Effect of intermittent fasting and refeeding on insulin action in healthy men

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Halberg, Nils, Morten Henriksen, Nathalie Söderhamn, Bente Stallknecht, Thorkil Ploug, Peter Schjerling, and Flemming Dela. Effect of intermittent fasting and refeeding on insulin action in healthy men. J Appl Physiol 99: 2128–2136, 2005. First published July 28, 2005; doi:10.1152/japplphysiol.00683.2005.—Insulin resistance is currently a major health problem. This may be because of a marked decrease in daily physical activity during recent decades combined with constant food abundance. This lifestyle collides with our genome, which was most likely selected in the late Paleolithic era (50,000–10,000 BC) by criteria that favored survival in an environment characterized by fluctuations between periods of feast and famine. The theory of thrifty genes states that these fluctuations are required for optimal metabolic function. We mimicked the fluctuations in eight healthy young men [25.0 ± 0.1 yr (mean ± SE); body mass index: 25.7 ± 0.4 kg/m2] by subjecting them to intermittent fasting every second day for 20 h for 15 days. Euglycemic hyperinsulinemic (40 mU·min⁻¹·m⁻²) clamps were performed before and after the intervention period. Subjects maintained body weight (86.4 ± 2.3 kg; coefficient of variation: 0.8 ± 0.1%). Plasma free fatty acid and β-hydroxybutyrate concentrations were 347 ± 18 and 0.06 ± 0.02 mM, respectively, after overnight fast but increased (P < 0.05) to 423 ± 86 and 0.10 ± 0.04 mM after 20-h fasting, confirming that the subjects were fasting. Insulin-mediated whole body glucose uptake rates increased from 6.3 ± 0.6 to 7.3 ± 0.3 mg·kg⁻¹·min⁻¹ (P = 0.03), and insulin-induced inhibition of adipose tissue lipolysis was more prominent after than before the intervention (P = 0.05). After the 20-h fasting periods, plasma adiponectin was increased compared with the basal levels before and after the intervention (5,922 ± 991 vs. 3,860 ± 784 ng/ml, P = 0.02). This experiment is the first in humans to show that intermittent fasting increases insulin-mediated glucose uptake rates, and the findings are compatible with the thrifty gene concept.

Euglycemic clamp; adiponectin

Our genome was probably selected during the Late-Paleolithic era (50,000–10,000 BC), during a time humans existed as hunter-gatherers (6). At that time there were no guarantees in finding food, resulting in intermixed periods of feast and famine. In addition, physical activity had to be a part of our ancestors’ daily living as forage and the hunt for food must have been done through physical activity (15). Cycling between feast and famine, and thus oscillations in energy stores, as well as between exercise and rest, was characteristic in the Late-Paleolithic era and might have driven the selection of genes involved in the regulation of metabolism (30).

Thus our genotype selected centuries ago to favor an environment with oscillations in energy stores still exists with few if any changes. The modern sedentary lifestyle common in the westernized countries is characterized by constant high food availability and low physical activity, and it has led to an imbalance between our genotype and the environment in which we live today. This may predispose our potential “thrifty” genes to misexpress metabolic proteins, manifesting in chronic diseases (e.g., Type 2 diabetes) in the industrialized part of the world.

It is well known that physical training increases insulin action (10). The molecular events leading to an exercise-mediated increase in insulin action are not fully characterized. In addition, energy usage during each exercise bout in the training regimen with subsequent eating creates oscillations in energy stores. These oscillations are probably not as massive as the oscillations seen between periods of feast and famine for the Late-Paleolithic people, but some similarities might exist, and we speculated whether exercise-induced oscillations in energy stores could be mimicked by intermittent fasting. This study was undertaken to test the hypothesis that 14 days of intermittent fasting and refeeding improves insulin-stimulated glucose disposal.

Materials and Methods

Subjects

Eight healthy young Caucasian men (age 25.0 ± 0.1 yr, body mass index 25.7 ± 0.4 kg/m²) gave their written consent according the declaration of Helsinki to participate in the study. The study was approved by the local Danish ethical committee (KF 01-109/04).

