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ORIGINAL ARTICLE

PPARγ activity in subcutaneous abdominal fat tissue and fat mass gain during short-term overfeeding

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Objective: As the peroxisome proliferator-activated receptor γ (PPARγ) plays a central role in fat mass regulation, we investigated whether initial subcutaneous PPARγ activity is related to fat mass generation during overfeeding.
Subjects: Fourteen healthy female subjects (age 25 ± 4 years, BMI 22.1 ± 2.3 kg/m^2).
Design and measurements: Subjects were overfed with a diet supplying 50% more energy than baseline energy requirements for 14 days. Fasting blood samples were analyzed for leptin, insulin and glucose. Fasting subcutaneous abdominal fat biopsies were obtained for analysis of PPARγ1, PPARγ2, aP2 and UCP2 mRNAs.
Results: Initial PPARγ1 and 2, aP2 and UCP2 mRNAs were not related to fat gain ($P > 0.12$). However, PPARγ1, PPARγ2 and aP2 mRNA changes were positively related to changes in plasma leptin ($P < 0.05$) and, except aP2 ($P = 0.06$), to fat gain ($P < 0.05$). PPARγ and aP2 mRNA changes were positively related ($P < 0.01$), indicating that PPARγ mRNA levels reflected PPARγ activity.
Conclusion: These data suggest that the ability to increase PPARγ activity might be involved in the susceptibility to gain weight during a positive energy balance.
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Keywords: peroxisome proliferator-activated receptor γ; uncoupling protein 2; adipogenesis; lipogenesis; humans

Introduction

Obesity is characterized by an excessive fat mass. However, within a normal weight population also, large differences in fat mass are observed. Overfeeding identical twins with a fixed amount of energy resulted in at least three-fold more variance in gains in body weight and fat mass between pairs than within pairs,[1] which indicates that body weight and composition are largely genetically controlled. An interaction between environmental factors (e.g. food intake) and genes involved in adipose tissue metabolism may therefore determine the susceptibility to obesity.

The peroxisome proliferator-activated receptor γ (PPARγ) has been shown to be both sufficient and obligatory for fat generation.[2] In addition, PPARγ has an important role in fat storage (leading to an increased adipocyte volume) and metabolism in differentiated adipocytes by stimulating the expression of adipocyte-specific genes.[2] PPARγ is a member of the nuclear hormone receptor superfamily and is expressed as two isoforms, PPARγ1 and PPARγ2, generated from the same gene by alternative promoter usage and alternative splicing. Both isoforms are expressed at high levels in adipose tissue.[3–5] PPARγ activity requires the formation of heterodimers and the binding of ligands. These heterodimers are formed with another nuclear hormone receptor, the retinoid X receptor (RXR), which binds to PPAR response elements in the promoter region of target genes and activates DNA transcription upon ligand binding.[4] Several ligands and agonists of PPARγ are known, including certain polyunsaturated fatty acids and eicosanoids and the anti-diabetic thiazolidinedione (TZD) class of drugs.[6]

As PPARγ plays a central role in fat mass regulation, we investigated whether initial PPARγ activity is related to fat mass gain during overfeeding. In this context, it is important to know whether increased PPARγ mRNA levels reflect an increase in PPARγ activity. The adipocyte-specific fatty acid-binding protein (aP2) is primary a PPARγ response gene and is therefore commonly used as an adipocyte-specific marker *in vitro*.[7,8] In addition, PPARγ is involved in the regulation of the uncoupling protein 2 (UCP2) as PPARγ agonists are shown to increase UCP2 expression in adipose tissue.[9–11] UCP2 mRNA levels are reduced in adipose tissue from obese compared to lean persons,[12] which suggests a role for UCP2 in obesity by influencing energy metabolism although a

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specific uncoupling function has not been confirmed in humans.[10] We investigated whether UCP2 influences fat gain during overfeeding.

Subjects and methods

Subjects

Male and female subjects were recruited through advertisements in the university and the university hospital. However only female subjects responded, therefore 14 female subjects, aged 19–36 years, participated in the study. Characteristics of the subjects are shown in Table 1. Selection criteria included being healthy, between the age of 18 and 40 years, Caucasian, non- or light (<10 cigarettes/day) smoker, with a BMI between 18.5 and 27 kg/m^2 and weight stable (body weight changes $\leqslant$2 kg) for at least a year at the start of the study. Before participating in the study, subjects were medically screened by a detailed health questionnaire, only subjects in good health were included. All subjects received verbal and written information and signed a written consent form. The study was approved by the Ethics Committee of Maastricht University.

