Prolonged fasting and the effects on biomarkers of inflammation and on adipokines in healthy lean men.

Prolonged Fasting and the Effects on Biomarkers of Inflammation and on Adipokines in Healthy Lean Men

Authors
N. A. van Herpen[1, 2], H. Sell[3], J. Eckel[3], P. Schrauwen[1, 2], R. P. Mensink[1, 2]

Affiliations
[1] Department of Human Biology, NUTRIM – School for Nutrition, Toxicology and Metabolism, Maastricht University Medical Center, Maastricht, The Netherlands
[2] TI Food and Nutrition, Wageningen, The Netherlands
[3] Paul Langerhans Group for Integrative Physiology, German Diabetes Center, Düsseldorf, Germany

Key words
- free fatty acids
- cytokines
- adipokines

Abstract

Obesity and insulin resistance are associated with low-grade systemic inflammation, which is related to increased concentrations of plasma FFAs, glucose, or insulin. Prolonged fasting induces insulin resistance due to elevated plasma FFAs, but is not accompanied by hyperinsulinemia or hyperglycemia. This makes it possible to study effects of physiologically increased FFA concentrations on inflammatory markers, when insulin and glucose concentrations are not increased. In random order, 10 healthy young lean men (mean BMI: 22.8 kg/m^2) were fasted or fed in energy balance for 60 h with a 2-week wash-out period. Subjects stayed in a respiration chamber during the 60-h periods. Blood samples were taken after 12, 36, and 60 h. Then, a hyperinsulinemic-euglycemic clamp was performed.
Fasting decreased insulin sensitivity by 45 % and increased FFA concentrations 5-fold. Fasting did not change concentrations of the inflammatory cytokines TNF-α, IL-1β, IL-6 and IL-8, or of hs-CRP. Effects on vascular endothelial growth factor (VEGF) – which may positively relate to insulin resistance, and on chemerin and leptin – adipokines related to obesity, and obesity-related pathologies, were also studied. At t = 60 h, VEGF concentrations were significantly increased during the fasted period ($p < 0.05$). At the same time point, chemerin ($p < 0.01$) and leptin ($p < 0.01$) were significantly decreased after fasting. For leptin, this decrease was also significant after 36 h ($p < 0.01$). Adiponectin levels remained unchanged. In healthy young lean men, fasting-induced increases in FFAs leading to insulin resistance do not cause changes in concentrations of the inflammatory cytokines. VEGF concentrations increased and those of chemerin decreased.

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Correspondence
R. P. Mensink
Department of Human Biology
NUTRIM School for Nutrition, Toxicology and Metabolism
Maastricht University Medical Center
P.O. Box 616
6200 MD Maastricht
The Netherlands
Tel.: +31/43/3881 621
Fax: +31/43/3670 976
r.mensink@hb.unimaas.nl

Introduction

Obese subjects have increased concentrations of free fatty acids (FFAs), which may explain the frequently observed associations between increased body fat content and insulin resistance. Indeed, increasing plasma FFA concentrations by lipid infusion causes insulin resistance [1]. However, increased plasma FFA concentrations are also related to other metabolic complications such as low-grade systemic inflammation, which on its turn may cause insulin resistance, hyperinsulinemia, and hyperglycemia [2–4]. Indeed, several studies have demonstrated that insulin resistance is associated with elevated levels of high-sensitive C-reactive protein (hs-CRP) and interleukin-6 (IL-6), and decreased levels of the anti-inflammatory adipokine adiponectin [5], independent of obesity [5, 6]. Moreover, infusion of insulin in humans increased leptin concentrations, which has also been linked to inflammation [7], and decreased adiponectin concentrations [8]. Finally, hyperglycemia can contribute to activation of NF-κB [9]. Thus, a complex interplay exists between FFA concentrations, insulin resistance, and inflammation. Further, it is unknown if insulin resistance per se is more important than hyperinsulinemia or hyperglycemia to explain the effects on inflammation.
Recently, we have shown that in lean healthy men prolonged fasting (60 h) was accompanied by elevated plasma FFA levels and reduced insulin sensitivity [10]. Importantly, fasting-induced insulin resistance was not accompanied by hyperinsulinemia or hyperglycemia, which makes it possible to study the effects of physiologically increased FFA concentrations apart from those of increased insulin and glucose concentrations on

Table 1 Subject characteristics.

