The metabolic response to fasting in humans: physiological studies

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7 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 rapamycine (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/m^2; 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 thermo-regulated (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-$^2H_2$] glucose, [1,1,2,3,3-$^2H_5$]glycerol and L-[1-^{13}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-$^2H_2$] glucose (prime, 8.8 µmol/kg; continuous, 0.11 µmol/kg·min), [1,1,2,3,3-$^2H_5$]glycerol (prime, 1.6 µmol/kg; continuous, 0.11 µmol/kg·min) and L-[1-^{13}C]valine (prime, 13.7 µmol/kg; continuous, 0.15 µmol/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/m^2·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-$^2H_2$]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/m^2·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.

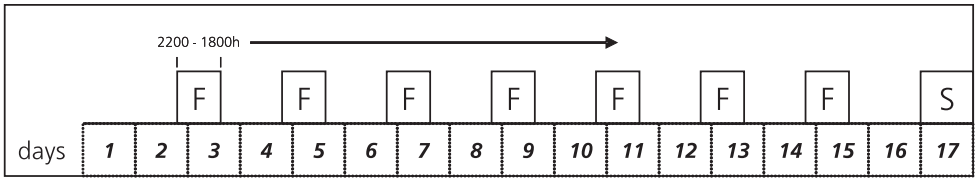

Figure 1 Schedule intermittent fasting: F = fasting, S = study day.

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 (VO_2) and CO_2 production (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-$^2H_2$]glucose and [1,1,2,3,3-$^2H_5$] glycerol enrichment were determined as described earlier (18). L-[1-^{13}C]valine, [1-^{13}C] α-ketoisovalerate (α-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}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^{2448} total anti-AKT and total anti-eIF4E (loading control) were from Cell Signaling (Boston, MA, USA). Phosphospecific anti-AS160-thr^{642} 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 ($MCR_{glucose}$) 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_2 consumption and CO_2 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 1 Subject characteristics at inclusion

Age (yr)	23.5 [20 - 30]
Weight (kg)	74.6 [58.4 - 85.0]
Height (cm)	188 [176 - 192]
BMI (kg/m2)	21.3 [18.2 - 24.7]
Lean body mass (%)	85.2 [75.3 - 87.9]
Fat mass (%)	14.8 [12.1 - 24.7]
FPG (mmol/liter)	5.0 [4.7 - 5.3]
OGTT (mmol/liter)	3.8 [3.4 - 5.4]
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.

Table 2 Indirect Calorimetry

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

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

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

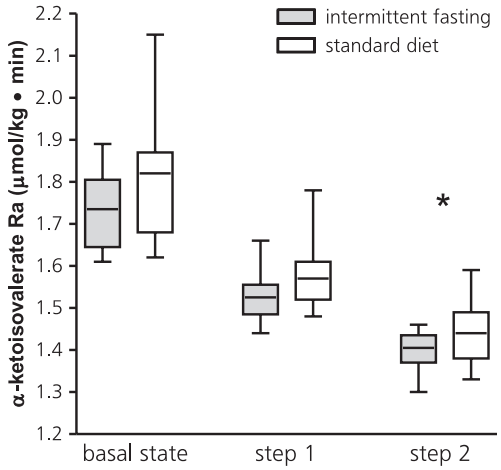

FIGURE 2 Differences between IF (grey box plots) and SD (open box plots) subjects in α-KIV Ra (proteolysis) during the basal state (P = 0.24) and during step 1(P = 0.13) and step 2 of the clamp, *P = 0.043. N = 7.

	hyperinsulinaemic euglycaemic clamp		
	IF (n = 8)	SD (n = 8)	P
	1784 [1427 - 2003]	1864 [1774 - 2147]	0.33
	19.3 [12.0 - 34.8]	20.1 [12.1 - 24.3]	0.78
	0.4 [0 - 0.82]	0.47 [0 - 1.6]	0.48
	0.93 [0.86 - 1.15]	0.93 [0.81 - 1.00]	0.78

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). $MCR_{glucose}$ 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

KIV Ra (expressing proteolysis) was not different between IF and SD in the basal state or step 1 of the clamp (Figure 2). 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.

Table 3 Glucose and lipid kinetics and glucoregulatory hormones

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

Data are presented as median [minimum - maximum]. N = 8. EGP, endogenous glucose production; Rd, rate of disappearance; NOGD, non oxidative glucose disposal. [a]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-ser^{473} in the basal state was not different between IF and SD, but during the clamp pAKT-ser^{473} tended to be higher after IF compared to SD (Figure 3). pAKT-ser^{473} increased significantly during both clamps.

