as described previously (8). Chemicals for enhanced chemiluminescence were from Sigma (St Louis, MO). Phosphospecific anti-AKTser473, phosphospecific anti-glycogen synthase kinase-3-ser9 (GSK, β-isofrom), anti-mTORser2448, and anti-mTORser2461, total anti-AKT, and total anti-elf4E (loading control) were from Cell Signaling (Boston, MA). Phosphospecific anti-AS160-thr642 was from GeneTex Inc (San Antonio, TX). Total anti-GSK3 and anti-mTOR were from Oncogene (Cambridge, MA) and Campro Scientific (Veenendaal, Netherlands). The results are presented as the fold increase compared with the control.

Calculations and statistics

Endogenous glucose production (EGP) and the peripheral glucose uptake of glucose (Rd) were calculated by using modified versions of the Steele Equations, as described previously (17, 21). EGP and Rd were expressed as µmol · kg⁻¹ · min⁻¹. Glucose metabolic clearance rates (MCRglucose) were calculated as MCR = rate of appearance (Ra) of glucose/[glucose].

Total triglyceride hydrolysis/lipolysis (glycerol turnover) was calculated by using formulas for steady state kinetics adapted for stable isotopes (22) and was expressed as µmol · kg⁻¹ · min⁻¹ and as µmol/kcal (23). Proteolysis was calculated by using the reciprocal pool model (α-KIV Ra), as described earlier (24).

All subjects served as their own controls. All data were analyzed with nonparametric tests. A 2-factor analysis of variance with repeated measures with factor 1 (time), factor 2 (IF compared with SD), and interaction was performed for EGP and Rd and for other substrate concentrations, kinetics, and hormone concentrations when these were assessed at all 3 occasions (basal, step 1 and 2 of the clamp) to detect differences between both diets. If significant effects were detected for factor 2 and 3, pairwise comparisons were performed with Wilcoxon’s signed-rank test.
interaction (or interaction alone), a post hoc Bonferroni test was performed, taking into account multiple comparisons to assess differences between SD and IF. REE, thyroid hormones, and muscle biopsies in the basal state and step 2 of the clamp were analyzed with Wilcoxon’s signed-rank test. Correlations were expressed as Spearman’s rank correlation coefficients (\( \rho \)). The SPSS statistical software program (version 14.0.2; SPSS Inc, Chicago, IL) was used for the statistical analysis. Data are presented as median (minimum–maximum).

RESULTS

Anthropometric characteristics

The subject characteristics at inclusion are presented in Table 1. Weight after 2 wk of IF (74.8 kg; 58.7–85.3 kg) was not significantly different from that after the SD (74.5 kg; 58.4–85.7 kg) \((P = 0.58)\). Fat mass and LBM were not significantly different after the IF and the SD: fat mass: 13.3% (11.7–20.9%) and 12.8% (9.9–13.5%), respectively \((P = 0.46)\); LBM: 86.7% (76.5–88.2%) and 87.3% (84.1–90.1%) respectively \((P = 0.46)\).

REE, glucose, and fatty acid oxidation

After 2 wk of IF, REE was significantly lower in the basal state than after 2 wk of the SD: median difference of –59 (–201–26) kcal/d (Table 2). During the clamp, no differences in REE were found. CHO and FAO were not different between the IF and SD groups during either the basal state or the clamp. When CHO and FAO were expressed per LBM, similar results were found (data not shown). Also, changes in CHO and FAO during the clamp were not different between the IF and SD groups (data not shown).

Glucose, lipid, and valine measurements

Plasma glucose concentrations and EGP in the basal state were not statistically different after the IF compared with after the SD (Table 3). MCRglucose in the basal state was not different between the IF and SD groups [2.4 (1.9–2.8) \( \text{mL} \cdot \text{kg}^{-1} \cdot \text{min}^{-1} \) compared with 2.3 (2.0–2.9) \( \text{mL} \cdot \text{kg}^{-1} \cdot \text{min}^{-1} \), respectively; \( P = 0.58 \)]. Basal state plasma FFA concentrations and lipolysis after 2 wk of IF were not different from those after the SD. When lipolysis was expressed as \( \mu \text{mol}/\text{kcal} \), no differences were found between the IF and SD groups in the basal state: 104 (58–143) \( \mu \text{mol}/\text{kcal} \) and 81 (45–211) \( \mu \text{mol}/\text{kcal} \), respectively (\( P = 0.26 \)).

During step 1 of the clamp (Table 3), plasma glucose concentrations, EGP, and Rd were not different between the IF and SD groups. Likewise plasma FFA concentrations and lipolysis were not different between the IF and SD groups.