Parameter	Mean ± SEM
Age (years)	23.3 ± 0.8
Body weight (kg)	79.6 ± 2.5
Fat free mass (kg)	66.6 ± 2.0
Height (m)	1.87 ± 0.03
Body mass index (kg/m^2)	22.8 ± 0.5
Maximal aerobic capacity (ml O_2/kg_{FFM}/min)	57.8 ± 1.6

Values refer to 10 male subjects

inflammatory markers. Therefore, the aim of the present study was to examine effects of FFA and insulin resistance, without the presence of increased glucose and insulin concentrations, on plasma biomarkers related to low-grade systemic inflammation and on plasma adipokines. Effects on vascular endothelial growth factor (VEGF) – which may positively relate to insulin resistance [11], and on chemerin – an adipokine related to obesity, and obesity-related pathologies [12], whose synthesis may be affected by TNF-α [13], were also studied.

Methods

Subjects

Eleven healthy lean male volunteers without family history of diabetes mellitus or any other endocrine disorder participated in this study (**Table 1**). None of the subjects engaged in sports activities for more than 2 h per week. Body composition and maximal aerobic capacity were measured as described previously [10]. The study protocol was reviewed and approved by the Medical Ethical Committee of Maastricht University Medical Center and all subjects gave their written informed consent before participating in the study.

Experimental design

Using a randomized crossover design with a 2-week wash-out period, subjects participated in 2 experimental periods: a 60-h fast and a 60-h normal fed condition. In the fast condition, subjects were fasted for 60 h (calorie-free drinks only), while in the other condition diets were adjusted individually to match energy expenditure (50, 35, 15% of energy as carbohydrates, fat, and protein, respectively) [14]. Before the start of each experimental period, a standardized evening meal was provided. Subject stayed in a respiration chamber during the entire 60-h period to ensure compliance to the strict dietary protocols and to allow for the measurement of 24-h substrate oxidation and energy expenditure. In the respiration chamber subjects followed an activity protocol as previously described [15, 16]. Sleep as such was not monitored, but movements were [14, 15]. During each 60-h period, blood samples were taken after 12, 36, and 60 h – after an overnight fast of 10 h in the case of the fed condition.

Hyperinsulinemic-euglycemic clamp

After leaving the respiration chamber on the morning of the third day, a hyperinsulinemic-euglycemic clamp procedure was performed, as described [10].

Blood analyses

Blood was collected in EDTA containing tubes and immediately centrifuged at high speed. Plasma was transferred to Eppendorf tubes, snap frozen in liquid nitrogen, and stored at –80 °C until further analysis. Plasma FFAs (Wako Nefa C test kit; Wako Chemicals, Neuss, Germany) and glucose (hexokinase method; LaRoche, Basel, Switzerland) were measured with enzymatic assays. Insulin was determined using a radioimmunoassay (Linco Research, St. Charles, MO, USA).

Plasma markers of inflammation (TNF-α (tumor necrosis factor-alpha), IL-1β, IL-6, IL-8 and hs-CRP) were measured with a commercially available Multi Spot ELISA kit (Meso Scale Discovery, Gaithersburg, MD, USA). ELISA kits for leptin, adiponectin, and chemerin analyses were purchased from Biovendor (Heidelberg, Germany) and those for VEGF from Millipore (Schwalbach, Germany). All samples for one subject were analyzed in the same analytical run. The intra- and interassay coefficients of variation (CV) varied slightly for the various measurements, but were all less than <5% and <7%, respectively.

Statistics

Data are reported as means ± SEM. Statistical analyses were performed using SPSS 16.0 for Mac OS X. Statistical comparisons between the 2 conditions (fed vs. fast) were performed using the paired Student's *t*-test. Plasma parameters over time were compared by 2-way repeated measures ANOVA for investigation of treatment and time (treatment × time) interactions. When the interaction reached statistical significance, we performed post-hoc testing to determine the exact location of the difference. Differences were considered statistically significant when $p < 0.05$.

Results

Results of one subject were removed from the statistical analysis, because of a hs-CRP concentration at the start of the fed period >50 mg/dl, as opposed to a value of <1 mg/dl at the start of the fasted period. Characteristics of the remaining 10 subjects are presented in **Table 1**. Results on insulin sensitivity have been described in detail previously [10]. In summary, the glucose infusion rate for all subjects was decreased after the 60-h fasted period as compared with the fed period. Also the insulin sensitivity index (S_I) was reduced by ~45% ($p < 0.001$). The reduction in whole body insulin sensitivity was mainly accounted for by a reduction in insulin-stimulated glucose disposal. Insulin-induced suppression of endogenous glucose production was only marginally affected by fasting and was almost complete in both conditions. During the stay in the respiration chamber, body weight decreased by 0.3 ± 0.4 kg during the fed period and by –1.2 ± 0.2 kg during the fasted period (**Table 2**).

Blood parameters

Concentrations of all parameters were not significantly different at the start of the fed and fasted periods.