No differences were found in pAS160-thr^{642} in the basal state or clamp between IF and SD (Figure 3). After both IF and SD, pAS160-thr^{642} tended to increase during the clamp.

pGSK-3-ser^{9} was higher after IF compared to SD in the basal state as well as during the clamp. pGSK-3-ser^{9} increased significantly within both clamps.

pmTOR-ser^{2448} tended to be lower in the basal state after IF compared to SD, whereas pmTOR-ser^{2448} was significantly lower during the clamp after IF compared to SD (Figure 3). pmTOR-ser^{2448} increased significantly within both clamps.

	clamp (step 1)			clamp (step 2)		
	IF	SD	P	IF	SD	P
	4.9 [4.7 - 5.3]	5.0 [4.7 - 5.4]	0.83	5.2 [4.8 - 5.5]	4.9 [4.8 - 5.3]	0.14
	2.6 [1.7 - 5.9]	1.9 [0 - 6.7]	0.67	-a	-a	-
	27.3 [20.4 - 36.5]	21.4 [16.5 - 35.7]	0.050	64.3 [49.4 - 89.2]	64.1 [38.9 - 81.3]	0.14
	-	-	-	28.5 [20.4 - 50.5]	31.7 [19.6 - 45.2]	0.78
	0.03 [0.01 - 0.07]	0.04 [0.01 - 0.14]	1	-a	-a	-
	0.5 [0.4 - 0.7]	0.6 [0.4 - 0.8]	0.091	0.4 [0.33 - 0.7]	0.4 [0.4 - 1.0]	0.61
	-	-	-	27 [14 - 45]	26 [21 - 50]	0.74
	103 [80 - 159]	115 [87 - 149]	0.94	408 [370 - 487]	409 [344 - 436]	0.12
	39 [34 - 72]	48 [37 - 82]	0.063	33 [28 - 60]	38 [27 - 81]	0.40
	177 [127 - 328]	201 [169 - 237]	0.33	169 [88 - 273]	76 [96 - 306]	0.48
	0.07 [0.03 - 0.31]	0.07 [0.03 - 0.48]	0.74	0.09 [0.03 - 0.18]	0.04 [0.03 - 0.47]	0.5
	0.60 [0.12 - 1.30]	0.79 [0.03 - 1.09]	0.4	0.48 [0.03 - 1.34]	0.69 [0.03 - 1.80]	0.025
	8.5 [4.6 - 15.8]	7.2 [4.1 - 16.5]	0.67	7.9 [3.9 - 15.0]	6.9 [4.3 - 15.4]	0.60

