

Leptin and the proinflammatory state associated with human obesity

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Leptin and the Proinflammatory State Associated with Human Obesity

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It has been suggested that elevated leptin levels underlie the low grade proinflammatory state in human obesity. We reasoned that if elevated leptin levels are an important factor in the proinflammatory state in obesity, then exogenous leptin administration during weight loss should counteract the concurrent beneficial effects of weight loss on the proinflammatory state. We therefore determined whether long-acting pegylated recombinant leptin (PEG-OB) prevents the decrease in cellular and humoral inflammation parameters during a very low calorie diet in healthy overweight young men. Except for B cells, PEG-OB treatment did not influence the decline in total leukocyte count and mononuclear subfractions during the diet. Weight loss decreased the humoral in-

flammation parameters TNF α , tissue plasminogen activator, and von Willebrand factor ($P < 0.05$), but in combination with PEG-OB treatment, a significant decrease was shown for inflammation markers as a whole ($P < 0.014$) and that of the individual parameters tissue plasminogen activator, von Willebrand factor, plasminogen activator inhibitor type 1, and intercellular adhesion molecule-1 ($P < 0.05$). The increase in C-reactive protein levels ($P < 0.05$) was the sole indication for a humoral proinflammatory action of leptin. Although PEG-OB treatment significantly increased weight loss ($P < 0.03$), the data do not support a proinflammatory role of leptin in human obesity. (*J Clin Endocrinol Metab* 89: 1773–1778, 2004)

HUMAN OBESITY IS associated with a chronic, low grade, proinflammatory state as indicated by leukocytosis (1), increased plasma levels of the proinflammatory cytokines IL-6 (2) and TNF α , (3) elevated acute phase proteins (4, 5), and increased levels of markers of endothelial cell dysfunction and activation (6). Weight loss attenuates this proinflammatory state (7).

Leptin is an adipocyte-derived polypeptide hormone that controls body weight through central regulation of food intake and energy expenditure (8). Abundance of leptin mRNA in adipose tissue and concentrations of leptin in the circulation are strongly positively correlated with body weight and adiposity in man; and human obesity is characterized by elevated circulating leptin levels (9). Both leptin and its receptor share structural and functional similarities with the IL-6 family of cytokines (10), and leptin appears to play a critical role in the inflammatory response. Defective immune responses are present in both leptin-deficient mice (11, 12) and infants (13) as well as during starvation and malnutrition, two conditions characterized by low levels of circulating leptin (12). CD 34⁺ hemopoietic stem cells (14) and most leukocytes express the leptin receptor (15), and the stimulating effects of leptin on leukocyte proliferation has been

well established *in vitro* (10). Further evidence from *in vitro* and animal studies suggests that leptin is also involved in regulation of the humoral inflammatory response through its direct effects on T cells (10), monocytes (10, 16), neutrophils (17), and endothelial cells (18, 19).

Weight loss reduces circulating leptin levels (20) and concomitantly lowers the plasma levels of inflammation markers associated with obesity (7). The question thus arises whether raised leptin levels underlie the proinflammatory state in human obesity (21). In this paper we sought evidence for a proinflammatory role of leptin in obesity through maintaining elevated plasma leptin levels during weight loss. To that end, moderately obese men were subjected to profound weight loss induced by a very low calorie diet (VLCD) and either received weekly injections of long-acting pegylated human recombinant leptin (PEG-OB) (22, 23) or placebo. It was reasoned that if elevated leptin levels are an important factor in inducing an inflammatory state in obese humans, exogenous leptin administration during weight loss would counteract the concurrent beneficial effects on the proinflammatory state.

