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Microarray profiling of human white adipose tissue after exogenous leptin injection

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Abstract

Background Leptin is a secreted adipocyte hormone that plays a key role in the regulation of body weight homeostasis. The leptin effect on human white adipose tissue (WAT) is still debated.

Objective The aim of this study was to assess whether the administration of polyethylene glycol-leptin (PEG-OB) in a single supraphysiological dose has transcriptional effects on genes of WAT and to identify its target genes and functional pathways in WAT.

Materials and methods Blood samples and WAT biopsies were obtained from 10 healthy nonobese men before treatment and 72 h after the PEG-OB injection, leading to an approximate 809-fold increase in circulating leptin. The WAT gene expression profile before and after the PEG-OB injection was compared using pangenomic microarrays. Functional gene annotations based on the gene ontology of the PEG-OB regulated genes were performed using both an 'in house' automated procedure and GenMAPP (Gene Microarray Pathway Profiler), designed for viewing and analyzing gene expression data in the context of biological pathways.

Results Statistical analysis of microarray data revealed that PEG-OB had a major down-regulated effect on WAT gene expression, as we obtained 1822 and 100 down- and up-regulated genes, respectively. Microarray data were validated using reverse transcription quantitative PCR. Functional gene annotations of PEG-OB regulated genes revealed that the functional class related to immunity and inflammation was among the most mobilized PEG-OB pathway in WAT. These genes are mainly expressed in the cell of the stroma vascular fraction in comparison with adipocytes.

Conclusion Our observations support the hypothesis that leptin could act on WAT, particularly on genes related to inflammation and immunity, which may suggest a novel leptin target pathway in human WAT.

Keywords Adipose tissue, inflammation and gene profiling, PEG-OB.

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Abbreviations: Body, mass index (BMI); false, discovery rate (FDR); gene, ontology (GO); GenMAPP, (Gene Map Annotator Pathway Profiler); high, sensitive C reactive protein (hsCRP); interleukin, (IL); microarray, pathway profiles (MAPPs); polyethylene, glycol-leptin (PEG-OB); real-time, quantitative PCR (RT-PCR); serum, amyloid A (SAA); significance, analysis microarray (SAM); stroma, vascular fraction (SVF); white, adipose tissue (WAT).

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Table 1 Clinical and biochemical characteristics of nonobese men treated with a single supraphysiological dose of polyethylene glycol-OB protein

	Before PEG-OB injection (day 0)	After PEG-OB injection (day 3)
Number	10	10
Age (y)	25.9 ± 4.1	–
Weight (kg)	77.65 ± 3.07	77.5 ± 3.1
BMI (kg m ⁻²)	23.1 ± 0.65	23.1 ± 0.62
Leptin (µmol L ⁻¹)	2.61 ± 0.75	2111.4 ± 175.53*
Insulin (U mL ⁻¹)	5.82 ± 1.08	6.09 ± 1.36
Adiponectin (µg mL ⁻¹)	7.61 ± 0.93	7.19 ± 0.91
HsCRP (mg dL ⁻¹)	0.134 ± 0.053	0.293 ± 0.13
SAA (µg mL ⁻¹)	5.31 ± 1.58	17.82 ± 7.06

Data are expressed as mean ± SEM.

PEG-OB, polyethylene glycol-OB protein.

*Mean fold increase of circulating leptin is approximately 809-fold.

Introduction

The leptin hormone is mainly produced by the white adipose tissue (WAT) and plays a key role in body weight homeostasis [1]. The changes in circulating leptin levels reflect the nutritional status and provides a feedback signal from the periphery to the central nervous system [1,2]. Thus, leptin satisfies the criteria for the long-sought 'Lipostat' molecule of the adipostatic model, which was postulated to explain the relative stability of weight over time. In healthy animals [3], as in humans [4], circulating concentrations of leptin are highly correlated with body fat mass; it crosses the blood–brain barrier and interacts with neurones known to decrease food intake and stimulate thermogenesis [5]. In addition to its central action on food intake and energy expenditure, leptin may also be endowed with direct peripheral effects on nonadipose tissue (i.e. muscle, liver, haematopoietic cells) to exert a role on different functional pathways such as angiogenesis, inflammation and immunity as well as fat oxidation [6–9]. However, the peripheral effect of leptin on adipose tissue physiology is still debated [10,11].

Leptin appears as a survival hormone aimed at maintaining the energy balance, representing the first strategy for obesity treatment. Unfortunately, except for the rare forms of monogenic obesity with leptin deficiency [12], the majority of obesity cases are associated with high levels of circulating leptin, and the emerged idea was that obese patients are resistant to the action of leptin [13]. Human trials performed with leptin treatment have shown that most subjects are unlikely to respond to pharmacological treatment with this hormone [14–16]. The widespread occurrence of leptin resistance might reflect the fact that the inability to store energy efficiently at times of abundance is evolutionarily disadvantageous. According to this alternative view, leptin has a broader physiological role and evolved as a signal whose reduction indicates starvation [17]. In some cases, leptin induces moderate fat loss especially under conditions of energy restriction indicating some action of leptin on WAT [14,18]. The physiological functions related to this feedback

signal, which can eventually be modulated in adipose tissue by leptin treatment, are mostly unknown.