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

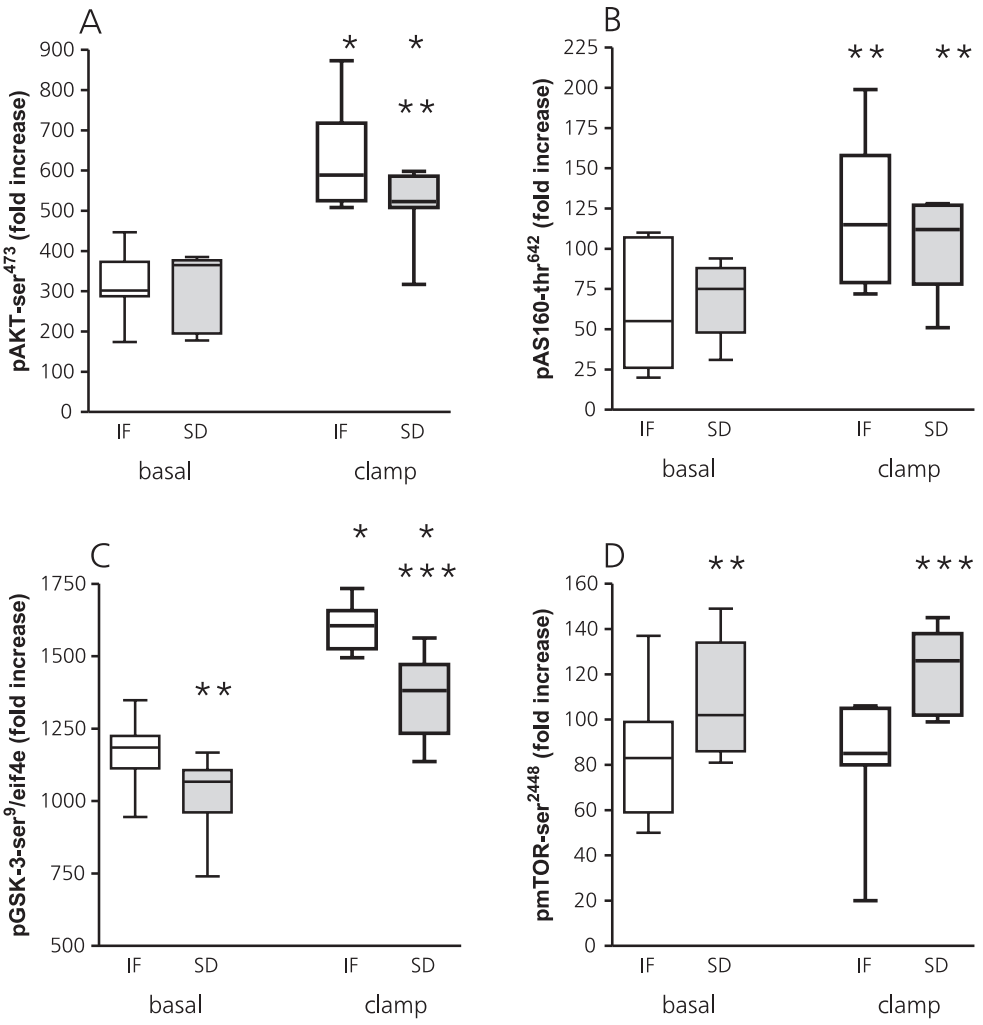

FIGURE 3 Panel A pAKT-ser^{473} (n = 7) in the basal state (P = 1.0) and during the hyperinsulinemic euglycemic clamp (**P = 0.063) after intermittent fasting (IF) (open box plots) and standard diet (SD) (grey box blots). *P = 0.018 and 0.028 for the difference in pAKT-ser^{473} between basal and clamp after IF and SD respectively.
Panel B pAS160-thr^{642} (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-thr^{642} between basal and clamp after IF and SD respectively.
Panel C pGSK-3-ser^{9} (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-ser^{9} between basal and clamp after IF and SD respectively.
Panel D pmTOR-ser^{2448} (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-ser^{2448} 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-ser^{473} and AS160-thr^{642} 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-ser^{473} 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-thr^{642}, 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-ser^{9} in the basal state nor during hyperinsulinemia (1). However, the higher basal and clamp pGSK-3-ser^{9} 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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Reference List

1. Halberg N, Henriksen M, Soderhamn N et al. Effect of intermittent fasting and refeeding on insulin action in healthy men. J Appl Physiol 2005; 99(6):2128-2136.
2. Chakravarthy MV, Booth FW. Eating, exercise, and "thrifty" genotypes: connecting the dots toward an evolutionary understanding of modern chronic diseases. J Appl Physiol 2004; 96(1):3-10.
3. Neel JV. Diabetes mellitus: a "thrifty" genotype rendered detrimental by "progress"? Am J Hum Genet 1962; 14:353-62.:353-362.