A significant treatment × time interaction ($p < 0.001$) was observed for plasma FFAs, glucose, and insulin concentrations [10]. Plasma FFA increased steadily, up to 2016 ± 118 µmol/l in de fasted condition vs. 421 ± 39 µmol/l in the fed condition ($p < 0.001$ for the difference in changes). Plasma glucose values remained unchanged throughout the fed condition, but gradually decreased to 3.73 ± 0.15 mmol/l after 60 h of fasting ($p < 0.001$). Baseline plasma insulin levels did not change in the fed condition, but were markedly reduced in the fast condition to 7.3 ± 0.7 µU/ml at t = 36 h ($p < 0.001$) and were maintained at this lower level (7.0 ± 0.89 µU/ml) at t = 60 h ($p < 0.001$).

Table 2 Effects of fasting on plasma markers of inflammation, VEGF, chemerin, leptin, and adiponectin.

	Fed period			Fasted period			p-Value
n = 10	t = 2 h	t = 36 h	t = 60 h	t = 12 h	t = 36 h	t = 60 h	Treatment × Time interaction
TNF-α (pg/ml)	4.33 ± 0.34	4.70 ± 0.26	4.43 ± 0.35	4.94 ± 0.38	4.87 ± 0.32	4.48 ± 0.30	0.156
Changes		0.37 ± 0.19	0.10 ± 0.30		−0.07 ± 0.18	−0.46 ± 0.21	
IL-1β (pg/ml)	0.79 ± 0.25	0.84 ± 0.26	0.71 ± 0.17	0.90 ± 0.21	0.81 ± 0.16	2.02 ± 0.67	0.288
Changes		0.05 ± 0.25	−0.08 ± 0.23		−0.09 ± 0.23	1.12 ± 0.55	
IL-6 (pg/ml)	0.62 ± 0.08	0.60 ± 0.05	0.57 ± 0.04	0.87 ± 0.17	0.66 ± 0.06	0.96 ± 0.14	0.038
Changes		−0.02 ± 0.07	−0.05 ± 0.08		−0.21 ± 0.14	0.09 ± 0.22	
IL-8 (pg/ml)	2.73 ± 0.30	2.87 ± 0.32	2.75 ± 0.27	3.82 ± 0.97	3.10 ± 0.42	3.01 ± 0.20	0.310
Changes		0.14 ± 0.35	0.02 ± 0.41		−0.72 ± 0.73	−0.81 ± 0.89	
hs-CRP (mg/dl)	0.51 ± 0.17	0.43 ± 0.18	0.32 ± 0.12	0.97 ± 0.55	1.23 ± 0.67	1.39 ± 0.56	0.405
Changes		−0.07 ± 0.05	−0.19 ± 0.06		0.26 ± 0.53	0.42 ± 0.46	
VEGF (pg/ml)	18.6 ± 7.7	17.4 ± 7.6	81.8 ± 11.2*	25.5 ± 9.3	40.7 ± 15.5	134.3 ± 12.1	0.042
Changes		−1.2 ± 4.7	63.2 ± 10.1*		15.2 ± 16.5	108.8 ± 14.5	
Chemerin (ng/ml)	129 ± 12	120 ± 8	135 ± 8	114 ± 7	112 ± 7	88 ± 6	0.002
Changes		−8.1 ± 7.5	6.1 ± 8.7+		−2.5 ± 3.5	−26.5 ± 3.8	
Leptin (ng/ml)	4.02 ± 1.19	3.91 ± 1.32	3.80 ± 1.07	3.89 ± 0.85	0.75 ± 0.23	0.19 ± 0.08	0.003
Changes		−0.10 ± 0.33+	−0.22 ± 0.29+		−3.14 ± 0.63	−3.70 ± 0.84	
Adiponectin (μg/ml)	8.41 ± 0.44	8.34 ± 0.38	8.41 ± 0.50	8.40 ± 0.37	8.29 ± 0.46	8.35 ± 0.36	0.991
Changes		−0.07 ± 0.35	0.00 ± 0.31		−0.12 ± 0.28	−0.05 ± 0.21	

VEGF concentrations could not be measured in one subject due to analytical problems.

* $p < 0.05$ and + $p < 0.01$ indicate a statistically significant difference from the change at the same time point during the fasted period

Plasma TNF-α concentrations did not significantly change during the fasted or fed condition. Similar conclusions could be drawn for IL-1β, IL-8, adiponectin, and hs-CRP. For IL-6, however, a statistically significant treatment × time effect was found ($p = 0.038$), but pair wise comparisons did not reveal any statistical differences between the various time points.