Two days before both clamp experiments (see Experimental Procedure), the subjects were instructed to eat at least 250 g of carbohydrate each day and to avoid strenuous exercise.

Throughout the intervention, the subjects were instructed to uphold their normal exercise habits, to maintain their usual macronutrient mixing of their meals, and to eat sufficient quantities of food on the nonfasting days to ensure that their body weight was stable. The subject characteristics are given in Table 1.

Experimental Procedure

The subjects were examined on two occasions: before and after 14 days of fasting every second day for 20 h, giving seven fasting periods. Each fasting period started at 2200 and ended at 1800 the following day (for protocol see Fig. 1). During the fasting periods the subjects were allowed to drink water and were instructed to maintain habitual activities.

On the day of clamp experiments, the subjects arrived at the laboratory at 0800, after an overnight fast. The subjects were weighed and had their height measured and were placed in a bed position.

A microdialysis catheter was inserted in the subcutaneous fat on the abdomen (see below), and a small subcutaneous depot of 133Xe was placed in close proximity (~5 cm) to the microdialysis catheter. One

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The relative recovery was calculated as \( \frac{dpmp}{dpmd} \), where \( dpmp \) and \( dpmd \) are the \(^{14}\)C activity in the perfusate and dialysate, respectively. This method involved the internal reference calibration technique (37).

During the intervention period the subjects recorded their heart rate (Ultima, Cardiosport, Denmark) 24 h a day and measured their body weight in the morning before breakfast (on nonfasting days). Additionally, after the fasting on day 10 a muscle biopsy was taken (Bergström needle method modified to apply suction). In addition, smaller biopsies were obtained from the mid portion of vastus lateralis after the fourth fasting period (i.e., day 10). The biopsy was then obtained with a Tru-core I biopsy needle and instrument (Medical Device Technology, Gainesville, FL).

Muscle biopsies were quickly cleaned from visible blood and frozen in liquid nitrogen (within 15 s) and stored at \(-80°C\) until further analysis. Before analysis, the biopsies were freeze dried and carefully dissected free from connective tissue, blood, and fat. A sample of the muscle powder was used to determine glycogen content by the hexokinase method (25). Another part of the muscle powder was used to determine intramuscular triglyceride (IMTG) content by the chemical extraction method (18, 33). Briefly, the samples were homogenized in methanol and chloroform and the supernatant containing the lipids was removed and mixed with water. The lipids contained in the chloroform phase were then removed and hydrolysis was accomplished by adding tetraethylammoniumhydroxide (20%) and ethanol (1:28). After 30 min at 60°C, the reaction was stopped with HCl. The released acyl-glycerol was finally determined on a CMA 600 analyzer (CMA/microdialysis) and the triacylglycerol content was calculated.

GLUT4 expression. Expression of GLUT4 protein was measured by Western blot in a muscle biopsy obtained during the fasting condition before each clamp. Muscle biopsies were quickly cleaned from visible blood and/or fat, frozen in liquid nitrogen, and stored at \(-80°C\). The muscle tissue was subsequently homogenized with a Polytron PT 3100 (Kinematica, Littau-Luzern, Switzerland) at maximum speed for \( \sim 10 \) s in 10 vol of \(-55°C\) buffer (4% SDS, 10 mM pyrophosphate, 2 mM sodium orthovanadate, 10 mM EDTA, 25 mM Tris-HCl, pH 6.8). Samples were sonicated for \( \sim 5 \) s to break DNA strands, and total protein concentrations were determined by the bicinchoninic acid method using BSA as standard. For Western blot, 10 μg of protein were separated by SDS-PAGE on 10% gels (Criterion system, Bio-Rad, Hercules, CA) and electrophoretically transferred to polyvinylidene difluoride membranes for 45 min at 100 V by using a tank buffer system (Bio-Rad). Transfer buffer contained 25 mM Tris, 192 mM glycine, and 20% methanol. Membranes were...
blocked in 1% defatted milk powder plus 5% BSA in TS buffer [10 mM Tris (pH 7.4), 150 mM NaCl] for 90 and 60 min with primary and horseradish peroxidase-labeled secondary antibodies, respectively, and diluted in blocking solution. Antigen-antibody complexes were visualized and quantitated by a LAS 3000 imaging system (Fuji Film). Monoclonal antibody F-27 was used for detection of GLUT4 (35). Signals were normalized against amount of mRNA to be constitutively expressed (14). To validate this assumption, another unrelated “constitutive” RNA, GAPDH mRNA, was observed.