4. Heilbronn LK, Civitarese AE, Bogacka I, Smith SR, Hulver M, Ravussin E. Glucose tolerance and skeletal muscle gene expression in response to alternate day fasting. Obes Res 2005; 13(3):574-581.
5. Heilbronn LK, Smith SR, Martin CK, Anton SD, Ravussin E. Alternate-day fasting in nonobese subjects: effects on body weight, body composition, and energy metabolism. Am J Clin Nutr 2005; 81(1):69-73.
6. Klein S, Sakurai Y, Romijn JA, Carroll RM. Progressive alterations in lipid and glucose metabolism during short-term fasting in young adult men. Am J Physiol Endocrinol Metab 1993; 265(5):E801-E806.
7. Bergman BC, Cornier MA, Horton TJ, Bessesen DH. Effects of fasting on insulin action and glucose kinetics in lean and obese men and women. Am J Physiol Endocrinol Metab 2007; 293(4):E1103-E1111.
8. Soeters MR, Sauerwein HP, Dubbelhuis PF et al. Muscle adaptation to short-term fasting in healthy lean humans. J Clin Endocrinol Metab 2008;jc.
9. Bjorkman O, Eriksson LS. Influence of a 60-hour fast on insulin-mediated splanchnic and peripheral glucose metabolism in humans. J Clin Invest 1985; 76(1):87-92.
10. Fery F, d'Attellis NP, Balasse EO. Mechanisms of starvation diabetes: a study with double tracer and indirect calorimetry. Am J Physiol Endocrinol Metab 1990; 259(6):E770-E777.
11. van der Crabben SN, Allick G, Ackermans MT, Endert E, Romijn JA, Sauerwein HP. Prolonged Fasting Induces Peripheral Insulin Resistance, Which Is Not Ameliorated by High-Dose Salicylate. J Clin Endocrinol Metab 2008; 93(2):638-641.
12. Fryburg DA, Barrett EJ, Louard RJ, Gelfand RA. Effect of starvation on human muscle protein metabolism and its response to insulin. Am J Physiol Endocrinol Metab 1990; 259(4):E477-E482.
13. Tsalikian E, Howard C, Gerich JE, Haymond MW. Increased leucine flux in short-term fasted human subjects: evidence for increased proteolysis. Am J Physiol Endocrinol Metab 1984; 247(3):E323-E327.
14. Afolabi PR, Jahoor F, Jackson AA et al. The effect of total starvation and very low energy diet in lean men on kinetics of whole body protein and five hepatic secretory proteins. Am J Physiol Endocrinol Metab 2007; 293(6):E1580-E1589.
15. Lecker SH, Goldberg AL, Mitch WE. Protein Degradation by the Ubiquitin-Proteasome Pathway in Normal and Disease States. J Am Soc Nephrol 2006; 17(7):1807-1819.
16. Report of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus. Diabetes Care 2003; 26(90001):5S-20.
17. Finegood DT, Bergman RN, Vranic M. Estimation of endogenous glucose production during hyperinsulinemic-euglycemic glucose clamps. Comparison of unlabeled and labeled exogenous glucose infusates. Diabetes 1987; 36(8):914-924.
18. Soeters MR, Sauerwein HP, Groener JE et al. Gender-Related Differences in the Metabolic Response to Fasting. J Clin Endocrinol Metab 2007; 92(9):3646-3652.
19. Geukers VG, Oudshoorn JH, Taminiau JA et al. Short-term protein intake and stimulation of protein synthesis in stunted children with cystic fibrosis. Am J Clin Nutr 2005; 81(3):605-610.
20. STEELE R. Influences of glucose loading and of injected insulin on hepatic glucose output. Ann N Y Acad Sci 1959; 82:420-30.:420-430.
21. Ackermans MT, Ruiter AF, Endert E. Determination of glycerol concentrations and glycerol isotopic enrichments in human plasma by gas chromatography/mass spectrometry. Anal Biochem 1998; 258(1):80-86.
22. Koutsari C, Jensen MD. Thematic review series: patient-oriented research. Free fatty acid metabolism in human obesity. J Lipid Res 2006; 47(8):1643-1650.
23. Bisschop PH, de Sain-van der Velden, Stellaard F et al. Dietary Carbohydrate Deprivation Increases 24-Hour Nitrogen Excretion without Affecting Postabsorptive Hepatic or Whole Body Protein Metabolism in Healthy Men. J Clin Endocrinol Metab 2003; 88(8):3801-3805.

24. Frayn KN. Calculation of substrate oxidation rates in vivo from gaseous exchange. J Appl Physiol 1983; 55(2):628-634.
25. Barthel A, Schmoll D. Novel concepts in insulin regulation of hepatic gluconeogenesis. Am J Physiol Endocrinol Metab 2003; 285(4):E685-E692.
26. Boden G, Lebed B, Schatz M, Homko C, Lemieux S. Effects of Acute Changes of Plasma Free Fatty Acids on Intramyocellular Fat Content and Insulin Resistance in Healthy Subjects. Diabetes 2001; 50(7):1612-1617.
27. Holland WL, Knotts TA, Chavez JA, Wang LP, Hoehn KL, Summers SA. Lipid Mediators of Insulin Resistance. Nutrition Reviews 2007; 65:39-46.
28. Varady KA, Roohk DJ, Loe YC, Evoy-Hein BK, Hellerstein MK. Effects of modified alternate-day fasting regimens on adipocyte size, triglyceride metabolism, and plasma adiponectin levels in mice. J Lipid Res 2007; 48(10):2212-2219.
29. Corssmit EPM, Stouthard JML, Romijn JA, Endert E, Sauerwein HP. Sex differences in the adaptation of glucose metabolism to short-term fasting: Effects of oral contraceptives. Metabolism 1994; 43(12):1503-1508.
30. Ahima RS. Adipose Tissue as an Endocrine Organ. Obesity 2006; 14(S8):242S-249S.
31. Merl V, Peters A, Oltmanns KM et al. Serum adiponectin concentrations during a 72-hour fast in over- and normal-weight humans. Int J Obes (Lond) 2005; 29(8):998-1001.
32. Horber FF, Horber-Feyder CM, Krayer S, Schwenk WF, Haymond MW. Plasma reciprocal pool specific activity predicts that of intracellular free leucine for protein synthesis. Am J Physiol Endocrinol Metab 1989; 257(3):E385-E399.
33. Dann SG, Thomas G. The amino acid sensitive TOR pathway from yeast to mammals. FEBS Letters 2006; 580(12):2821-2829.