Subjects and Methods

Study population

The study was part of a larger study into the effects of PEG-OB treatment on weight loss. Twenty-four overweight and moderately obese Caucasian men (body mass index, 25–32 kg/m²) were recruited through advertisements in local newspapers. All subjects had a stable weight and were healthy according to medical history, clinical examination, and routine laboratory findings. To achieve balanced treatment groups, patients were stratified and matched in pairs according to age, body mass index, and plasma leptin and insulin concentrations. Ran-

Abbreviations: CRP, C-Reactive protein; HDL, high-density lipoprotein; ICAM, intracellular adhesion molecular; LDL, low-density lipoprotein; PAI-1, plasminogen activator inhibitor type 1; PEG-OB, pegylated recombinant leptin; sTNFR, soluble TNF receptor; tPA, tissue plasminogen activator; VLCD, very low calorie diet; vWF, von Willebrand factor.

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domization numbers were generated and incorporated into the double-blind labeling by an independent third party. Within the first week of the study, two subjects in the placebo group dropped out voluntarily because they were unable to sustain their diet; this did not influence baseline characteristics of the study population. The study was approved by the medical ethical committee of University of Maastricht, and all participants gave written informed consent.

Weight loss and treatment

Weight loss was induced through a very low calorie diet (2 MJ/d; Modifast, Novartis, Breda, The Netherlands). The dietary prescription was discussed weekly with a dietician, and compliance with the diet was verified by measurement of weight loss. Body weight was measured on a calibrated digital scale accurate to 0.1 kg.

During the study, patients either received weekly sc injections of 80 mg PEG-OB (10 mg/ml; produced and provided by Hoffmann-La Roche, Inc., Nutley, NJ) or matching placebo (8 ml) in the paraventricular region. PEG-OB treatment was well tolerated. No indication could be found for the occurrence of significant amounts of PEG-OB neutralizing antibodies at the end of the study (d 46).

Blood sampling and biochemical analysis

Blood samples and plasma samples were obtained on d 1 (before the start of the diet and PEG-OB treatment), 8, 15, 25, and 46 of the study. Blood sampling was performed in the morning after at least an 8-h overnight fast, immediately before study medication. Samples were directly placed on melting ice and centrifuged within 1 h of sampling. The extracted plasma was recentrifuged, frozen in liquid nitrogen, and stored at -80°C until further analysis. All assays were performed in duplicate.

Glucose was measured by the hexokinase method (Roche, Basel, Switzerland), and insulin levels were measured at the certified central laboratory of University Hospital Maastricht (Maastricht, The Netherlands). Estimated insulin sensitivity was assessed by homeostasis model assessment (24): (fasting glucose \times fasting insulin)/22.5.

Total cholesterol was measured by the CHOD-PAP method (Roche, Mannheim, Germany), and the lipase method (Sigma-Aldrich Corp., St. Louis, MO) was used to measure triglycerides. High density lipoprotein (HDL) cholesterol was measured at the certified central laboratory of the University Hospital Maastricht, The Netherlands. Low density lipoprotein (LDL) cholesterol was calculated with the Friedwald formula (25): LDL cholesterol = total cholesterol $- (0.45 \times \text{triglycerides}) - \text{HDL cholesterol}$.

Total leukocytes and mononuclear subpopulations were analyzed on d 1 and 25 by fluorescence-activated cell sorting analysis. Plasma leptin levels were measured by a double-antibody sandwich ELISA using a monoclonal antibody specific for human leptin. This assay measures both pegylated and free leptin (26). Plasma IL-1, IL-6, TNF α , intercellular

adhesion molecular -1 (ICAM-1), and tissue plasminogen activator (tPA) were assessed by commercially available ELISA kits: PeliKane compact kit (CLB, Amsterdam, The Netherlands) for IL-6, HS Quantikine (R&D Systems, Abingdon, UK) for IL-1, TNF α -1, and ICAM-1, and Imulyse-1 (Biopool, Umea, Sweden) for tPA. C-Reactive protein (CRP), vWF, and fibrinogen were measured by in-house ELISAs using commercially available antibodies (DakoCytomation BV, Heverlee, Belgium) as described previously (27–29). Details of the ELISAs for soluble p55 and p75 TNF α receptors (sTNFR55 and sTNFR75) have been described previously (30). Plasminogen activator inhibitor type 1 (PAI-1) antigen levels were assessed using a specific in-house sandwich ELISA that measures latent, active, and complexed forms. The linearity of this assay ranges from 2–30 ng/ml. The coefficients of variation for these assays were less than 10%. The within- and between-day coefficients of variation were 2.9% and 7.2% for CRP, 1.7% and 6.3% for fibrinogen, and 6% and 12% for PAI-1, respectively.