In this study, we hypothesized that even if leptin administration had a limited effect on body fat loss, it could act on gene expression of known or unknown targets in WAT. Our purpose was to identify gene targets and functional pathways of leptin in human adipose tissue. To this end, we compared gene expression profiles in WAT of seven lean male subjects before and after 72 h of administration leading to a high peak of leptin in the circulation. In order to capture functions that might be significantly affected by PEG-OB treatment, different methods of gene annotation were used. Our results showed that functional processes related to immunity and inflammation were among the most affected functions after PEG-OB treatment in WAT of healthy nonobese subjects.

Materials and methods

PEG-OB

Modification of proteins through covalent linkage of polyethylene glycol polymers to the proteins results in reduced immunogenicity and increased serum half-life for many proteins. Recombinant native human PEG-OB, expressed and purified from *Escherichia coli*, was chemically conjugated to a species of branched PEGs with an average molecular mass of 42 kDa in a 1 : 1 ratio. PEG is not metabolized and excreted completely. The result was a globular PEG–native human PEG-OB polymer with increased molecular size. A volume of 1.3 mL PEG-OB at a concentration of 10 mg mL⁻¹ was placed in sterile glass vials (Hoffmann-La Roche Inc., Nutley, NJ). Preclinical studies with PEG-OB indicate an extended half-life (> 48 h) and efficacy for reduction of food intake and body weight in animals [19]. Our previous study in obese male subjects clearly showed sustained elevated blood concentrations after weekly subcutaneous dosing of PEG-OB in humans. Mean peak serum PEG-OB concentrations were achieved 72 h after dosing, followed by a return to the elevated predose concentrations after 1 week [15].

Subjects and study design

Ten nonobese caucasian men (age 21–27 years, body mass index $20\text{--}26\text{ kg m}^{-2}$) were recruited through a local advertisement. All subjects had a stable weight and were healthy according to their medical history, clinical examination and routine laboratory findings. The study was approved by the medical Ethics Committee of the University of Maastricht, and all participants gave written informed consent. The leptin treatment consisted of an 80-mg PEG-OB injection in the para-umbilical region. Blood and WAT biopsies were obtained from fasted subjects on day 0 (before the PEG-OB treatment) and 72 h after the injection. At this time, PEG-OB concentration was approximately 809-fold higher compared with basal levels (Table 1).

Biochemical measures

Plasma leptin levels were measured by a double-antibody sandwich ELISA using a monoclonal antibody specific for human leptin. This assay measures total leptin [15]. Serum insulin (mU L^{-1}) was measured with immunoradiometric assay (IRMA) (Bi-INSULINE IRMA CisBio International, Gif-sur-Yvette, France). Serum adiponectin concentrations were determined using standard kits (Linco Research, Saint Louis, MI). High sensitive C reactive protein circulating levels were measured using an IMAGE automatic immunoassay system (Beckmann-Coulter, Fullerton, CA).

Total RNA preparation

Human white subcutaneous adipose tissues were homogenized in lysis buffer (RLT buffer, Qiagen, Cour taboeuf, France) using a rotor-stator, followed by a chloroform delipidation step. The upper aqueous phase was processed for total RNA extraction using silica-based spin columns (RNeasy Mini Kit, Qiagen Protocol). The yield of WAT total RNA was $3\text{--}7\text{ }\mu\text{g g}^{-1}$ WAT. The RNA concentration was determined by measuring absorbance at 260 nm and the purity was estimated by 260/280 nm absorbance ratio. Total RNA integrity was verified by agarose gel electrophoresis or using an Agilent 2100 Bioanalyser (Agilent, Paris, France). We obtained high-quality RNA, mandatory for microarray in seven subjects.

A reference pool containing adipose tissue RNAs from 14 healthy subjects was used to compare data from different experiments. Equivalents of $15\text{ }\mu\text{g}$ of total RNA from each of the 14 healthy subjects were mixed together in order to constitute a pool of total RNA [20].

RNA amplification and microarray experiments

RNA preparations from the seven subjects were amplified using MessageAmp aRNA protocol (Ambion, Cambridge-shire, UK) in order to generate a sufficient amount of labelled hybridization samples to cDNA microarrays [20]. Briefly, the procedure consists of reverse transcription of

