The metabolic response to fasting in humans: physiological studies

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Intermittent fasting differentially affects glucose, lipid and protein metabolism.

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Submitted
Abstract

Context: 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: To assess effects of IF on intermediary metabolism and energy expenditure.

Main Outcome Measures and Design: Glucose, glycerol and valine fluxes were measured after two weeks of IF and a standard diet (SD), before and during a two-step hyperinsulinemic euglycemic clamp with assessment of energy expenditure and phosphorylation of muscle protein kinase B (AKT/PKB), glycogen synthase kinase (GSK) and mTOR. We hypothesized that IF selectively increases peripheral glucose uptake and lowers proteolysis thereby protecting protein stores.

Results: There were no differences in body weight after IF vs. SD. Peripheral glucose uptake during step 1, but not step 2, was significantly higher after IF while hepatic insulin sensitivity did not differ. Insulin-mediated suppression of lipolysis tended to be increased after IF. Proteolysis was significantly lower after IF, but only at higher insulin levels. IF decreased resting energy expenditure. Finally, IF tended to increase phosphorylation of AKT, and significantly increased phosphorylation of glycogen synthase kinase.

Conclusion: IF increases peripheral, but not hepatic insulin sensitivity and tends to increase the anti-lipolytic effect of insulin. Proteolysis is lower under hyperinsulinemic conditions. The decrease in REE after IF warns for increasing weight during IF when caloric intake is not adjusted. Whether IF is beneficial in improving peripheral insulin resistance in obese insulin resistant subjects remains elusive.
Introduction

Intermittent fasting (IF) has been suggested to mimic cycles of feast and famine (food abundance and food shortage) as may have been physiological in the late Palaeolithic 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 during fasting while replenishing fuel stores during refeeding (2;3).

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

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 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 unknown whether IF affects protein metabolism via changes in activity of mTOR, at present.

In this study, we measured glucose, glycerol and valine fluxes after two weeks IF and after two weeks of a standard isocaloric diet (SD) both in the basal state and during a two-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. It was our hypothesis 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 yr; 3) Caucasian race; 4) BMI 20-25 kg/m²; 5) normal oral glucose tolerance test according to the ADA criteria (16); 6) normal routine blood examination; 7) no excessive sport activities, i.e. < 3 times per week; and 8) no medication. Additionally, subjects who were not in the habit of eating breakfast every day were excluded. At inclusion, indirect calorimetry and dietary interviews were performed by a dietician to assess daily energy requirements.

Written informed consent was obtained from all subjects after explanation of purposes, 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
Subjects were studied twice in balanced assignment (cross over-design) to two weeks of intermittent fasting or two weeks of a standard diet. Study days were separated by at least four weeks to minimize influences of the previous diet. Subjects were fasting from 2000 h the evening before the study days. They were allowed to drink water only.

After admission at the Metabolic Unit at 0730 h, 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 (60ºC) plexiglas box for sampling of arterialized venous blood. Saline was infused as NaCl 0.9% at a rate of 50 mL/h to keep the catheters patent. \([6,6-2\text{H}_2]\) glucose, \([1,1,2,3,3-2\text{H}_5]\)glycerol and \(L-[1-13\text{C}]\)valine were used as tracers (>99% enriched; Cambridge Isotopes, Andover, USA) to study glucose kinetics, lipolysis (total triglyceride hydrolysis), and proteolysis respectively.

At \(T = 0\) h (0800 h), blood samples were drawn for determination of background enrichments. Then a primed continuous infusion of isotopes was started: \([6,6-2\text{H}_2]\) glucose (prime, 8.8 \(\mu\)mol/kg; continuous, 0.11 \(\mu\)mol/kg·min), \([1,1,2,3,3-2\text{H}_5]\)glycerol (prime, 1.6 \(\mu\)mol/kg; continuous, 0.11 \(\mu\)mol/kg·min) and \(L-[1-13\text{C}]\)valine (prime, 13.7 \(\mu\)mol/kg; continuous, 0.15 \(\mu\)mol/kg·min) and continued until the end of the study. After an equilibration period of two hours (14 h of fasting), 3 blood samples were drawn for isotope enrichments and 1 for glucoregulatory hormones and FFA. Thereafter (\(T = 3.0\) h) a two-step hyperinsulinemic euglycemic clamp was started: step 1 included an infusion
of insulin at a rate of 10 mU/m²·min (Actrapid 100 IU/ml; Novo Nordisk Farma B.V., Alphen aan den Rijn, the Netherlands) to assess hepatic insulin sensitivity. Glucose 10% was started to maintain a plasma glucose level 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 levels were measured every 5 minutes at the bedside. After 2 h (T = 5 h), 5 blood samples were drawn at 5 minute intervals for determination of glucose concentrations and isotopic enrichments. Another blood sample was drawn for determination of glucoregulatory hormones and FFA. Hereafter insulin infusion was increased to a rate of 40 mU/m²·min (step 2) to assess peripheral insulin sensitivity. After another 2 h (T = 7 h), blood sampling was repeated.