**Blood sampling and analysis.** Arterialized blood for measurement of hormones, metabolites, and cytokines was sampled from the catheter in the hand vein at basal and at termination of the clamp. Blood was collected in iced tubes and immediately centrifuged at 4°C. Plasma FFA analysis was performed by an enzyme color assay (ACS-ACOD, WAKO) and performed according to manufacturer’s instructions (insulin: DakoCytomation; glveloper, Glostrup, Denmark).

**Indirect calorimetry.** Expiratory gases were measured on the Oxycon Pro Online Ventilated hood system (Jaeger). The measured values were averaged over 10 min of steady state.

**Statistics.** All data are presented as means ± SE, except muscle mRNA and protein as well as plasma hormones (excluding insulin), which were log transformed before statistical analysis and are presented as geometric means ± back-transformed SE.

Two-way ANOVA for repeated measurements was used for detection of differences between the glucose infusion rates before and after the intermittent fasting. When a significant main effect was observed, the Student-Newman-Keuls test was used post hoc. In comparison of a single parameter before, during, and after the experiment, a one-way ANOVA for repeated measures was used. Comparison of a single parameter before and after the fasting intervention was performed with Student’s paired t-test. The SigmaStat version 2.03 software package was used for all statistical analysis. P < 0.05 was considered statistically significant in two-tailed testing.

## RESULTS

**Weight, Body Composition, and Indexes of Physical Activity**

The body weight was maintained stable throughout the experiment (86.4 ± 2.3 kg, 0.8 ± 0.1% coefficient of variation) and percent body fat was also unchanged before compared with after the fasting intervention (Table 1).

The level of habitual daily physical activity did not decrease during fasting days. Thus the average heart rate during daytime was not different during fasting (79 ± 3 min⁻¹) compared with nonfasting days (80 ± 3 min⁻¹).

**Whole Body Glucose Metabolism**

Plasma glucose concentration during both clamps was kept constant (Fig. 2), with a coefficient of variance of 4.4% ± 1.3% mmol/l during the last hour of the clamps.

The glucose infusion rate was significantly increased during fasting days. Thus the average heart rate during daytime was not different during fasting (79 ± 3 min⁻¹) compared with nonfasting days (80 ± 3 min⁻¹).

**Glycerol Metabolism in Adipose Tissue**

There was no effect of intermittent fasting in either the adipose tissue blood flow (2.4 ± 0.5 vs. 2.9 ± 0.7 ml·100 g⁻¹·min⁻¹ at basal and 2.6 ± 0.5 vs. 3.1 ± 0.5 ml·100 g⁻¹·min⁻¹ at the insulin-stimulated state) or the absolute interstitial glycerol concentrations (Fig. 3A) during the clamps. However, the interstitial glycerol concentrations decreased exponentially with the insulin infusion (R² = 0.96 before and R² = 0.99 after the fasting intervention), and the negative slopes of the curves were larger after the fasting intervention compared with before (P = 0.05) (Fig. 3B). This indicates that insulin had an enhanced inhibitory effect on lipolysis after intermittent fasting compared with before.

**Substrates and Metabolites**

Fasting (8 h) plasma glucose concentrations were similar before (5.0 ± 0.1 mM) and after (5.1 ± 0.1 mM) the intermittent fasting period. After 20-h fasting, i.e., days 4, 6, and 10, plasma glucose concentrations were lower (4.6 ± 0.1, 4.6 ± 0.1, and 4.7 ± 0.1 mM, respectively) compared with the shorter fasting periods (8 h) (P < 0.05).