$5\text{ }\mu\text{g}$ total RNA using Primer Oligo dT bearing a T7 promoter sequence, followed by *in vitro* T7 RNA polymerase transcription of cDNA to generate RNA antisense copies of each messenger RNA. This amplification procedure is now generally accepted and it has been demonstrated that it does not distort the relative abundance of a single mRNA within a RNA population [21]. Briefly, $3\text{ }\mu\text{g}$ antisense RNA was labelled by incorporating Cyanine dyes (Amersham Pharmacia Biotech, Orsay, France) during random reverse transcription with Cyscript (Cyscribe first-strand cDNA labelling kit, Amersham Pharmacia Biotech). Amplified RNA from the seven healthy men before and after PEG-OB treatment was labelled with Cy5, whereas amplified RNA from the reference pool was labelled with Cy3. The labelled mixture (Cy5 and Cy3) was purified and concentrated using Microcon YM 30 column (Millipore, Bedford, MA) after the addition of human cot-1, yeast tRNA and poly A. After denaturation of probes, the mixture was hybridized overnight on 14 slides (seven slides both before and after treatment) at $65\text{ }^{\circ}\text{C}$ in a sealed humidified hybridization chamber, and then washed in 0.03 SDS , 2XSSC , 1XSSC and 0.2 SSC solutions for 2 min. After washing, the arrays were immediately scanned using a Genepix 4000 A Scanner (Axon Instrument, Union City, CA). Images were analyzed using Genepix Pro 3 software where spots with bad quality were omitted. Data files generated by Genepix were entered in Stanford microarray database. The spots with an average intensity less than 2.5-fold above the background were removed. A uniform scale factor was applied to all measured intensities in order to normalize signal intensities between Cy5 and Cy3. The data of 14 slides were normalized in log-space using *Locally Weighted Linear Regression* (Lowess) [22]. In order to identify the genes that were significantly regulated by PEG-OB injection, we used the *Significance Analysis Microarray* (SAM) procedure, a validated statistical technique for identifying differentially expressed genes across high-density microarrays. SAM is a nonparametric method by which a modified *t*-statistic $d(i) = x(i)/[s(i) + s_0]$ is calculated for the i^{th} gene, where $x(i)$ is the mean of the \log_2 ratio data across all seven experiments and $s(i)$ is the appropriately scaled standard deviation. The quantity s_0 is an adjustment factor derived from the data which attempts to make $d(i)$ independent of $s(i)$. Because the null hypothesis was that there was no treatment effect, we tested whether the expected value of the \log_2 ratio is 0 in the statistic $d(i)$. The FDR (False Discovery Rate) method controls the expected value of the number of false positives to the total number of genes. For this experiment the FDR was set at a value of 5%.

Quantification of mRNA levels by real-time PCR

Total RNA was extracted using Rneasy, as described earlier. Reverse transcription was performed with $1\text{ }\mu\text{g}$ total RNA for each biopsy, and 10 ng cDNA was used as template for RT-PCR as recommended by the manufacturer (Applied Biosystems, Foster City, CA). We used 18S ribosomal RNA as control to normalize gene expression (Applied Biosystems).

The PCR was performed on the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Minneapolis, MN) using the TaqMan Reverse Universal PCR Master Mix and Assays-on-Demand Gene Expression probes (Applied Biosystems) which contain a mixture of forward and reverse oligonucleotide primers and a specific TaqMan probe (Applied Biosystems).

The probe was labelled at the 5' end with the reporter dye, 6-carboxy-fluorescein (FAM) and at the 3' end with the quencher, 6-minor groove binder (MGB). The assay identification number of the probes used are available on the Applied Biosystem website.

Each reaction well contained cDNA, TaqMan PCR Master Mix, 900 nmol L⁻¹ of each primer and 250 nmol L⁻¹ of TaqMan probe. Cycling parameters were standardized as 50 °C for 2 min, 95 for 10 min, then 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

TaqMan reagent-based chemistry uses a fluogenic probe to enable detection of a specific PCR product as it accumulates during PCR cycles. When both quencher and reporter are attached to the probe, reporter dye emission is quenched. During each extension cycle at 60 °C, the polymerase cleaves the reporter dye from the probe. After being separated from the quencher, the reporter dye emits its characteristic fluorescence.

A standard curve for each primer pair was obtained using serial dilutions of WAT cDNA. A standard curve for serial dilutions of 18S rRNA was similarly generated. The Ct represents the threshold cycle number at which the fluorescence signal is linearly increasing above background. The Ct readings for unknown samples were used to calculate the amount of each target gene relative to 18 S. The relative standard curve method (Applied Biosystems) was used to calculate the amplification difference between the before and after PEG-OB treatment for each primer set.

Isolation of human white adipocytes and stroma vascular fraction

To determine whether differentially expressed genes were mainly expressed in adipocytes, stroma vascular fraction (SVF) or equally in adipocytes and SVF, we performed sets of experiments aimed at isolating adipocytes from the stromal cells, as described [20]. Briefly, stroma vascular fraction and adipocytes were separated after adipose tissue digestion and centrifugation. Total RNAs obtained from adipocytes and SVF cells were extracted and a set of microarray experiments were realized to distinguish genes expressed in adipocyte from those expressed in SVF cells [20].

Capture of enriched functions after PEG-OB treatment

A functional profiling of PEG-OB regulated genes was realized by using the Gene Ontology Consortium (GO, <http://www.geneontology.org/>), which provides a controlled vocabulary to describe gene and gene product attributes in any organism [23]. We used GO through an 'in-house' automated annotation procedure (Henegar C, *Bioinformatics in*