Intermittent fasting and standard diet
The two weeks of IF were equal to the study of Halberg et al (1); subjects were fasting every second day for 20 h (Figure 1). Fasting started at 2200 and ended at 1800 h the following day. In total, subjects fasted seven times. During SD, subjects were not allowed to skip meals. The day before the study days, subjects were not fasting, but consumed their diet like on non fasting days.

The caloric intake during both diet periods was equal to avoid energy restriction with secondary effects on metabolism. Diversion of calories between carbohydrates, fat and protein were kept equal as well. To increase comparability, volunteers ate mainly bread, fruits and dairy products (60% daily energy intake), supplemented with liquid meals (40% daily energy intake): Nutridrink® (Nutricia Advanced Medical Nutrition, Zoetermeer, the Netherlands), per serving (200ml): 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 was measured with indirect calorimetry at inclusion. Then total energy requirements were calculated by a dietician based on REE, dietary history and activity score, resulting in an average of advised caloric intake of 130 - 140% of REE. We aimed at preventing weight loss and the diets were adjusted in case of a one kilogram weight change. However, this did not occur.

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 two times per week.

Body composition, indirect calorimetry and muscle biopsies
Body composition, oxygen consumption (\(\text{VO}_2\)) and \(\text{CO}_2\) production (\(\text{VCO}_2\)) were measured as described earlier (18). Muscle biopsies were performed as described previously in the basal state and during step 2 of the clamp (18).

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 determined with an enzymatic colorimetric method (NEFA-C test kit, Wako Chemicals GmbH, Neuss, Germany): intra-assay variation: 1%; inter-assay variation: 4-15%; detection limit: 0.02 mmol/L. 
\([6,6\text{-}2\text{H}_2]\text{glucose}\) and \([1,1,2,3,3\text{-}2\text{H}_5]\text{glycerol}\) enrichment were determined as described earlier (18). \(\text{L-[1-}^{13}\text{C]valine}\), \([1-^{13}\text{C}\) \(\alpha\)-ketoisovalerate (\(\alpha\)-KIV) enrichments were measured as described previously (19).

Glucoregulatory hormones
Insulin was determined on an Immulite 2000 system (Diagnostic Products Corporation, Los Angeles, USA) with a chemiluminescent immunometric assay, intra-assay variation: 3-6%, inter-assay variation: 4-6%, detection limit: 15pmol/L. Glucagon was determined with the Linco \(^{125}\text{I}\) radioimmunoassay (St. Charles, USA), intra-assay variation: 9-10%, inter-assay variation: 5-7% and detection limit: 15ng/L. Cortisol, norepinephrine, epinephrine and adiponectin were determined as described earlier (18).

Immunoblotting
Muscle tissue was prepared and separated by electrophoresis on a polyacrylamide gel and immunoblots were visualized by enhanced chemi-luminescence (ECL) as described previously (8). Chemicals for ECL were from Sigma (St.Louis, MO, USA). Phosphospecific anti-AKTser\(^{473}\), phosphospecific anti-glycogen synthase kinase-3-ser\(^9\) (GSK), anti-
mTORser<sup>2448</sup> total anti-AKT and total anti-eIF4E (loading control) were from Cell Signaling (Boston, MA, USA). Phosphospecific anti-AS160-thr<sup>642</sup> was from GeneTex Inc. (San Antonio, TX, USA). Results are presented as fold increase compared to control.

Calculations and statistics
Endogenous glucose production (EGP) and peripheral glucose uptake of glucose (rate of disappearance/Rd) were calculated using the modified forms of the Steele Equations as described previously (17;20). EGP and Rd were expressed as μmol/kg-min. Glucose metabolic clearance rates (MCR<sub>glucose</sub>) were calculated as MCR = Ra glucose/[glucose].