Step 2 of the clamp showed no differences in glucose Rd between the IF and SD groups (EGP being suppressed). NOGD was not different between the IF and SD groups during step 2 of the clamp: 28.5% (20.4–50.5%) and 31.7% (19.6–45.2%), respectively \((P = 0.78)\). Also, no differences were observed in lipolysis between the IF and SD groups during step 2 of the clamp. When lipolysis was expressed as \( \mu \text{mol}/\text{kcal} \), no differences were found between the IF and SD groups during step 2 of the clamp: 27 (14–45) \( \mu \text{mol}/\text{kcal} \) and 26 (21–50) \( \mu \text{mol}/\text{kcal} \) respectively (\( P = 0.74 \)). KIV Ra (expressing proteolysis) was not significantly different from that after the SD (74.5 kg; 58.4–85.7 kg) \((P = 0.46)\); LBM: 86.7% (76.5–88.2%) and 87.3% (84.1–90.1%) respectively \((P = 0.46)\).

**Table 3**

| Glucose and lipid kinetics and glucoregulatory hormones in the intermittent fasting (IF) and standard diet (SD) groups. |
|---------|---------|---------|---------|
| Glucose (mmol/L) | 4.8 (4.3–4.7) | 4.9 (4.7–5.3) | 4.7 (4.0–4.9) | 4.5 (4.2–4.7) | 5.2 (4.8–5.5) | 4.9 (4.4–5.3) |
| EGP (\( \mu \text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1} \)) | 11.4 (10.0–13.5) | 10.4 (9.6–12.5) | 11.6 (10.6–13.5) | 11.6 (10.6–13.5) | 11.6 (10.6–13.5) | 11.6 (10.6–13.5) |
| Rd (\( \mu \text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1} \)) | 4.8 (3.8–5.8) | 4.8 (3.8–5.8) | 4.8 (3.8–5.8) | 4.8 (3.8–5.8) | 4.8 (3.8–5.8) | 4.8 (3.8–5.8) |
| Lipolysis (\( \mu \text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1} \)) | 1.74 (1.61–1.89) | 1.74 (1.61–1.89) | 1.74 (1.61–1.89) | 1.74 (1.61–1.89) | 1.74 (1.61–1.89) | 1.74 (1.61–1.89) |
| KIV Ra (\( \mu \text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1} \)) | 34 (26–42) | 34 (26–42) | 34 (26–42) | 34 (26–42) | 34 (26–42) | 34 (26–42) |
| Insulin (\( \mu \text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1} \)) | 3.8 (2.5–5.3) | 3.8 (2.5–5.3) | 3.8 (2.5–5.3) | 3.8 (2.5–5.3) | 3.8 (2.5–5.3) | 3.8 (2.5–5.3) |
| Glucagon (\( \mu \text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1} \)) | 0.18 (0.12–0.25) | 0.18 (0.12–0.25) | 0.18 (0.12–0.25) | 0.18 (0.12–0.25) | 0.18 (0.12–0.25) | 0.18 (0.12–0.25) |
| Adiponectin (ng/mL) | 8.7 (4.5–15.6) | 8.7 (4.5–15.6) | 8.7 (4.5–15.6) | 8.7 (4.5–15.6) | 8.7 (4.5–15.6) | 8.7 (4.5–15.6) |

All values are medians (minimum–maximum); \( \alpha \), \( \beta \), and \( \gamma \) = 0.05. EGP, endogenous glucose production; Rd, rate of disappearance; KIV , \( \alpha \)-ketoisovalerate; Ra, rate of appearance; FFA, free fatty acid.
different between the IF and SD groups in the basal state or during step 1 or step 2 of the clamp (Table 3).

Glucoregulatory and thyroid hormones

There were no differences in insulin, glucagon, cortisol, adrenaline, norepinephrine, or adiponectin between the IF and SD groups in the basal state or during step 1 and step 2 of the clamp (Table 3). There were no differences in TSH, T₄, T3, or rT3 between the IF and SD groups in the basal state or step 2 of the clamp (Table 4).

Immunoblotting

pAKT-ser⁴⁷³ was not different between the IF and SD groups during the basal state or during the clamp (Figure 2). pAKT-ser⁴⁷³ increased significantly during both clamps, but this increase was not significantly different between the IF and SD groups (data not shown). No differences were found in pAS160-thr⁶⁴² in the basal state or during the clamp between the IF and SD groups (Figure 2). After both IF and the SD, pAS160-thr⁶⁴² showed no significant increase during the clamp.

pGSK-3-ser⁹ was higher after IF than after the SD in the basal state and during the clamp (Figure 2). pGSK-3-ser⁹ increased significantly during both clamps. pmTOR-ser²⁴⁴⁸ was not different between the IF and SD groups in the basal state but was significantly lower during the clamp after IF than after the SD (Figure 2). pmTOR-ser²⁴⁴⁸ increased significantly during both clamps. We found no differences in the total muscle content of AKT, GSK-3, or mTOR (Figure 3).