revision). In order to assure a correct over-representation analysis, LocusLink numbers were used as the standard gene accession system (SGA), allowing us to map gene identifiers to GO functional annotations in an unequivocal way. The GO hierarchical structure allows the characterization of genes with a variable biological term precision (generic terms and/or more specific terms based on the hierarchical levels), and then the annotation procedure aimed to favour the selection of the most specific GO categories against more general ones. A gene-enrichment measure was computed for each GO category by restricting annotation inheritance only to the genes directly related to its direct subsumes within the ontological hierarchy. The significance of the gene enrichment for each GO category was assessed by calculating a *P*-value. This *P*-value estimates the probability that the observed number of differentially regulated genes (annotated by a specific GO category) could have resulted from random distribution between the tested list of differentially regulated genes in the experiment, for which at least one GO annotation was identified, and a reference list. The reference list included all the genes available on the entire microarray, which were tested for differential regulation during the experiment and which had at least one GO annotation. As many of the identified GO categories had less than five annotations, a unilateral Fisher exact test was used for *P*-value calculations. Only GO categories showing significant gene enrichment (after *P*-value correction for a 5% false discovery rate) were selected and used for further processing. In order to facilitate the analysis of functional profiles, we clustered together all related GO categories, based on the sharing of a significant number of annotated genes (Henegar C, *Bioinformatics in revision*). As for the individual GO categories, a *P*-value of gene enrichment significance was calculated for each of the identified clusters, by using a unilateral Fisher exact test, and then the clusters were ranked based on the statistical significance of the gene space coverage. Gene annotation procedure was realized separately for each of the three available ontologies: molecular function, biological process and cellular component. As 'biological process' annotations were the more exhaustive and immediately comprehensive, they are presented in this study.

The second method used is the visualization tool GenMAPP (Gene Map Annotator Pathway Profiler) version 2.0 (<http://www.genmapp.org/>). This is a program for viewing and analyzing microarray data on microarray pathway profiles (MAPPs) representing biological pathways or any other functional grouping of genes [24]. Microarray pathway profiles are generated from several public and commercial pathway resources currently in existence, including:

- GO database and G-protein Coupled Receptor Database (<http://www.gpcr.org/>, database on G-protein and their interactions with their receptors) [25];
- a small number of MAPPs based on the KEGG database (<http://www.genome.ad.jp/kegg/>, database integrating knowledge on molecular interaction networks in biological processes, the information about the universe of genes and proteins, and the information about the universe of chemical compounds and reactions) [26];
- MAPPs specifically designed for GenMAPP.

Table 2 Validation of microarray results using quantitative real-time quantitative PCR

Response to leptin	UG cluster	Name	Ratio array	Ratio RT-PCR	P-value
Down	Hs 234734	Lysozyme	-2.39	-2.22	0.01
	Hs 82848	Selectin L	-2.13	-1.88	0.1
	Hs 181301	Cathepsin S	-1.91	-2.00	0.04
	Hs 196352	Neutrophile cytosolic factor 4	-1.64	-1.56	0.07
	Hs 76930	Synuclein alpha	-2.07	-1.85	0.01
	Hs 241392	Chemokines CCL5	-1.45	-1.63	0.1
	Hs 75703	Chemokines CCL4	-1.45	-2.85	0.03
	Hs 174228	Chemokine XCL1	-1.87	-2.63	0.03
	Hs 371856	TNF superfamily 3 ligand	-1.86	-2.27	0.09
	Nonsignificant	Hs 389700	Microsomal glutathione s transferase	1.16	-1.3

Fold changes in mRNA levels of 10 randomly chosen genes were determined by real-time PCR (RT-PCR) on total RNA from the seven subjects.

P-values were calculated using the nonparametric Wilcoxon test.

Microarray experiments were performed on amplified RNA from seven subjects. Microsomal glutathione S-transferase (MGST1) is a control gene of which the fold change did not significantly change in real-time PCR and microarray experiments.

The MAPPs from the G-protein Coupled Receptor Database, KEGG database and the specifically designed MAPPs are called the local MAPPs. We imported the median gene expression data of the significant gene group, sorted by SAM, into the program and used GenMAPP to illustrate pathways containing differentially expressed genes. The obtained illustrations show presence and expression changes for the genes presented on the microarray based on the analysis of PEG-OB treatment versus control. All the gene expression data are dynamically linked to the MAPPs with a tool called MAPPFinder [27]. For all the imported genes MAPPFinder calculates the percentage of genes measured that meet a user-defined criterion (fold change > 1.4 or < -1.4) and a z-score. The z-score is a standardized difference-score using the expected value and standard deviation of the number of genes meeting the criterion on a MAPP. The z-score attempts to take into account that a larger fraction of changed genes is less likely to occur in a large MAPP than is a small one.

Statistical analysis

Data are expressed as mean \pm SEM. Statistical analysis was performed with JMP statistic software (SAS Institute Inc., Cary, NC). Differences in means of clinical and biochemical parameters of subjects before and after PEG-OB treatment were determined using the Wilcoxon nonparametric paired test. Significance of correlation between circulating levels of CRP and SAA was examined using the nonparametric Spearman's rank.

Results

Effect of PEG-OB injection on clinical and biochemical parameters

The baseline characteristics of the subjects and changes after 72 h of PEG-OB injection are shown in Table 1. While

the circulating leptin increased approximately 809-fold, the weight of the subjects, insulin as well as the adipokine adiponectin did not change after the PEG-OB injection (Table 1). The inflammatory factors CRP and SAA tended to increase after the PEG-OB injection. However, these increases were not significant (CRP fold increase = 2.18, $P = 0.27$; SAA fold increase = 3.35, $P = 0.055$). There was a strong positive correlation between variations of circulating CRP and SAA levels under the PEG-OB injection ($r = 0.93$, $P < 0.0001$).