Statistics

Results are expressed as means (SD) or medians (range) where indicated. Differences between start (d 1) and end of diet (d 46) values were determined by paired *t* test or Wilcoxon rank-order test for paired observations when applicable. For clarity, uncorrected *P* values are provided in the text, but a Bonferroni correction should be considered when interpreting the data.

The effects of weight loss on the proinflammatory state in the placebo and PEG-OB groups were assessed by combining the individual proinflammatory parameters in a single model. To that end, plasma PAI-1, IL-6, tPA, fibrinogen, and ICAM-1 values on d 1, -8 , -15 , -25 , and -46 were logarithmically transformed and incorporated into a single model, and the effect was analyzed as a multivariate response by multivariate analysis of covariation, with time as the independent variable.

Results

Baseline characteristics of the study population and the effects of weight loss on metabolic parameters are shown in Table 1. The VLCD resulted in a rapid and substantial weight loss and a significant decline in serum leptin levels in the placebo-treated group ($P < 0.0001$). Compensating for the fall in serum leptin levels by PEG-OB treatment resulted in a total (*i.e.* pegylated and endogenous) leptin level of 3980 ± 704 ng/ml. Weight loss was significantly higher in the PEG-OB group than in the placebo group (14.6 ± 2.7 vs. 11.7 ± 2.9 kg, respectively; $P < 0.03$). Baseline leptin serum concentrations immediately before leptin injection ranged

TABLE 1. Metabolic characteristics of placebo- and PEG-OB-treated groups before and after weight loss

	Placebo (n = 10)		PEG-OB (n = 12)	
	Before weight loss (d 0)	After weight loss (d 46)	Before weight loss (d 0)	After weight loss (d 46)
Age (yr)	36.4 (2.8)		34.6 (4.2)	
Weight (kg)	96.6 (11.8)	84.8 (12.7) ^a	97.9 (6.6)	83.3 (5.6) ^a
Weight loss (kg)		11.8 (2.9)		14.6 (2.7) ^b
BMI (kg/m ²)	28.8 (1.8)	25.4 (2.3) ^a	28.9 (1.3)	24.8 (1.5) ^a
Leptin (ng/ml)	7.10 (2.37)	2.01 (0.75) ^c	7.61 (4.22)	3980 (704) ^c
Glucose (mmol/liter)	5.2 (0.2)	4.7 (0.5) ^a	5.3 (0.3)	4.7 (0.3) ^c
Insulin (mU/liter)	9.4 (3.2)	3.6 (2.2) ^c	7.2 (2.8)	2.0 (1.0) ^c
HOMA	2.2 (0.8)	0.8 (0.5) ^c	1.7 (0.6)	0.4 (0.2) ^c
Cholesterol (mmol/liter)	4.8 (0.9)	3.7 (0.5) ^a	4.7 (0.8)	3.2 (0.5) ^a
LDL cholesterol (mmol/liter)	3.2 (0.8)	2.4 (0.3) ^a	2.8 (0.5)	1.8 (0.3) ^c
HDL cholesterol (mmol/liter)	1.1 (0.1)	1.1 (0.3)	1.2 (0.3)	1.1 (0.2)
Triacylglycerol (mmol/liter)	1.15 (0.60)	0.58 (0.22) ^a	1.13 (0.91)	0.48 (0.21) ^a

Values are given as means (SD). BMI, Body mass index.

^a $P < 0.05$.

^b Significant difference between placebo and PEG-OB-treated group, $P < 0.03$.

^c $P < 0.001$.

from 950–3700 ng/ml. Peak concentrations, measured 72 h after sc PEG-OB injection, ranged from 2300–6050 ng/ml.