Total triglyceride hydrolysis/lipolysis (glycerol turnover) was calculated by using formulas for steady state kinetics adapted for stable isotopes (21) and was expressed as μmol/kg-min and as μmol/kcal (18;22). Proteolysis was calculated using the reciprocal pool model (α-KIV Ra) as described earlier (23).

REE, carbohydrate oxidation (CHO) and FAO rates were calculated from O<sub>2</sub> consumption and CO<sub>2</sub> production as reported previously (24). Nonoxidative glucose disposal (NOGD) was calculated as: NOGD = Rd - CHO.

All subjects served as their own controls. All data were analyzed with non parametric tests. Comparisons were performed with the Wilcoxon Signed Rank test. Correlations were expressed as Spearman's rank correlation coefficient (ρ). The SPSS statistical software program version 14.0.2 (SPSS Inc, Chicago, IL) was used for statistical analysis. Data are presented as median [minimum-maximum].

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Subject characteristics at inclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>23.5 [20 - 30]</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>74.6 [58.4 - 85.0]</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>188 [176 - 192]</td>
</tr>
<tr>
<td>BMI (kg/m&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>21.3 [18.2 - 24.7]</td>
</tr>
<tr>
<td>Lean body mass (%)</td>
<td>85.2 [75.3 - 87.9]</td>
</tr>
<tr>
<td>Fat mass (%)</td>
<td>14.8 [12.1 - 24.7]</td>
</tr>
<tr>
<td>FPG (mmol/liter)</td>
<td>5.0 [4.7 - 5.3]</td>
</tr>
<tr>
<td>OGTT (mmol/liter)</td>
<td>3.8 [3.4 - 5.4]</td>
</tr>
</tbody>
</table>

Dietary requirements

| Energy requirement (kcal/day) | 2653 [2395 - 2860] |
| Protein (gram/day) | 101 [89 - 130] |
| Fat (gram/day) | 88 [64 - 93] |
| Carbohydrate (gram/day) | 366 [306 - 403] |

Data are presented as median [minimum - maximum]. BMI, body mass index; FPG, fasting plasma glucose; OGTT, oral glucose tolerance test.
Results

Anthropometric characteristics

The subject characteristics at inclusion are presented in Table 1. Weight after two weeks of IF was not different compared to SD (74.8 [58.7 - 85.3] vs. 74.5 [58.4 - 85.7] kg respectively, \( P = 0.58 \)). Fat and lean body mass (FM and LBM) were not different after IF compared to SD (FM 13.3 [11.7 - 20.9] % vs. 12.8 [9.9 - 15.9] % respectively, \( P = 0.46 \) and LBM 86.7 [79.1 - 88.2] % vs. 87.3 [84.1 - 90.1] % respectively, \( P = 0.46 \)).

Resting energy expenditure, glucose and fatty acid oxidation

After two weeks of IF, REE was significantly lower in the basal state compared to two weeks of SD: median difference of -59 [-201 - 26] kcal/day (Table 2). During the clamp, no differences in REE were found. CHO and FAO were not different between IF and SD during either basal state or clamp. When CHO and FAO were expressed per LBM, similar

<table>
<thead>
<tr>
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<th>IF (n = 8)</th>
<th>SD (n = 8)</th>
<th>( P )</th>
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<tbody>
<tr>
<td>REE (kcal/day)</td>
<td>1698 [1280 - 1754]</td>
<td>1716 [1478 - 1901]</td>
<td>0.036</td>
</tr>
<tr>
<td>CHO (μmol/kg•min)</td>
<td>4.4 [1.5 - 9.2]</td>
<td>6.5 [2.3 - 13.9]</td>
<td>0.58</td>
</tr>
<tr>
<td>FAO (μmol/kg•min)</td>
<td>1.5 [1.0 - 1.8]</td>
<td>1.4 [0.8 - 2.2]</td>
<td>0.89</td>
</tr>
<tr>
<td>RQ</td>
<td>0.76 [0.72 - 0.83]</td>
<td>0.79 [0.68 - 0.87]</td>
<td>0.67</td>
</tr>
</tbody>
</table>

Data are presented as median [minimum - maximum]. REE, resting energy expenditure; CHO, carbohydrate oxidation; FAO, fatty acid oxidation RQ, respiratory quotient.

FIGURE 2 Differences between IF (grey box plots) and SD (open box plots) subjects in α-KIV Ra (proteolysis) during the basal state (\( P = 0.24 \)) and during step 1(\( P = 0.13 \)) and step 2 of the clamp, \( ^*P = 0.043 \), \( N = 7 \).
results were found (data not shown). Also changes during the clamp in CHO and FAO were not different between IF and SD (data not shown).