PEG-OB treatment mobilized genes in human WAT

Our gene profiling experiment was performed with total RNA preparation from WAT of seven healthy men before and after 72 h of PEG-OB treatment, leading to a 809-fold increase in circulating serum leptin. We started to analyze the expression of 40 844 cDNAs printed on a glass slide. After normalization, filtering procedures and use of the SAM program, we found 1922 cDNAs with significant changes using an estimated FDR of 5%. These cDNAs included 1822 downy expressed and 100 overexpressed genes.

Using RT-PCR, we confirmed the changes in expression of nine genes, including chemokines (CCL5, CCL4, XCL1), TNF superfamily factor 3 and other inflammatory-related factors like cathepsin S, selectin L and neutrophile cytosolic factor 4 (Table 2). In agreement with microarray findings, MGST1 gene expression was not changed in RT-PCR experiments.

Enriched functions of PEG-OB mobilized genes in WAT

We further searched for the functional classes that were significantly captured by the PEG-OB injection in WAT. Although no significant cluster of enriched functions was identified in the group of overexpressed genes (data not

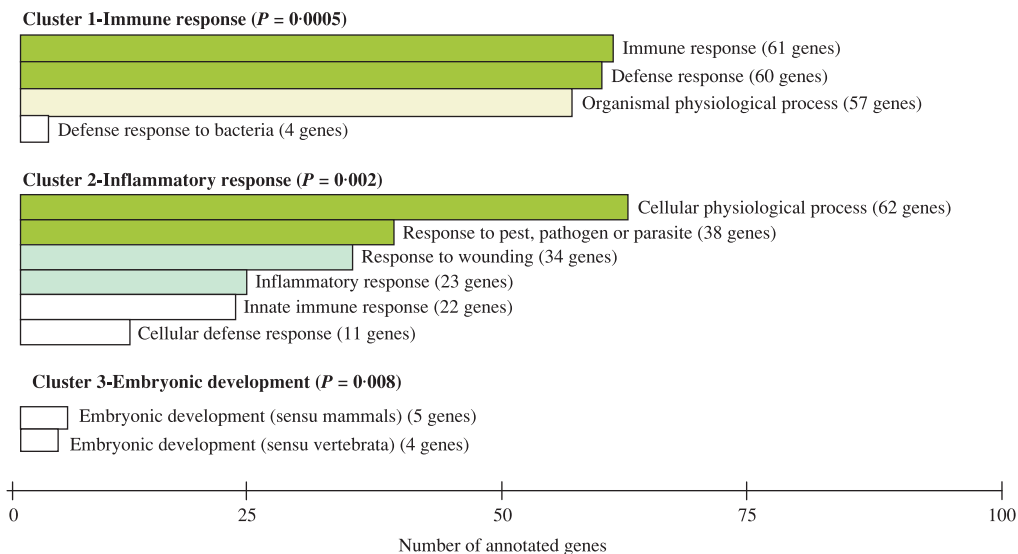


Figure 1 Schematic representation of enriched functions in the group of polyethylene glycol-leptin down-regulated genes. The clusters of enriched functions are listed by P -value score (after adjustment with the Benjamini & Hochberg FDR method) and the figure shows the three significant clusters of genes. The number of genes annotated by a given gene ontology function (biological process) is indicated in the parenthesis.

shown), the GO biological process based on automatic annotation revealed different sets of enriched functions in the group of down-regulated genes. Three significant functional clusters were enlightened as illustrated in Fig. 1. The first two significant clusters included genes related to immunity and inflammatory functions (Fig. 1). The third cluster was related to embryonic development functions.

For examination of more specific changes after the PEG-OB injection, the functional grouping of genes modified by the PEG-OB injection was systematically evaluated for differential regulation using the visualization tool GenMAPP. We found genes significantly modified by the PEG-OB injection in eight local MAPPs (Table 3). In agreement with our first functional analysis, the inflammatory response

pathway was among the local pathways most down-regulated after the PEG-OB injection (Table 3).

Other local MAPPs identified were nucleotide metabolism, peptide GPCRs, apoptosis, TGF beta signalling, synthesis and degradation of ketone bodies, pentose phosphate pathway and proteasome degradation (Table 3). The MAPPs were ranked with a decreasing z -score. More positive z -scores suggest with more confidence that the correlation between the expression changes of the genes in this MAPP are not occurring by chance alone. Taken together, these results indicate that adipose tissue genes related to inflammation and immunity were significantly down-regulated by the supraphysiological dose of the PEG-OB injection.