Weight loss led to a significant decrease in fasting glucose levels ($P < 0.03$ and $P < 0.0001$ in the placebo and PEG-OB groups, respectively) and fasting insulin levels ($P < 0.001$ and $P < 0.0001$ in the control and PEG-OB groups, respectively), resulting in an improved estimated insulin sensitivity in both the placebo group ($P < 0.03$) and the PEG-OB group ($P < 0.001$).

Total plasma cholesterol, LDL cholesterol, and triglycerides were significantly lower after weight loss ($P < 0.02$ in both study groups). HDL cholesterol was not significantly influenced by the VLCD.

The effects of the VLCD and PEG-OB treatment on the cellular and humoral components of the immune system have been summarized in Tables 2 and 3. In general, these parameters show a gradual decline over the 46-d study period, indicating that the fall in proinflammatory parameters is not simply related to the VLCD (see also Fig. 1.)

Table 2 shows the effects of VLCD and PEG-OB treatment on circulating cellular components of the immune system at the end of the 46-d study period. Weight loss significantly lowered total leukocyte count in both the placebo and PEG-OB groups ($P < 0.01$ and $P < 0.001$, respectively). The fall in total leukocytes was, with one exception, evenly distributed over the various mononuclear subfractions. Monocytes, $CD4^+$, $CD8^+$, and NK cells decreased proportionally;

only $CD19^+$ cells (B lymphocytes) showed a small, but significant ($P < 0.04$), increase in the PEG-OB group.

Data on the evaluation of humoral inflammatory markers are shown in Table 3. Of the parameters tested, only the vascular endothelial cell activation markers tPA ($P < 0.004$) and vWF ($P < 0.03$) and the proinflammatory cytokine TNF α ($P < 0.04$) significantly decreased upon weight loss. Compared with baseline values, no significant change was found for IL-1, IL-6, sTNFR-55, sTNFR-75, CRP, fibrinogen, PAI-1, or ICAM-1. Overall assessment of humoral inflammatory markers in a model incorporating all relevant inflammatory parameters showed a tendency to decline upon weight loss ($P < 0.09$).

The VLCD in combination with PEG-OB treatment resulted in significant changes in the endothelial activation markers tPA ($P < 0.001$), vWF ($P < 0.002$), and ICAM-1 ($P < 0.009$) and in sTNFR-75 ($P < 0.044$) and PAI-1 ($P < 0.002$). No significant changes in TNF α , sTNFR-55, or fibrinogen were observed. Overall assessment of all relevant parameters by multivariate analysis of covariation revealed a gradual, significant decline over time in the proinflammatory parameters ($P < 0.014$).

Although the VLCD generally induced a decrease in humoral inflammatory markers, two exceptions were noticed (Fig. 1). First, in the placebo group, but not in the PEG-OB group, the VLCD induced a significant ($P < 0.02$) 2-fold increase in median IL-6 values on d 8, which had disappeared

TABLE 2. Effect of weight loss and PEG-OB-treatment on cellular inflammatory parameters

	Placebo (n = 10)		PEG-OB (n = 12)	
	Before weight loss (d 0)	After weight loss (d 46)	Before weight loss (d 0)	After weight loss (d 46)
Leucocytes (10E9/L)	7.7 (3.6)	4.7 (0.7) ^a	6.8 (1.7)	4.7 (1.3) ^b
Monocytes (%)	8.4 (2.9)	8.6 (1.8)	8.2 (1.1)	8.8 (3.0)
$CD4^+$ lymphocytes (%)	60.8 (10.9)	62.1 (8.2)	57.5 (7.8)	58.0 (6.9)
$CD8^+$ lymphocytes (%)	34.0 (8.3)	32.3 (7.7)	37.2 (6.1)	37.8 (5.8)
CD4/CD8 ratio	1.96 (0.96)	2.10 (0.90)	1.60 (0.47)	1.60 (0.45)
$CD19^+$ cells (%)	10.2 (4.4)	10.6 (3.6)	12.7 (3.0)	13.8 (3.1) ^a
NK cells (%)	12.2 (5.1)	7.8 (5.1)	14.8 (6.9)	11.1 (3.9)

Values are given as means (SD).