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**Table 2. Primers for real-time RT-PCR**

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Sense Primer</th>
<th>Antisense Primer</th>
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<tbody>
<tr>
<td>PGC-1α</td>
<td>TCAAGACCAGCAAAACACGGGAC</td>
<td>TCAAGGAGGACAAACCCATCACCA</td>
</tr>
<tr>
<td>GAPDH</td>
<td>CTCCTGCAGGAGAACGTCTTT</td>
<td>GAGGGGCCATCCACAGTCTTCT</td>
</tr>
<tr>
<td>RPLP0</td>
<td>GCCAAGTCGATCCCTGCTT</td>
<td>GCCAAGACCTGTTTGTAAGTCGTM</td>
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Fasting (8 h) plasma β-hydroxybutyrate, FFA, and glycerol concentrations were similar before and after the intermittent fasting period, and all decreased \( (P < 0.05) \) with insulin infusion (Fig. 4). After 20-h fasting, i.e., days 4, 6, and 10, plasma FFA and glycerol concentrations were increased compared with the shorter fasting periods \( (P < 0.05) \) whereas the increase in β-hydroxybutyrate did not attain statistical significance \( (P = 0.07) \) (Fig. 4).

**Hormones**

Fasting (8 h) plasma insulin concentrations were similar before (33 ± 5 pM) and after (38 ± 7 pM) the intermittent fasting period, and concentrations increased \( (P < 0.05) \) with insulin infusion (to 439 ± 63 and 404 ± 18 pM, respectively). After 20-h fasting, i.e., days 4, 6, and 10, plasma insulin concentrations were unchanged (24 ± 4, 24 ± 5, and 16 ± 4 pmol/l) compared with the shorter fasting period (Fig. 4).

Plasma adiponectin concentrations did not change with insulin infusion and were similar on the 2 clamp days (Fig. 5). However, after 20-h fasting (days 6, 10, and 14) a 37% increase was seen compared with the shorter fasting days \( (P = 0.02) \) (Fig. 4).

Plasma leptin concentrations were similar on the 2 clamp days and did not change with insulin infusion (Fig. 5). However, after 20-h fasting (days 6, 10, and 14) a 37% increase was seen compared with the shorter fasting days \( (P = 0.02) \) (Fig. 5).

No significant differences were observed in either TNF-α or IL-6 concentrations during this study (Fig. 5).

**Muscle Triglyceride, Glycogen, GLUT4, and PGC-1α mRNA**

No overall changes were observed in concentrations of IMTG \( (P = 0.11) \), glycogen \( (P = 0.26) \), or mRNA content of PGC-1α \( (P = 0.18) \) when measured before and after each clamp and after fasting on day 10 (Figs. 6 and 7). However, with insulin stimulation (data from both clamps are included), we observed a significant decrease \( (P = 0.04) \) in the IMTG...
concentration. Furthermore, total muscle GLUT4 protein content did not change with the fasting intervention \((P = 0.66)\) (Fig. 7).

**Respiratory Exchange Ratios**

Respiratory exchange ratios (RER) were similar at basal (after 8-h fasting) on the 2 clamp days. With insulin stimulation RER increased at both occasions (Fig. 8). No differences were observed in RER values between the overnight and the 20-h fasted state (Fig. 8).

**DISCUSSION**

In the present study we have used a very simple intervention protocol with the aim of mimicking the perturbations in energy stores that are inherent in a physical active lifestyle with regular exercise sessions. In a wider perspective we have tried to unravel the significance of genes that may be responsible for an evolutionary selection process, i.e., the thrifty genes. In this context the used intervention seems inevitably small. Nevertheless, by subjecting healthy men to cycles of feast and famine we did change the metabolic status to the better, implying that the mismatch between our ancient genotype and the lifestyle of the westernized individual of today became smaller. To our knowledge this is the first study in humans in which an increased insulin action on whole body glucose uptake and adipose tissue lipolysis has been obtained by means of intermittent fasting. This result is in accordance with previously reported in rodents (2, 32). In these studies, fasting every second day increased the insulin sensitivity approximately sevenfold according to the homeostatic model assessment (2) and decreased the incidence of diabetes (32).