Table 3 GenMAPP pathways affected by leptin treatment

Pathway MAPP name	z -score	Genes changed	Reporters present	Genes on MAPP
Hs_inflammatory response pathway	1.588	3	5	31
Hs_nucleotide metabolism	1.588	3	5	16
Hs_peptide GPCRs	1.377	4	8	69
Hs_apoptosis	0.977	2	4	43
Hs_TGF beta signalling pathway	0.831	5	13	51
Hs_synthesis and degradation of ketone bodies	0.692	1	2	5
Hs_pentose phosphate pathway	0.692	1	2	7
Hs_proteasome degradation	0.285	5	16	42

The table shows a ranked list of processes with a relatively high number of gene expression values found to be changed. The z -score is calculated as the number of genes changed (fold change > 1.4) in a pathway minus the number of expected changes based on the number of genes present in the pathway MAPP and the total fraction of genes changed for all MAPPs, divided by the standard deviation of the observed number of changes in all MAPPs. High z -scores indicate pathways where relatively many changes occurred. Note that the z -score also depends on the coverage of the pathway genes by the successfully annotated microarray reporter content. Next to the number of changed genes, the number of reporters for that pathway on the array and the total number of genes in the pathway MAPP are also given (cf. Methods).

In our study in healthy nonobese males, we observed a decreased expression of factors bearing proinflammatory properties in human adipose tissue under PEG-OB treatment. Several of these genes encoded for inflammatory factors are potentially secreted, such as chemokines with Cys (XCL1), Cys-Cys (CCL 2, 4, 5, 18, 19, 26) Cys-x-Cys (CXCL 7, 4, 16) and Cys-x3-Cys (CX3CL1) motifs. These molecules have well-known chemotactic properties and proinflammatory effects. Other secreted factors are cytokines or related factors such as several members of the tumour necrosis factor superfamily ligand, interleukins (IL6, IL15,

IL16, IL13), interferon gamma (INF γ) and interferon inducible protein. These changes are associated with a decreased expression of many chemokines and interleukin receptors. Other genes encoded proteins with protease (cathepsin S, lysozyme) activities that have the ability to degrade antigens presented to immune cells. Genes encoding proteins that enable adhesion of immune cells, such as integrin alpha X, selectin L, selectin P ligand, carcino-embryonic antigen-related cell adhesion, CD4 and CD3 antigens, were also mobilized (Table 4).

To determine whether the major down-regulated PEG-OB

Table 4 Transcriptional effects of leptin on inflammatory related genes

G cluster	Gene name	Fold change
	Chemokines	
Hs.81564	platelet factor 4 (chemokine (C-X-C motif) ligand 4)	-1.32
Hs.82407	chemokine (C-X-C motif) ligand 16	-1.33
Hs.80420	chemokine (C-X3-C motif) ligand 1	-1.41
Hs.50002	chemokine (C-C motif) ligand 19	-1.43
Hs.75703	chemokine (C-C motif) ligand 4	-1.45
Hs.131342	chemokine (C-C motif) ligand 26	-1.46
Hs.241392	chemokine (C-C motif) ligand 5	-1.46
Hs.2164	pro-platelet basic protein (chemokine (C-X-C motif) ligand 7)	-1.49
Hs.16530	chemokine (C-C motif) ligand 18 (pulmonary and activation-regulated)	-1.76
Hs.174228	chemokine (C motif) ligand 1	-1.88
	Chemokine receptors	
Hs.421986	chemokine (C-X-C motif) receptor 4	-1.42
Hs.458436	chemokine (C-C motif) receptor-like 2	-1.47
Hs.301921	chemokine (C-C motif) receptor 1	-1.51
Hs.395	chemokine (C-C motif) receptor 2	-1.57
Hs.78913	chemokine (C-X3-C motif) receptor 1	-1.80
	Cytokines, TNF superfamily and interferons	
Hs.105656	small inducible cytokine subfamily E, member 1 (endothelial monocyte-activating)	-1.31
Hs.130210	interleukin 6 (interferon, beta 2)	-1.34
Hs.168132	interleukin 15	-1.36
Hs.58831	regulator of Fas-induced apoptosis	-1.37
Hs.856	interferon, gamma	-1.38
Hs.170359	interleukin 16 (lymphocyte chemoattractant factor)	-1.38
Hs.415839	tumour necrosis factor (ligand) superfamily, member 12	-1.44
Hs.83795	interferon regulatory factor 2	-1.49
Hs.387871	tumour necrosis factor (ligand) superfamily, member 10	-1.56
Hs.845	interleukin 13	-1.58
Hs.169274	interferon-induced protein with tetratricopeptide repeats 2	-1.60
Hs.376208	lymphotoxin beta (TNF superfamily, member 3)	-1.87
	Cytokines and TNF superfamily receptors	
Hs.143527	interleukin 1 receptor accessory protein	-1.35
Hs.272410	toll-like receptor 8	-1.42
Hs.362807	interleukin 7 receptor	-1.43
Hs.144748	interferon (alpha, beta and omega) receptor 1	-1.44
Hs.1314	tumour necrosis factor receptor superfamily, member 8	-1.47
Hs.74647	T-cell receptor alpha locus	-1.80
	Adhesion	
Hs.13572	calcium modulating ligand	1.56
Hs.10247	activated leucocyte cell adhesion molecule	-1.35
Hs.2257	vitronectin (serum spreading factor, somatomedin B, complement S-protein)	-1.36
Hs.17483	CD4 antigen (p55)	-1.47
Hs.3003	CD3E antigen, epsilon polypeptide (TiT3 complex)	-1.48
Hs.54517	ficolin (collagen/fibrinogen domain containing lectin) 2 (hucolin)	-1.48
Hs.381568	immunoglobulin J polypeptide, linker protein for immunoglobulin alpha and mu polypeptides	-1.49