Glucose and lipid measurements
Plasma glucose concentrations and EGP in the basal state were not statistically different after IF compared to SD (table 3). $\text{MCR}_{\text{glucose}}$ in the basal state was not different between IF and SD [2.4 (1.9 –2.8) ml/kg x min vs. 2.3 (2.0–2.9) ml/kg x min, respectively; $P = 0.58$]. Basal state plasma FFA levels and lipolysis after two weeks of IF were not different from SD.

During step 1 of the clamp (Table 3), plasma glucose concentrations and EGP were not different after IF compared to SD. Glucose Rd during step 1 was significantly higher after IF compared to SD. Plasma FFA levels were not different during step 1, but lipolysis tended to be lower after IF compared to SD.

Step 2 of the clamp revealed no differences in glucose Rd between IF and SD (EGP being suppressed). Also no differences were observed in lipolysis between IF and SD during step 2 of the clamp.

Valine measurements
$\text{KIV Ra}$ (expressing proteolysis) was not different between IF and SD in the basal state or step 1 of the clamp (Figure 2). $\text{KIV Ra}$ was significantly lower after IF compared to SD during step 2 of the clamp.

Glucoregulatory hormones
In the basal state plasma glucagon levels were significantly lower after IF compared to SD (Table 3). Insulin, cortisol, epinephrine, norepinephrine and adiponectin were not different between IF and SD.

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**Hyperinsulinaemic euglycaemic clamp**

<table>
<thead>
<tr>
<th></th>
<th>IF (n = 8)</th>
<th>SD (n = 8)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>1784 [1427 - 2003]</td>
<td>1864 [1774 - 2147]</td>
<td>0.33</td>
</tr>
<tr>
<td>Lipid</td>
<td>19.3 [12.0 - 34.8]</td>
<td>20.1 [12.1 - 24.3]</td>
<td>0.78</td>
</tr>
<tr>
<td>Protein</td>
<td>0.4 [0 - 0.82]</td>
<td>0.47 [0 - 1.6]</td>
<td>0.48</td>
</tr>
<tr>
<td>Fat</td>
<td>0.93 [0.86 - 1.15]</td>
<td>0.93 [0.81 - 1.00]</td>
<td>0.78</td>
</tr>
</tbody>
</table>
During step 1 of the clamp, glucagon levels tended to be lower after IF compared to SD, whereas the other glucoregulatory hormones and adiponectin were not different. During step 2 of the clamp, no differences were found in glucoregulatory hormones and adiponectin except for norepinephrine that was lower after IF compared to SD (Table 3).

**Table 3** Glucose and lipid kinetics and glucoregulatory hormones

<table>
<thead>
<tr>
<th></th>
<th>IF</th>
<th>SD</th>
<th>P</th>
</tr>
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<tbody>
<tr>
<td>Glucose (mmol/liter)</td>
<td>4.8 [4.3 - 4.7]</td>
<td>4.7 [4.0 - 4.9]</td>
<td>0.09</td>
</tr>
<tr>
<td>EGP (μmol/kg•min)</td>
<td>11.4 [10.0 - 13.5]</td>
<td>10.4 [9.6 - 13.9]</td>
<td>0.16</td>
</tr>
<tr>
<td>Rd (μmol/kg•min)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NOGD (%)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>FFA (mmol/liter)</td>
<td>0.31 [0.20 - 0.44]</td>
<td>0.33 [0.12 - 0.55]</td>
<td>0.89</td>
</tr>
<tr>
<td>Lipolysis (μmol/kg•min)</td>
<td>1.6 [0.9 - 2.3]</td>
<td>1.3 [0.8 - 3.3]</td>
<td>0.24</td>
</tr>
<tr>
<td>Lipolysis (μmol/kcal)</td>
<td>104 [58 - 143]</td>
<td>81 [45 - 211]</td>
<td>0.26</td>
</tr>
<tr>
<td>Insulin (pmol/liter)</td>
<td>34 [&lt;15 - 66]</td>
<td>32 [&lt;15 - 63]</td>
<td>0.87</td>
</tr>
<tr>
<td>Glucagon (ng/liter)</td>
<td>46 [32 - 83]</td>
<td>63 [38 - 83]</td>
<td>0.036</td>
</tr>
<tr>
<td>Cortisol (nmol/liter)</td>
<td>273 [184 - 381]</td>
<td>291 [155 - 458]</td>
<td>0.67</td>
</tr>
<tr>
<td>Epinephrine (pmol/liter)</td>
<td>0.06 [0.03 - 0.32]</td>
<td>0.065 [0.03 - 0.38]</td>
<td>0.75</td>
</tr>
<tr>
<td>Norepinephrine (nmol/liter)</td>
<td>0.67 [0 - 0.98]</td>
<td>0.60 [0.03 - 1.46]</td>
<td>0.67</td>
</tr>
<tr>
<td>Adiponectin (ng/ml)</td>
<td>8.7 [4.5 - 15.6]</td>
<td>7.7 [4.0 - 16.2]</td>
<td>0.44</td>
</tr>
</tbody>
</table>