Prolonged fasting for 72 h with minimal physical activity has previously been shown to increase IMTG levels in humans (46). With the present fasting protocol and maintenance of habitual daily physical activity in the fasting periods, we had expected to detect a decrease in IMTG content in the skeletal muscle. The fact that this was not seen and that muscle glycogen content was unchanged could suggest that skeletal muscle is not immediately involved in recognition of acute energy oscillations. There is no doubt, however, that fasting for 20 h while maintaining normal daily physical activity must cause a temporary negative energy balance larger than normally experienced in a daily basis. This is also indicated by our finding of decreased plasma glucose concentrations after 20-h fasting. We did not have the possibility to estimate the hepatic glycogen stores, but from animal studies (17) we must infer that liver glycogen probably also decreased considerably during the 20-h fasting periods. It has previously been suggested that usage of muscle energy depots during fasting would be an evolutionary disadvantage, because it would lessen the capacity for physical performance and hence the ability to provide food (i.e., to hunt and gather) during periods of fasting (6, 45). The present findings support this view.

In contrast to the findings in skeletal muscle, the adipose tissue responded to the changes in energy balance as intermittent fasting changed the plasma concentrations of the adipocyte-specific hormones leptin and adiponectin. However, because we did not measure the energy stores in the adipose...
tissue during the intervention (e.g., by fat cell size), we cannot
determine whether the change in adipokine release is merely a
secondary response to intermittent fasting or whether the adi-
pose tissue is an active recognizer of energy oscillations.

Blood sampling for measurement of adipokines at basal
levels before and after the fasting intervention was performed
at 1000 whereas the three samples on days 6, 10, and 14 were
taken at 1700. The amount of circulating adiponectin is con-
stant or slightly decreased during daytime (20). Hence, the
boosts of 37% we observed after each fasting period are not
due to nocturnal variation. Because the plasma adiponectin
concentration is positively correlated to insulin sensitivity in
humans (8, 23, 29) and adiponectin administration in rodents
increases insulin action (9, 38, 48), it seems likely that our
finding of increases in circulating adiponectin after each fasting
period would be able to exert an insulin-sensitizing effect.

Skeletal muscle content of GLUT4 protein after the over-
night fast did not differ before and after the fasting interven-
tion. Future studies will have to determine whether the insulin
signaling, e.g., phosphorylation of the insulin receptor sub-
strate, is influenced by fluctuations in energy stores and thereby
accounts for the increase insulin action as measured by the
clamp method reported herein.

Because 36 h passed between the last fasting period and the
last clamp, it seems most likely that the potential insulin-
sensitizing effects of adiponectin were due to adiponectin-
induced changes in gene expression. This could in turn be
mediated through an AMPK activation that further activates
several transcription factors including myocyte enhancing fac-
tor that increases GLUT4 expression (24, 27). Another posi-
bility is that the adiponectin boosts peroxisome proliferated-
activated receptor-γ (PPAR-γ) expression as seen in 3T3-L1
adipocytes (1). In addition, because PPAR-γ induces adiponec-
tin expression (16), it can be speculated that fasting starts a
positive feedback loop that results in increased levels of both
circulating adiponectin and PPAR-γ. Both are known to in-
crease the insulin sensitivity.

A considerable increase in plasma FFA concentrations (5-
fold) may raise the amount of circulating adiponectin slightly
(43), and glucocorticoids positively regulate adiponectin gene
expression (21). FFA and glucocorticoid increase during fast-
ing, but in previous studies no effect of fasting on circulating
adiponectin was seen (19, 49). Apart from differences in
increases of FFA and glucocorticoids, different analysis meth-
ods used [RIA vs. ELISA (present study)] may recognize
different isoforms of adiponectin and thereby account for the
discrepancy.

Leptin exhibits nocturnal differences with a peak during the
night (2400–0800), whereas there is no difference between
1000 and 1700; if anything, plasma leptin concentrations are
slightly higher at 1000 (40). In accordance with previous
findings (19, 41, 49), we found a decrease in circulating leptin
after 8–20 h of fasting. This decrease most likely reflects a
state of energy deficiency and is probably not involved in the
increased insulin action we have found in the present study.