DISCUSSION

In this study we investigated the effect of 2 wk of IF on hepatic and peripheral insulin sensitivity (EGP and glucose uptake, respectively), lipolysis, and proteolysis in comparison with 2 wk of an isocaloric SD in lean healthy subjects. The stable body weight and body composition in our study indicates that, in general, our findings cannot be attributed to weight loss in contrast with many earlier studies (4, 5).

The importance of the significant decrease in REE after IF (median decrease in REE: 59 kcal/d) is illustrated by the calculation that, on a year’s basis, such a decrease results in a difference of 21,535 kcal (approximating the caloric equivalent of 3 kg body fat). It has been postulated that a decreased REE reflects a calorie shortage per se (25). Our results strongly argue against such a mechanism because our participants were not calorie restricted and did not lose weight (albeit in a relative short period). Changes in REE have been shown after short-term fasting (26), but it is of interest that, already after repeated periods of IF, an adaptation apparently occurs that spares energy stores. This change in REE could not be attributed to changes in the traditional glucoregulatory hormones or adiponectin. Neither did we find changes in the thyroid hormones, but this does not take into account possible changes in intracellular differences in triiodothyronine production. The type 2 iodothyronine deiodinase has been implicated in the regulation of energy expenditure and insulin signaling (27). Whether iodothyronine deiodinase changes after IF, as described recently after short-term fasting by Heemstra et al (28), remains to be elucidated. Finally, a decrease in REE may also increase body weight if isocaloric IF diets are consumed for longer periods and/or if physical activity is unaltered. Our study period has not been long enough to increase body weight.

Although Halberg et al (1) showed an increased glucose infusion rate during the hyperinsulinemic clamp in their study we were not able to discern differences in peripheral or hepatic insulin sensitivity despite equal diets and a crossover design. The lack of an effect on peripheral insulin sensitivity was strengthened by the data on phosphorylated AS160-thr⁶⁴²—a downstream target of AKT that is involved in the translocation of GLUT4 to the plasma membrane. Insulin-mediated phosphorylation of AS160 was shown to be decreased in patients with type 2 diabetes and after short-term fasting (8, 29).

FFAs may be a factor in modulating peripheral insulin sensitivity because the interfering role of FFAs and their metabolites with the insulin signaling cascade is generally appreciated (30, 31). Although the 20-h fasting period in our study was long enough to stimulate lipolysis and FAO (32), we did not detect differences in (insulin-mediated supression of) lipolysis (expressed both as μmol · kg⁻¹ · min⁻¹ and μmol/kcal) or plasma FFA. Intriguingly, it was shown that an insulin-mediated decrease in interstitial glycerol concentrations (subcutaneous abdominal adipose tissue) was greater after IF (1). Whether these conflicting results are explained by adipose tissue depot-specific differences in lipolysis are of interest and remain to be elucidated. IF did not affect FAO in the present study, although FAO was higher in the study by Heilbronn et al (4). However, in that study, subjects were studied after a 36-h fast in contrast with the overnight fast in our study (4).

We previously showed that decreased peripheral insulin sensitivity during short-term fasting in lean healthy humans is explained by lower phosphorylation of muscle AKT-ser⁴⁷³ under hyperinsulinemic circumstances (8). AKT is a 56-kD serine/
threonine kinase and a mediator of many insulin effects with a complex regulation (33). We only measured phosphorylation of these signaling proteins during step 2 of the clamp and not during step 1. The data on AKT were in line with the results of both muscle AS160 content and the Rd during step 2 of the clamp, which show no effect of IF on peripheral glucose uptake. However in the present study, basal and clamp pGSK-3-ser9 values were remarkably higher after IF and suggest adaptation that favors glycogen replenishment. GSK is another downstream target of AKT that, once phosphorylated, stimulates glycogen synthesis (34, 35). We showed previously that short-term fasting does not influence the phosphorylation of GSK-3-ser9 in the basal state nor during hyperinsulinemia (8). The increased phosphorylation of GSK opposed to the unchanged phosphorylated AS160 suggests that divergent effects on downstream targets of AKT might be present. This can be explained by different AKT-isoforms that exist (36) or subcellular localization of the intermediate AKT itself. Additionally, we measured the activation on the serine473-residue, not taking into account possible phosphorylation of the threonine308-residue. Although we found no differences in whole-body NOGD between the IF and SD groups, we cannot exclude increased glycogen synthesis in the vastus lateralis muscle of our subjects.