Data are presented as median [minimum - maximum]. N = 8. EGP, endogenous glucose production; Rd, rate of disappearance; NOGD, non oxidative glucose disposal. *EGP and FFA were completely suppressed during step 2 of the clamp.

During step 1 of the clamp, glucagon levels tended to be lower after IF compared to SD, whereas the other glucoregulatory hormones and adiponectin were not different.

During step 2 of the clamp, no differences were found in glucoregulatory hormones and adiponectin except for norepinephrine that was lower after IF compared to SD (Table 3).

**Immunoblotting**

pAKT-ser473 in the basal state was not different between IF and SD, but during the clamp pAKT-ser473 tended to be higher after IF compared to SD (Figure 3). pAKT-ser473 increased significantly during both clamps.

No differences were found in pAS160-thr642 in the basal state or clamp between IF and SD (Figure 3). After both IF and SD, pAS160-thr642 tended to increase during the clamp.

pGSK-3-ser9 was higher after IF compared to SD in the basal state as well as during the clamp. pGSK-3-ser9 increased significantly within both clamps.

pmTOR-ser2448 tended to be lower in the basal state after IF compared to SD, whereas pmTOR-ser2448 was significantly lower during the clamp after IF compared to SD (Figure 3). pmTOR-ser2448 increased significantly within both clamps.
Discussion

In this study we investigated the effect of two weeks of IF on hepatic and peripheral insulin sensitivity (EGP and glucose uptake respectively), lipolysis and proteolysis in comparison with two weeks of an isocaloric SD in lean healthy subjects.

Body weight and body composition did not change in the period of IF versus two weeks of SD indicating that our findings cannot be attributed to weight loss in contrast to a number of earlier studies (4;5). Previous reports showed no differences in data on REE and substrate oxidation between IF and SD (1;5). Therefore, the decrease in REE after two weeks IF that we found, is intriguing. A decreased REE may spare energy stores but it may also increase body weight if isocaloric diets are consumed or if physical activity is unaltered. Our study period may not have been long enough to increase body weight.

The increased peripheral insulin sensitivity during step 1 of the clamp strengthens the data by Halberg et al. Moreover, we show that IF selectively increases peripheral insulin sensitivity and not hepatic insulin sensitivity since we found no effect on the latter. The reason why the insulin sensitizing effect of IF was lost during step 2 is unclear, but clamp duration is most likely to play a role since Halberg et al reported an increase in peripheral insulin sensitivity after a 2h clamp with an insulin infusion regimen comparable to step 2 in our study. The mechanism of the higher insulin-mediated peripheral glucose uptake...
FIGURE 3 Panel A pAKT-ser473 (n = 7) in the basal state (P = 1.0) and during the hyperinsulinemic euglycemic clamp (**P = 0.063) after intermittent fasting (IF) (open box plots) and standard diet (SD) (grey box blots). *P = 0.018 and 0.028 for the difference in pAKT-ser473 between basal and clamp after IF and SD respectively.

Panel B pAS160-thr642 (n = 7) in the basal state (P = 0.55) and during the hyperinsulinemic euglycemic clamp (P = 0.45) after IF (open box plots) and SD (grey box blots). **P = 0.091 and 0.063 for the difference in pAS160-thr642 between basal and clamp after IF and SD respectively.

Panel C pGSK-3-ser9 (n = 7) in the basal state (**P = 0.063) and during the hyperinsulinemic euglycemic clamp (P = 0.018) after IF (open box plots) and SD (grey box blots). *P = 0.018 and 0.018 for the difference in pGSK-3-ser9 between basal and clamp after IF and SD respectively.