Table 4 (*cont'd*)

G cluster	Gene name	Fold change
Hs.89575	CD79B antigen (immunoglobulin-associated beta)	-1.56
Hs.95327	CD3D antigen, delta polypeptide (TiT3 complex)	-1.58
Hs.407861	alpha-2-glycoprotein 1, zinc	-1.60
Hs.79630	CD79A antigen (immunoglobulin-associated alpha)	-1.63
Hs.838	CD80 antigen (CD28 antigen ligand 1, B7-1 antigen)	-1.99
Hs.434918	carcinoembryonic antigen-related cell adhesion molecule 1 (biliary glycoprotein)	-2.00
Hs.82848	selectin L (lymphocyte adhesion molecule 1)	-2.13
Hs.385521	integrin, alpha X (antigen CD11C (p150), alpha polypeptide)	-2.31
Hs.423077	selectin P ligand	-2.99
	Signal transduction	
Hs.753	formyl peptide receptor 1	-1.42
Hs.433300	Fc fragment of IgE, high affinity I, receptor for; gamma polypeptide	-1.46
Hs.46	platelet-activating factor receptor	-1.51
Hs.126384	Fc fragment of IgG, low affinity IIb, receptor for (CD32)	-1.53
Hs.44439	suppressor of cytokine signalling 4	-1.58
Hs.381027	colony stimulating factor 3 receptor (granulocyte)	-1.65
Hs.46348	bradykinin receptor B1	-1.84
	Transcriptional factors	
Hs.14453	interferon consensus sequence binding protein 1	1.83
Hs.374357	nuclear factor related to kappa B binding protein	-1.31
Hs.433759	barrier to autointegration factor 1	-1.41
Hs.83795	interferon regulatory factor 2	-1.49
Hs.25647	v-fos FBJ murine osteosarcoma viral oncogene homolog	-1.50
Hs.105434	interferon stimulated gene 20 kDa	-1.62
Hs.41691	basic leucine zipper transcription factor, ATF-like	-1.94
Hs.153837	myeloid cell nuclear differentiation antigen	-1.96
	Other related inflammatory genes	
Hs.79241	B-cell CLL/lymphoma 2	1.64
Hs.407442	Homo sapiens TCRgamma alternate reading frame protein (TCRg) mRNA, complete cds	-1.33
Hs.169998	bone marrow stromal cell antigen 1	-1.38
Hs.2253	complement component 2	-1.38
Hs.146559	angiopoietin-like factor	-1.39
Hs.381568	immunoglobulin J polypeptide, linker protein for immunoglobulin alpha and mu polypeptides	-1.49
Hs.406238	aldehyde oxidase 1	-1.50
Hs.2563	tachykinin, precursor 1 (substance K, substance P, neurokinin 1, neurokinin 2, neuromedin L, neurokinin alpha, neuropeptide K, neuropeptide gamma)	-1.55
Hs.100194	arachidonate 5-lipoxygenase-activating protein	-1.57
Hs.407861	alpha-2-glycoprotein 1, zinc	-1.60
Hs.46465	T cell, immune regulator 1, ATPase, H + transporting, lysosomal V0 protein a isoform 3	-1.62
Hs.196352	neutrophil cytosolic factor 4, 40 kDa	-1.64
Hs.414332	2'-5'-oligoadenylate synthetase 2, 69/71 kDa	-1.81
Hs.105407	ectodermal dysplasia 1, anhidrotic	-1.85
Hs.181301	cathepsin S	-1.91
Hs.449439	Homo sapiens TCR BV3 mRNA for T cell receptor beta chain (CDR3 region), partial cds, isolate:HTLV-1 myopathy case 3, clone:Tax tetramer-5.	-1.95
Hs.949	neutrophil cytosolic factor 2 (65 kDa, chronic granulomatous disease, autosomal 2)	-1.99
Hs.2200	perforin 1 (pore forming protein)	-2.30
Hs.234734	lysozyme (renal amyloidosis)	-2.39

effect in WAT was related to the decrease of PEG-OB receptor (Ob-Rb) expression under PEG-OB treatment we analyzed the individual variation in expression of Ob-Rb by RT-PCR in seven subjects. The microarray experiments did not reveal a significant change. The results obtained showed that the PEG-OB injection did not significantly change the Ob-Rb expression (fold change = 0.95, *P*-value > 0.05; data not shown).

Cellular origin of inflammatory mobilized genes in WAT

As adipose tissue contains not only adipocytes but also stromal-vascular cells, including fibroblastic connective tissue cells, preadipocytes (not yet filled with lipids) as well as leucocytes and macrophages [28], we further examined whether the inflammatory-related genes regulated by the

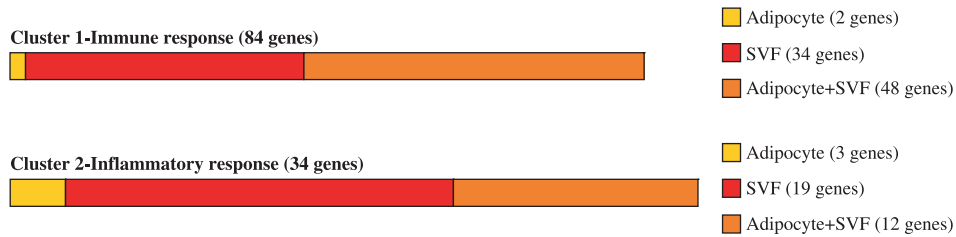


Figure 2 Cellular origin of inflammatory related genes in white adipose tissue. The two clusters represent the enriched functions isolated with the gene ontology annotation system. The yellow, red and orange boxes represent the number of genes overexpressed in the adipocytes, stroma vascular fraction (SVF) fraction and equally in both the adipocytes and SVF fraction, respectively.