^a $P < 0.05$.

^b $P < 0.001$.

TABLE 3. Effect of weight loss and PEG-OB treatment on humoral inflammatory parameters

	Placebo group (n = 10)		PEG-OB group (n = 12)	
	Before weight loss (d 0)	After weight loss (d 46)	Before weight loss (d 0)	After weight loss (d 46)
IL-1 (ng/liter)	ND	ND	ND	ND
IL-6 (ng/liter)	1.32 [0.23–3.43]	0.62 [0.18–3.64]	1.20 [0.30–8.90]	0.74 [0.14–3.45]
TNF α (ng/liter)	4.10 [1.96–5.30]	3.46 [1.46–4.74] ^a	3.04 [2.07–4.33]	2.72 [1.26–5.63]
sTNFR55 (μ g/liter)	0.33 (0.08)	0.31 (0.09)	0.30 (0.09)	0.28 (0.07)
sTNFR75 (μ g/liter)	1.08 (0.29)	1.08 (0.22)	1.35 (0.24)	1.15 (0.26) ^a
CRP (mg/liter)	2.75 [0.04–7.6]	0.93 [0.11–9.35]	1.30 [0.83–11.66]	2.66 [0.79–14.45] ^a
Fibrinogen (g/liter)	3.10 (1.11)	2.90 (0.93)	2.56 (0.77)	2.36 (0.52)
PAI-1 (μ g/liter)	20.7 (7.5)	13.5 (11.0)	21.5 (10.0)	6.9 (4.7) ^a
tPA (μ g/liter)	13.4 (10.6)	6.8 (7.1) ^a	11.9 (5.0)	4.03 (2.05) ^b
vWF (μ g/liter)	152.1 (39.6)	136.5 (38.0) ^a	160.8 (30.7)	136.1 (25.3) ^a
ICAM-1 (μ g/liter)	155.8 (13.2)	144.6 (23.9)	165.4 (34.1)	141.9 (31.4) ^a

Values are means (SD) or medians [range]. ND, Not detectable, below the detection limit of the assay ($\leq 0.2 \mu$ g/liter).

^a $P < 0.05$.

^b $P < 0.001$.