A statistically significant treatment × time effect was found for VEGF concentrations ($p = 0.042$). No differences were seen after 36 h. After 60 h, however, concentrations were increased with 63.2 ± 10.1 pg/ml during the fed period, which was significantly less than the increase of 108.8 ± 14.5 pg/ml during the fasted period ($p = 0.02$). The treatment × time effect also reached statistical significance for changes in chemerin concentrations ($p = 0.002$). In the fed condition, concentrations remained virtually unaffected, but were significantly decreased after 60 h of fasting ($p = 0.003$). Leptin concentrations were also affected and were significantly decreased after 36 and 60 h of fasting ($p = 0.004$ and $p < 0.001$, respectively).

Discussion

In the present study we have found that fasting-induced increases in FFA concentrations did not relate to changes in the concentrations of the inflammatory cytokines TNF-α, IL-1β, IL-6 and IL-8, or of hs-CRP. This extends the findings of a smaller study with 6 healthy lean men, that also found no proinflammatory effects of 60 h of fasting, as indicated by unchanged concentrations of IL-6, soluble TNF receptors I and II, and hs-CRP [17].

In our study, FFA concentrations were physiologically increased by fasting, which was accompanied by insulin resistance at reduced glucose and insulin concentrations [10]. In contrast, insulin resistance induced by longer-term lipid infusion does lead to hyperglycemia and hyperinsulinemia, and to increased concentrations of FFA and proinflammatory cytokines. This suggests that in normal weight men, insulin-resistance or increased FFA concentrations per se are not a prerequisite to induce a proinflammatory cytokine profile, but hyperglycemia or hyperinsulinemia – possibly in combination with high FFA levels – are. As prediabetic subjects, who are characterized by normal glucose concentrations and slightly elevated insulin concentrations, also have increased concentrations of markers and mediators of inflammation [18], it can be speculated that insulin may be more important in this respect. This is further supported by the findings of Stegenga et al. [19], who found that a short-term (6 h) lower-insulinemic hyperglycemic clamp decreased the expression of several proinflammatory cytokine in LPS-stimulated leucocytes. In contrast, after a hyperinsulinemic euglycemic clamp, expressions of most of the inflammatory cytokines were increased.

VEGF concentrations increased significantly during both conditions at t = 60 h for which we do not have an obvious explanation. Concentrations, however, were significantly more increased in the fasted situation. The association between insulin and glucose concentrations on VEGF is not clear. Loebig et al. [20] could not demonstrate a relationship between VEGF concentrations and the insulin sensitivity index, as measured under euglycemic clamp conditions in lean, overweight or obese male subjects. In contrast, Dandona et al. [21] found suppressed VEGF concentrations during insulin infusion and steady glucose concentrations. Hypoglycemia, however, may also increase plasma VEGF concentrations, as suggested by in vitro and in vivo studies [22–24]. In addition, a positive correlation between concentrations of circulating VEGF levels and BMI has been reported in healthy male subjects [20]. As BMI is also positively related to FFA concentrations [25], it can be speculated that fasting also increases plasma VEGF. In vitro, FFAs induce a significant release of VEGF from vascular smooth muscle cells that might contribute to increased circulating levels of this factor [26]. Thus, although insulin sensitivity per se does not relate to VEGF, further studies are needed on the exact determinants of plasma VEGF levels.

Chemerin concentrations decreased after fasting. In vitro, chemerin synthesis and secretion by 3T3-L1 adipocytes are upregulated by TNF-α [13]. Furthermore, we have shown a regu-

lation by TNF-α in human adipocytes [27]. Similar results have been found for IL-1β [28]. However, these 2 cytokines in our study were not affected by fasting and can therefore not have played a role in the decrease of plasma chemerin concentrations. Also, in vitro studies and studies in tissue explants have shown that insulin increases dose-and-time dependently chemerin secretion by adipocytes [29]. Further, a study in subjects with normal glucose tolerance has found positive relationships between plasma chemerin concentrations and various measures of the metabolic syndrome, although chemerin were not different between subjects with type 2 diabetes mellitus and controls [12]. At least for healthy subjects, this may suggest that insulin concentrations are an important determinant of plasma chemerin. To what extent plasma FFA concentrations play a role, is not known. It should be noted that the liver also secretes chemerin [30]. However, nothing is known about a possible regulation of hepatic chemerin release.

During fasting, plasma leptin levels decreased as expected [31], which was already evident after 24 h. Adiponectin concentrations were not affected by fasting, which agrees with other study in which subjects fasted for 48 or 72 h [31, 32]. These findings support the notion that circulating adiponectin concentrations observed after weight reduction do not result from a negative energy balance, but rather from a decreased body fat content [32].

In conclusion, our study with healthy male subjects indicates that fasting-induced increases in FFAs and insulin resistance do not lead to changes in concentrations of the inflammatory cytokines. VEGF concentrations increased and those of chemerin decreased.

Conflict of Interest

The authors do not have any conflict of interest.

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