PEG-OB injection in humans were expressed mostly in adipocytes or in the stroma vascular fraction. Mature adipocytes were separated from the SVF as described in the Materials and Methods section. Among the clusters (cluster 1 and 2, Fig. 1) related to immunity and inflammation pathways, we sorted either genes typical of adipocytes or genes mostly expressed in SVF or in both fractions. As shown in Fig. 2, most of the inflammatory related genes were mainly expressed in the SVF or equally expressed in adipocyte and SVF. The fraction of genes mainly expressed in adipose cells was faint.

Discussion

The existence of the leptin effect on WAT or isolated adipocytes is a matter of controversy. The leptin target genes in human WAT are unknown. Although both mRNA and protein of the leptin receptor (Ob-Rb) were detected on human adipocytes [29], leptin treatment in different systems led to divergent results. Several studies provide some evidences of an effect of leptin both *in vitro* on isolated murine adipocytes [30] and *in vivo* in WAT from rodents [31,32]. In contrast, in other studies no effect of leptin on WAT gene expression was found [33,34]. Our experiment showed that the supraphysiological PEG-OB injection in healthy individuals induced transcriptional changes in WAT and most of these genes were downly expressed. We also found that most of these genes belong to the inflammatory and immune functional class.

In vitro and *in vivo* studies have suggested a potential role for leptin in modulating the immune response [8]. The leptin receptor is structurally related to the class 1 cytokine receptor family and has been shown to have signalling capabilities of interleukin-6-type cytokine receptors [35]. The first observations were carried out on rodents with genetic leptin (ob/ob) or leptin receptor (db/db) deficiency. These mice elicit macrophage phagocytosis and the overexpression of proinflammatory cytokines [36]. Administration of exogenous leptin to ob/ob mice reversed the inflammation associated phenotype [36]. In most of the available publications, a proinflammatory function of leptin on immune cells (lymphocytes, monocytes) was described [37,38]. No study has demonstrated such effects in human adipose tissue or

adipocytes. In addition, in human monocytes, leptin was shown to induce the expression and the secretion of IL-1Ra, an interleukin widely recognized as an anti-inflammatory factor *in vitro* [39]. Another study showed that an increase in circulating leptin in monkey blunts the cytokine response to an inflammatory challenge [40].

One explanation of the down-regulated effect of PEG-OB in WAT might be associated with the supraphysiological dose of PEG-OB used in these healthy subjects. As expected, the PEG-OB injection induced very high plasma leptin levels in all the subjects. At this dose, many physiological pathways could be down-regulated. In this regard, we observed that most of the genes were down-regulated in our experience, including factors involved in metabolism, signal transduction, extracellular matrix and cytoskeleton.

To date, all human PEG-OB injection trials have not shown large effects. In the present study no other major physiological change on energy metabolism or hormonal status was observed. However, analyzing the pro-inflammatory status in one of our weight reduction studies using the PEG-OB injection, we found an additional significant decrease of inflammatory markers as a whole [41]. It was concluded that the apparently higher decline in inflammatory parameters in the PEG-OB treated group may be related to the higher weight loss in this group. The present study provides additional information regarding the anti-inflammatory effect of PEG-OB especially in WAT.

In our study, we observed a trend toward an increase of circulating levels of the pro-inflammatory factors (CRP and SAA) after the PEG-OB injection. The mechanism by which leptin increased CRP and SAA in this study is not known but could relate to an acute-phase response. There is no confirmation that CRP and SAA could be produced by the WAT in this context. Indeed, in nonobese subjects CRP and SAA are mainly produced by the liver [42]. The CRP and SAA are classical and highly sensitive acute-phase proteins; plasma levels that typically increase by 100-fold or more during inflammation. The twofold increase in CRP levels and threefold increase of SAA upon the PEG-OB injection are indicative of only a weak inflammatory response and may result from the direct interaction of leptin on hepatocytes via its receptor. This kind of interaction has been reported to induce IL-6-type responses [43,44]. These observations are in line with the strong correlation found between CRP and SAA changes upon a PEG-OB injection.

In our study, we showed a decreased gene expression 3 days after PEG-OB administration in WAT, but we cannot exclude that in the first few days the inflammatory gene expression responded differently to PEG-OB administration. To investigate this possibility, future kinetic studies might be of interest.

Conclusions

In this study, we have characterized the WAT gene expression profile after 3 days in response to the administration of PEG-OB in a single supraphysiological dose. Most of these genes showed a decreased expression after the PEG-OB injection. Sorting the PEG-OB regulated genes into functional classes, we observed that genes related to the inflammation and immunity classes were significantly affected by PEG-OB.

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