Experimental design

The study enclosed a 7-day baseline period (days 1–8) and a 14-day overfeeding period (days 8–22). During the baseline period subjects chose their diets from a variety of food items provided daily in weighed food packages, bringing back the leftovers the next day, for calculation of habitual energy intake. When subjects had not been in energy balance (i.e. body weight changed from day 1 to day 8), baseline energy requirements were calculated from basal metabolic rate and physical activity level. During the overfeeding period, diets contained 50% more energy than the baseline energy requirements (14.8 $\pm$ 1.6 MJ/day or a total of 207.2 $\pm$ 21.6 MJ). The excess energy intake was introduced gradually to allow the bowels to adjust to the increased amount of food. Overfeeding diets provided 7% of energy from protein, 40% from fat and 53% from carbohydrates (Dutch food composition table, Komeet, version 2.0d, 1996, B·ware Nutrition Software). All foods and drinks were provided daily in weighed food packages while subjects consumed dinner at the university. Alcohol consumption was not allowed during the study. Body weight was measured on days 1, 8 and 22.

Table 1 Baseline characteristics of the 14 female subjects

	Mean	*s.d.*
Age (years)	25	4
Height (m)	1.72	0.06
Body weight (kg)	64.8	7.0
Body mass index (kg/m^2)	22.1	2.3
Body fat (%)	27.5	4.4

Body composition was measured on days 8 and 22 and on the same days blood samples and fat biopsies were taken. Subjects maintained their normal lifestyles (i.e. work, education, sports participation) throughout the study.

Procedures

Anthropometry and body composition. Measurements were carried out in the morning after voiding and before breakfast. Body weight and height were measured to the nearest 0.01 kg and 0.1 cm, respectively. Body mass index (BMI, kg/m^2) was calculated as body weight (kg) divided by height (m) squared. Body composition was estimated using hydrodensitometry and isotope dilution. Body density was determined by underwater weighing with simultaneous measurement of residual lung volume with the helium dilution technique. Total body water (TBW) was determined with deuterium dilution following the Maastricht protocol.[13] Body composition was calculated from body density and TBW using the three-compartment model of Siri.[14]

Plasma glucose, insulin and leptin. After an overnight fast, blood samples were obtained and mixed with citrate to prevent clotting. Plasma was obtained by centrifugation (4°C, 3000 rpm, 10 min), frozen in liquid nitrogen and stored at −80°C until analysis of concentrations of glucose (hexokinase method, Glucose HK 125 kit, ABX diagnostics, Montpellier, France), insulin (ELISA, Mercodia, Uppsala, Sweden) and leptin (double-antibody RIA, human leptin specific RIA kit, Linco Research Inc., St Charles, USA).

Fat biopsy. Abdominal subcutaneous fat biopsies were obtained by needle liposuction under local anesthesia (lidocain 2% with adrenalin 1:80 000, AstraZeneca BV, Nederland) after an overnight fast. The tissue was immediately washed in cold saline, homogenized in 1 ml Trizol (Gibco BRL, Life Technologies) in a mini-beadbeater (Biospec Products, Bartlesville, OK, USA), frozen in liquid nitrogen and stored at −80°C until RNA extraction. Total RNA was isolated using the method of Chomczynski and Sacchi[15] and 10 μg glycogen was added to each sample. Extracted RNA was quantified and assessed for purity by UV spectrophotometry.

Quantitation of PPARγ1, PPARγ2 and aP2 mRNA expression by real-time RT-PCR. cDNA was synthesized from 500 ng RNA in a volume of 20 μl containing 2 μl RT buffer (Sensiscript RTkit, Qiagen, Hilden, Germany), 2 μl dNTPs, 1 μl oligo (dT) primer, 0.25 μl RNase inhibitor and 1 μl reverse transcriptase (Sensiscript RTkit, Qiagen). The RT reaction was performed at 37°C for 60 min.