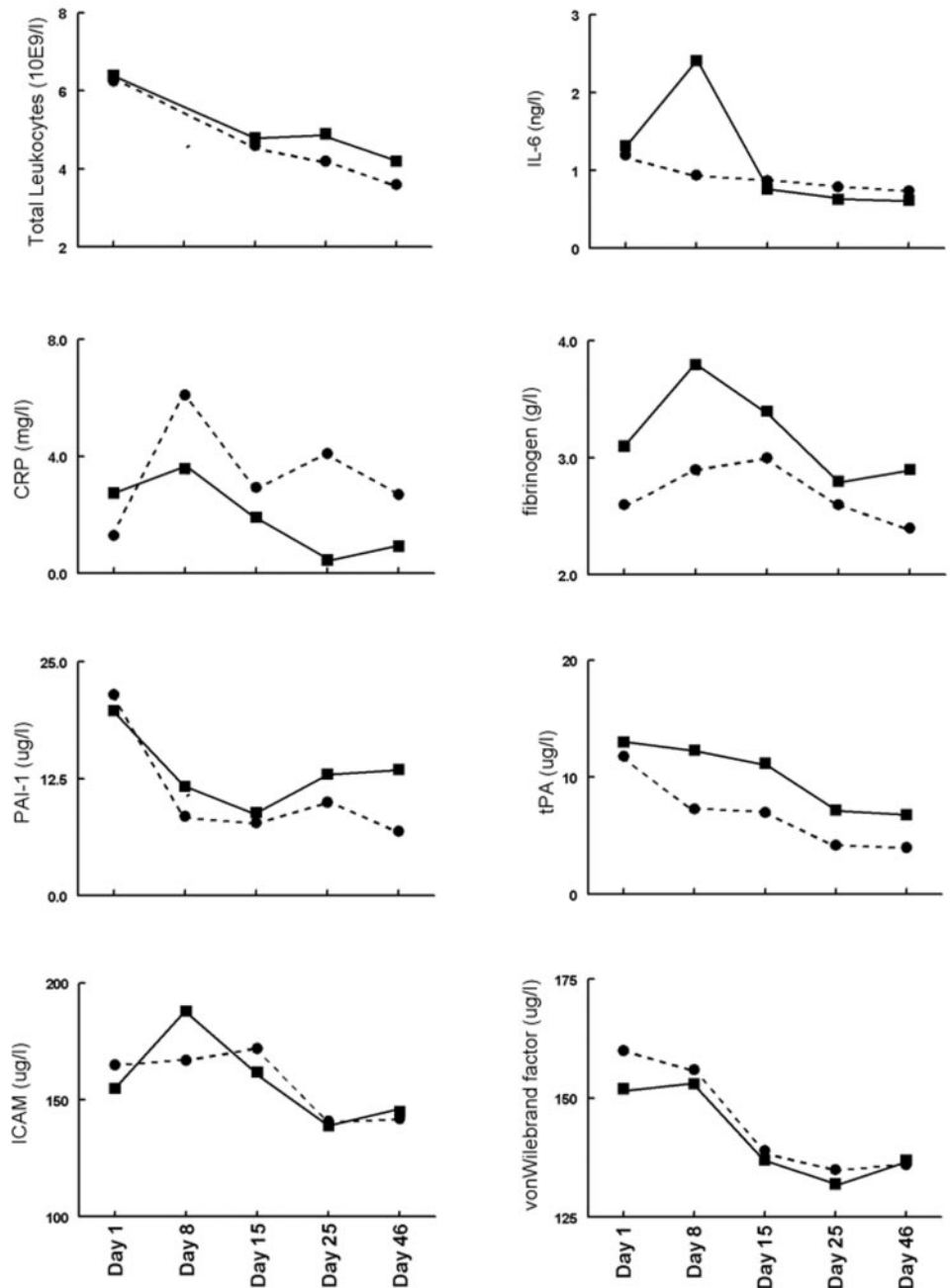


FIG. 1. Changes in the proinflammatory parameters [total leukocytes (mean), plasma IL-6 (median), CRP (median), fibrinogen (mean), PAI-1 (median), tPA (mean), ICAM (mean), and vWF (mean)] during weight loss in the placebo group (●) and the PEG-OB group (■).

by d 15. Second, in the PEG-OB group, CRP levels peaked on d 8 ($P < 0.004$) and remained elevated for the remainder of the study period.

Discussion

In addition to acting as a regulator of food intake and energy expenditure, (8), there is a growing body of evidence to suggest that leptin is a modulator of the immune and proinflammatory responses (10, 12). Consequently, it has been hypothesized that elevated leptin levels underlie the low grade proinflammatory state associated with human obesity (21). In the present study we found that high leptin levels produced by PEG-OB treatment of overweight young

men during a VLCD significantly increased weight loss (31), but our data do not support a proinflammatory role for leptin in human obesity. With the exception of small increases in CD19⁺ cells and plasma CRP levels, PEG-OB treatment, which compensated for the fall in serum leptin levels during weight loss, did not counteract the changes in plasma levels of cellular and humoral inflammatory markers during the VLCD period. In fact, PEG-OB treatment further strengthened the beneficial effects of the diet on the inflammatory state.

The additional weight loss in the PEG-OB group is in line with studies in rodents, which also showed enhanced weight loss upon leptin administration (8). The higher weight loss in

the PEG-OB-treated subjects appears primarily related to a suppression of appetite during the VLCD. Voluntary weight loss in man has been shown to be accompanied by an increase in baseline appetite (32) and in the relevant human studies reported to date a decrease in appetite, rather than an increase in energy expenditure, was found to underlie the enhanced weight loss in leptin-treated individuals (31, 33–35).