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Fig. 5. Plasma concentrations of adiponectin (A), leptin (B), IL-6 (C), and TNF-α (D) before and after clamps performed before and after 15 days of intermittent fasting, and after 20 h of fasting on days 6, 10, and 14 of the intermittent fasting intervention. ‡P < 0.05 between the sample taken after 20 h of fasting compared with basal samples taken after an overnight fast (8 h). Data are geometric means (GeoMean) ± back-transformed SE.
The mechanism by which physical training increases whole body insulin sensitivity is not known in detail. It has previously been shown that in muscle the effect is mediated via local contraction dependent mechanisms (11–13), and this could include exercise-induced oscillations in local energy stores. However, the insulin-sensitizing effects of exercise and intermittent fasting may not exert their effects via the same pathway. Although the local effect of exercise is well proven (there is no transfer of training-induced increase in insulin sensitivity to nontrained muscle), it is less likely that the effect of intermittent fasting is a local, muscle phenomenon. Thus even though we were not able to detect changes in muscle glycogen and triglyceride content after 20-h fasting, the intervention may still have exerted the effects via oscillations in other energy stores (e.g., in adipose tissue or liver). The finding of decreased leptin concentrations corresponding to the intermittent fasting verifies that adipocyte metabolism was influenced by the intervention.

We did not find an effect of intermittent fasting on muscle PGC-1α mRNA levels. In contrast, PGC-1α mRNA increases with acute exercise (34, 47) and is suggested to be involved in the enhancement of insulin-mediated glucose uptake after exercise training (28, 39). Thus PGC-1α may represent a step at which the insulin enhancement actions of exercise training and intermittent fasting diverge.

Whole body insulin-mediated glucose uptake was estimated by the euglycemic hyperinsulinemic clamp technique. Even though this method is a standard for measuring insulin action, day-to-day coefficient of variation has been reported to vary between 2.4 and 15% (4, 36, 42). Part of the observed effect of
the intervention may therefore be due to biological and instrumental variation.

It is important to note that, in the present study, the subjects maintained their body weight throughout the intervention period, and percent body fat did not change with intermittent fasting. Thus, in contrast to previous studies using alternate-day fasting (22), the subjects in the present study kept their body weight by following the dietary instructions of eating abundantly every other day. It is well known that insulin sensitivity can be influenced by long-term profound changes of macronutrients in the diet. However, because the subjects were instructed to maintain their usual diet habits (although increasing the amount of food), it is unlikely that eventual minor changes in the macronutrient mix during 8 nonconsecutive days (i.e., the nonfasting days) would influence insulin sensitivity.

Furthermore, the increased insulin action after the intervention was not the result of the last fasting period because from the last fasting period until the beginning of the overnight fast the subjects were allowed to eat for 30 h during which they consumed at least 250 g of carbohydrates. Muscle glycogen was not different between the pre- and postintervention clamps, testifying that carbohydrate loading was sufficient before each clamp experiment.

In keeping with previous findings (3), we observed a decrease in IMTG with insulin stimulation. At first glance this seems counterintuitive. However, during insulin stimulation the FFA supply to the skeletal muscle decreases dramatically, and because some skeletal muscle FFA oxidation is still the FFA supply to the skeletal muscle decreases dramatically, it seems counterintuitive. However, during insulin stimulation clamps, testifying that carbohydrate loading was sufficient before each clamp experiment.

In conclusion, the findings that intermittent fasting increases insulin sensitivity on the whole body level as well as in adipose tissue support the view that cycles of feast and famine are important as an initiator of thrifty genes leading to improvements in metabolic function (6). We suggest that a fasting-induced increase in circulating adiponectin is at least partly responsible for this finding. The change in adiponectin, together with changes in plasma leptin with fasting, underlines the important role of the adipose tissue in recognizing the oscillation in energy stores. Finally, the data indicate that intermittent fasting and physical training may increase insulin action via different mechanisms because muscle energy stores did not change with the present fasting intervention.

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