Panel D pmTOR-ser2448 (n = 7) in the basal state tended to be lower after IF (open box plots) compared to SD (grey box blots) (**P = 0.063) whereas pmTOR-ser2448 was significantly lower during the hyperinsulinemic euglycemic clamp (P = 0.028) after IF compared to SD.
remains to be elucidated. Glucoregulatory hormones were not different except for glucagon. However, glucagon seems to have its major effect on EGP and not Rd (25).

FFA may be an alternative factor in modulating peripheral insulin sensitivity since the interfering role of FFA and their metabolites with the insulin signaling cascade is generally appreciated (26;27). It was shown in mice that IF induces an increase in plasma FFA levels on the one hand and smaller adipocytes on the other suggesting increased triglyceride hydrolysis, but these animals lost weight during the study period (28). The authors questioned whether such increase in plasma FFA levels would be a beneficial adaptation. Although the 20 h fasting periods in our study may have been long enough to stimulate lipolysis and FAO (29), we did not detect changes in lipolysis or plasma FFA in the basal state after an overnight fast, thereby confirming previous human studies (1;5).

During step 1 of the clamp, lipolysis (expressed as μmol/kg x min) tended to be lower after IF compared with SD in keeping with the increased suppressibility of adipose tissue lipolysis after IF as shown earlier by Halberg et al (1). Although it is attractive to explain the increased insulin sensitivity after IF by lower lipolysis and thereby lower exposure to FFA, we cannot make a definite assumption.

Earlier we showed that decreased peripheral insulin sensitivity during short-term fasting in lean healthy humans is explained by lower phosphorylation of muscle AKT-ser⁴⁷³ and AS160-thr⁶⁴² under hyperinsulinemic circumstances (8). We only measured phosphorylation of these signaling proteins during step 2 of the clamp and not during step 1 when peripheral insulin sensitivity differed between IF and SD. The trend towards higher pAKT-ser⁴⁷³ suggests that changes in muscle insulin signaling are present after IF in lean healthy individuals that favor peripheral glucose uptake although we cannot make a definitive assumption. Moreover, this trend was not accompanied by a difference in pAS160-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 (18). Another downstream target of AKT is GSK that, once phosphorylated by AKT, stimulates glycogen synthesis (20). We showed previously that short-term fasting does not influence phosphorylation of GSK-3-ser⁹ in the basal state nor during hyperinsulinemia (1). However, the higher basal and clamp pGSK-3-ser⁹ levels after IF suggest adaptation that favors glycogen replenishment.

The adipokine adiponectin is thought to increase insulin sensitivity and fatty acid oxidation and is decreased in insulin resistant states as obesity and type 2 diabetes mellitus (30). Also, adiponectin was suggested to play a role in increased insulin sensitivity in IF although adiponectin levels were only increased at the end of fasting days and not in the basal state (1). In contrast it was shown that plasma adiponectin levels during short-term
fasting do not change within days when more frequent sampling was performed (31). The lack of change in adiponectin levels and FAO between IF and SD does not support a role for adiponectin in increasing peripheral insulin sensitivity during IF.

Protein metabolism in IF has not been investigated in humans before to our knowledge. Intracellular valine contributes to the citric acid cycle intermediate succinyl-CoA (via transamination to α-KIV that is converted to propionyl-CoA). α-KIV Ra is assumed to reflect the intracellular valine enrichment and proteolysis (32). We show that IF lowers α-KIV Ra during step 2 of the clamp. Collectively these data suggest that IF does not decrease proteolysis in the basal state but increases insulin mediated inhibition of proteolysis during higher insulin levels.

mTOR is involved in protein synthesis and activated by growth factors and nutrient sensitive pathways (e.g. amino acids) (33). Although we did not assess protein synthetic rates, the decrease in mTOR phosphorylation suggests that IF lowers protein synthesis as shown earlier for short-term fasting (14). Whether our results indicate maintenance of skeletal muscle mass during IF remains elusive.

In conclusion, IF increases insulin-mediated peripheral glucose uptake but not hepatic insulin sensitivity. Insulin sensitivity of adipose tissue tends to be augmented whereas insulin-mediated suppression of proteolysis was significantly higher. The observed changes in glucose fluxes may be mediated via changes in phosphorylation of muscle AKT and GSK. The decrease in REE after IF may precede weight gain during IF when caloric intake is not adjusted. Whether IF is beneficial in improving peripheral insulin resistance in obese, insulin resistant subjects remains to be established.

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