PCR reactions were performed in a final volume of 25 μl containing 0.2 × SYBR Green/fluorescein in DMSO (5 × SYBR Green/0.25 μM fluorescein), 1 × *Taq* buffer, 0.02 U/μl *Taq* polymerase, 0.2 mM dNTPs, 3 mM $MgCl_2$, 0.6 pmol/μl of each primer and 25 ng/μl cDNA. The PCR program was at

94°C for 2 min, followed by 45 cycles of 94°C for 15 s and 60°C for 1 min. After every cycle, the fluorescence of SYBR Green was measured, which represents the amount of double-stranded DNA amplified to that point in the PCR. Subsequently, melt curve analysis (45°C for 5 min, 100 cycles of 65°C for 10 s, +0.3°C after cycle 2) indicated specific product for each pair of primers. The primer sequences were as follows: PPARγ1 forward 5′-AAGGCCATTTTCTCAAACG A-3′, reverse 5′-AGGAGTGGGAGTGGTCT-TCC-3′; PPARγ2 forward 5′-CCATGCTGTTATGGGTG-AAA-3′, reverse 5′-TCAAAGGAGTGGGAGTGGTC-3′; aP2 forward 5′-GCATTC CAC-CACCAGTTTATC-3′, reverse 5′-CAGGAAAGTCAAGAG CACCAT-3′; β-actin forward 5′-AGAAAATCTGGCACCA CACC-3′, reverse 5′-AGAGGCGTACAGGGATA-GCA-3′. For target and housekeeping genes, standard curves were created from a specific PCR product. To account for differences in RNA loading, target mRNA was expressed relative to β-actin mRNA. The mRNA concentration of β-actin was not different between baseline and overfeeding (109±67 and 88±87, respectively, based on photospectrometry, $P=0.48$). The threshold cycle (C_T) is the cycle number at which the fluorescence is first recorded, statistically significant above background. The C_T always occurs during the exponential phase of the amplification and thus shows a negative linear relationship with the log cDNA: the more cDNA present at the beginning of the PCR, the fewer number of cycles it takes to reach the C_T. The PCR amplification efficiencies of the target and housekeeping genes were similar and therefore the amount of target mRNA relative to the amount of β-actin mRNA in each sample was calculated as follows: $\Delta C_T = C_T$ (target)$-C_T$ (β-actin), relative expression level $= 2^{-\Delta C_T}$.

Quantitation of UCP2 mRNA expression by RT-competitive PCR. Total RNA (0.2 μg) was reverse transcribed as described above using Tth DNA polymerase and specific primers. Target cDNA (0.2 μg) was coamplified with known amounts of the target cDNA competitor in the same tube. The PCR reaction was performed with 5′-ATGGACGCCTACAGAAC CAT-3′ as sense primer and 5′-ATAGGTGACGAACATCAC CACG-3′ as antisense primer, and specific competitor DNA was obtained by the deletion of 55 bp from a 290-nt long UCP2 cDNA fragment. The PCR program was at 94°C for 3 min, 35 cycles of 94°C for 1 min, 57°C for 45 s and 72°C for 2 min, followed by 74°C for 5 min. PCR products were analyzed with the ALF sequencer. The amount of UCP2 mRNA, expressed as amol/μg total RNA, was obtained from the point where the initial amount of target cDNA corresponds to the initial amount of competitor (log density ratio competitor/target cDNA = 0).

Statistical analysis

Results are presented as means±s.d.'s unless otherwise specified. The nonparametric paired Wilcoxon signed rank test (two-sided) was used to test the effect of the overfeeding on the parameters. Significance of correlations was tested with the nonparametric Spearman rank correlation (r_S). $P<0.05$ was considered as statistically significant. StatView statistical software (1992–1998, SAS Institute Inc., NC, USA) was used for the analysis.

Results

Overfeeding resulted in a mean body weight gain of 1.45±0.86 kg ($P<0.0001$), with a range from 0.19 to 3.00 kg. Fat mass increased by 1.05±0.75 kg ($P<0.001$) ranging from 0.12 to 2.65 kg.

Mean fasting plasma glucose, insulin and leptin concentrations before and after overfeeding are shown in Table 2. Changes in plasma insulin and leptin concentrations were positively correlated ($r_S=0.77$, $P<0.01$). Changes in plasma leptin concentrations were related to fat mass gain ($r_S=0.62$, $P=0.02$), changes in plasma insulin concentrations were not statistically significant related to fat mass gain ($P=0.08$).