The current data on the beneficial effects of weight loss on cellular and humoral markers of inflammation are in accordance with other reports. Field *et al.* (36) also reported that total leukocyte count and mononuclear subfractions decline during weight loss induced by a VLCD in moderately obese subjects. A beneficial effect of weight loss on circulating levels of TNF α in obesity has been shown by Dandona *et al.* (3) and Zahorska *et al.* (37). Heilbronn *et al.* reported a decline in plasma CRP levels in proportion to weight loss (38).

With the exception of a minor fractional enrichment in CD19⁺ lymphocytes and an increase in plasma CRP levels, we found that PEG-OB treatment enhanced, rather than antagonized, the beneficial effects of weight loss on proinflammatory markers. Our data are seemingly in contrast with previous reports that showed a proinflammatory role for leptin. Fantuzzi *et al.* (10) extensively reviewed a putative role of leptin in the modulation of the innate immune response, inflammation, and hemopoiesis. Also, Yamagishi *et al.* (18) and Bouloumié *et al.* (19) reported proinflammatory effects of leptin on endothelial cell activation. Wang *et al.* (39) and Dowidar *et al.* (40) showed direct effects of leptin on the hepatocyte. The discrepancies between the present results and those reported in the cited references are unlikely to be related to the high leptin levels in our study, because Zarkesh-Esfahani *et al.* (16) also used high leptin concentrations in their leukocyte studies. Also, the apparent efficacy of PEG-OB is less than that of native leptin (Campfield, L. A., unpublished observations), resulting in lower effective leptin concentrations. The discrepancies may be related, however, to *in vitro*/*in vivo* differences, species differences, (13), as well as differences in model systems. For example, several reports indicate that exogenous leptin induces a profound suppression of appetite in rodents (8), whereas exogenous leptin administration in humans induces only a very modest suppression of appetite (31, 33–35). Moreover, most data on the proinflammatory role of leptin were obtained in leptin-deficient animals and/or situations such as starvation, which are characterized by low circulating leptin levels (12). Apparently these findings cannot simply be extrapolated to the human situation, in which obesity is characterized by elevated plasma leptin levels and diminished leptin responsiveness (41).

The increase in plasma CRP levels in the PEG-OB-treated group is the only significant proinflammatory effect of leptin. CRP is a classical and highly sensitive acute phase protein, plasma levels of which typically increase 100-fold or more during inflammation. The 2-fold increase in CRP levels upon PEG-OB treatment is indicative of only a weak inflammatory response and may result from the direct interaction of leptin and its receptor on hepatocytes, which interaction has been reported to induce IL-6-type responses (12, 40, 41). CRP is a strong predictor of cardiovascular risk, and indirect evidence

suggests that CRP may directly be involved in atherosclerosis (42). No evidence for such a mechanism was found in this study, and the decline in the endothelial cell activation markers vWF and ICAM-1 was similar in both study groups.

An interesting finding is the increase in IL-6 concentration on d 8 in the placebo-group, an increase that was absent in the PEG-OB group. We are not aware of similar observations during acute (semi) starvation. An increased plasma IL-6 concentration has been reported upon strenuous exercise, and it was speculated that these elevated levels reflect a muscle-derived signal, indicating critically low muscle glycogen stores and signaling the liver to increase glucose output (43). The observed increase in IL-6 upon initiation of the very low calorie diet may serve similar purposes. The apparent suppression of the IL-6 surge by PEG-OB concurs with the proposed role of leptin as a peripheral signal, signaling the brain to initiate adaptive responses to starvation (44).

In conclusion, in this prospective study no evidence was found for a causative role of leptin in the proinflammatory state associated with human obesity. We found that high leptin levels, produced by PEG-OB treatment, increased weight loss in moderately obese men during a VLCD. The apparent higher decline in inflammatory parameters in the PEG-OB group may relate to the higher weight loss in this group.

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C.J.H. and J.H.N.L. contributed equally to this work and should both be considered as first authors.

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