**FIGURE 2.** pAKT-ser473 (n = 7) in the basal state (P = 1.0) and during the hyperinsulinemic euglycemic clamp (P = 0.063) (A), pAS160-thr642 (n = 7) in the basal state (P = 0.55) and during the hyperinsulinemic euglycemic clamp (P = 0.45) (B), pGSK-3-ser9 (n = 7) in the basal state (P = 0.063) and during the hyperinsulinemic euglycemic clamp (P = 0.018) (C), and pmTOR-ser2448 (n = 7) in the basal state (D) after intermittent fasting (IF) (white box plots) and the standard diet (SD) (gray box plots). A: *P = 0.018 and 0.028 for the difference in pAKT-ser473 between the basal state and the clamp after IF and the SD, respectively. B: $P = 0.091 and 0.063 for the difference in pAS160-thr642 between the basal state and the clamp after IF and the SD, respectively. C: *P = 0.018 and 0.018 for the difference in pGSK-3-ser9 between the basal state and the clamp after IF and the SD, respectively. D: pmTOR-ser2448 tended to be lower after IF than after the SD (P = 0.063) but was significantly lower during the hyperinsulinemic euglycemic clamp (***P = 0.028) after IF than after the SD. E, F: Representative bands of pAKT-ser473 (E) and of pmTOR-ser2448 (F) in the basal state and during the clamp after IF and the SD, respectively, in 3 volunteers. pGSK, phosphorylated glycogen synthase kinase; pmTOR, phosphorylated mammalian target of rapamycin; pAKT, phosphorylated muscle protein kinase B.
The adipokine adiponectin is thought to increase insulin sensitivity and FAO and is decreased in insulin-resistant states such as obesity and type 2 diabetes mellitus (37). In contrast with our findings, the peaks in adiponectin after each fasting period in the study by Halberg et al (1) were suggested to cause the observed increased insulin sensitivity. Our findings on adiponectin, however, were in line with the similar peripheral insulin sensitivity and FAO after IF and the SD.

Protein metabolism during IF has not been investigated in humans to our knowledge. Intracellular valine contributes to the citric acid cycle intermediate succinyl-CoA (via transamination to 2-KIV that is converted to propionyl-CoA). 2-KIV Ra is assumed to reflect intracellular valine enrichment and proteolysis (38). We could not detect an effect on whole-body protein metabolism during the clamps, which means that IF does not seem to protect protein stores. mTOR is a protein serine-threonine kinase that functions as a central element in signaling pathways involved in the control of cell growth and proliferation and is also involved in protein synthesis and activated by growth factors and nutrient-sensitive pathways (eg, amino acids) (39). Because we did not assess protein synthesis rates, we cannot support that the lower mTOR phosphorylation in the basal state after IF than after the SD reflects lower protein synthesis, as shown earlier for short-term fasting (14). LBM was not affected, although the duration of our study may have been insufficient to observe measurable effects on muscle mass.

**FIGURE 3.** Total muscle protein kinase B (AKT) in the basal state ($P = 0.29$) and during the hyperinsulinemic euglycemic clamp ($P = 0.58$) (A), total glycogen synthase kinase (GSK) in the basal state ($P = 0.26$) and during the hyperinsulinemic euglycemic clamp ($P = 0.67$) (B), and total mammalian target of rapamycin (mTOR) ($n = 7$) in the basal state ($P = 0.40$) and during the hyperinsulinemic euglycemic clamp ($P = 0.74$) after intermittent fasting (IF) (white box plots) and the standard diet (SD) (gray box plots). D: Representative bands of total AKT, GSK, and mTOR in the basal state and during the clamp after IF and the SD during the basal state (b) and during the clamp (c) in 2 volunteers.
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Of interest, mTOR plays a role in autophagia and probably life span (40). Indeed, alternate-day fasting has been suggested to increase life span, although caloric restriction may play a greater role (4, 5). Whether such mechanisms are important in IF remains to be elucidated.

In conclusion, IF does not change insulin-mediated peripheral glucose uptake, hepatic insulin sensitivity, insulin sensitivity of adipose tissue, or proteolysis. However IF affects muscle signaling pathways that may be beneficial in storing glycogen (GSK) or modulating nutrient signaling (mTOR). The decrease in REE after IF may precede weight gain during IF when caloric intake is not adjusted. Whether IF is beneficial at improving peripheral insulin resistance in obese, insulin-resistant subjects remains to be established.

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The authors’ responsibilities were as follows—MRS, NML, CFJ-S, HPS, and MJS: design of the study; MRS and NML: performance of experiments; MTA, PFD, and JMA: sample analysis; MRS, NML, MTA, and JMA: data analysis; and MRS, EF, JMA, MJS, and HPS: writing of the manuscript. There were no conflicts of interest.

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