Table 3 shows the median fasting mRNA levels of PPARγ1 and 2, aP2 and UCP2 in subcutaneous abdominal adipose tissue before and after overfeeding. PPARγ1 and 2 and aP2 mRNA levels at baseline were neither related to the initial fat mass ($P>0.30$) nor to the fat mass gain ($P>0.12$). UCP2 mRNA levels at baseline tended to be negatively related to the initial fat mass ($P=0.06$), but were not associated with fat mass gain ($P=0.79$). However, the increase in fat mass with overfeeding was related to changes in PPARγ1 ($r_S=0.71$, $P=0.01$; Figure 1a) and PPARγ2 ($r_S=0.64$, $P=0.02$; Figure 1b) mRNA with a trend towards a relation with changes in aP2 ($r_S=0.52$, $P=0.06$; Figure 1c) mRNA. Similar results were

Table 2 Fasting plasma concentrations of glucose, insulin and leptin at baseline and after overfeeding

	Baseline		*Overfeeding*	
	Mean	*s.d.*	*Mean*	*s.d.*
Glucose (mmol/l)	4.4	0.2	4.4	0.3
Insulin (μU/ml)	5.7	1.7	7.0	2.6
Leptin (ng/ml)	5.9	2.5	10.5[a]	5.3

[a]Significantly different from baseline using Wilcoxon's paired signed rank test ($P=0.001$).

Table 3 mRNA levels of PPARγ1, PPARγ2, aP2 and UCP2 in subcutaneous abdominal adipose tissue at baseline and after overfeeding

	Baseline		*Overfeeding*	
	Median	*Range*	*Median*	*Range*
PPARγ1/β-actin mRNA	1.55	(0.28–15.35)	1.96	(0.68–10.06)
PPARγ2/β-actin mRNA	0.27	(0.11–4.08)	0.54	(0.22–2.04)
aP2/β-actin mRNA	22.27	(3.39–362.04)	27.94	(10.34–76.11)
UCP2 (amol/μg RNA)	0.35	(0.01–2.18)	0.32	(0.05–1.54)

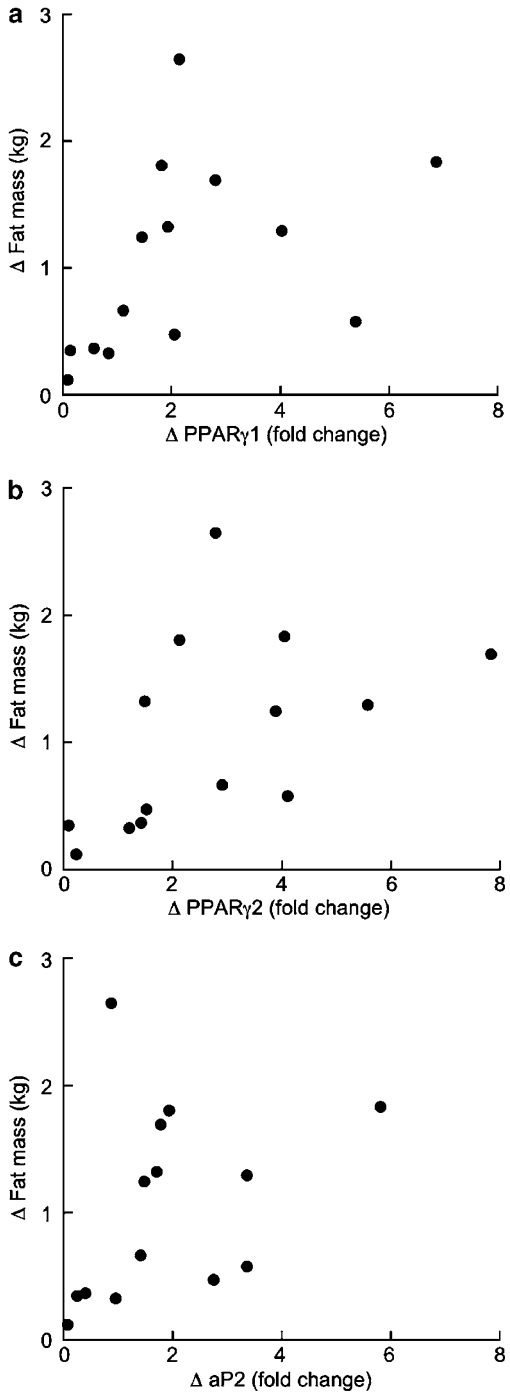

Figure 1 Relationship between changes in PPARγ1 (**a**), PPARγ2 (**b**) and aP2 (**c**) mRNA levels and fat mass gain.

obtained when fat mass gain was expressed as percent change of intial fat mass (data not shown). The increase in fat mass was not related to changes in UCP2 mRNA levels ($P=0.23$) and changes in PPARγ2 and UCP2 mRNA levels were not statistically significantly related ($P=0.08$).

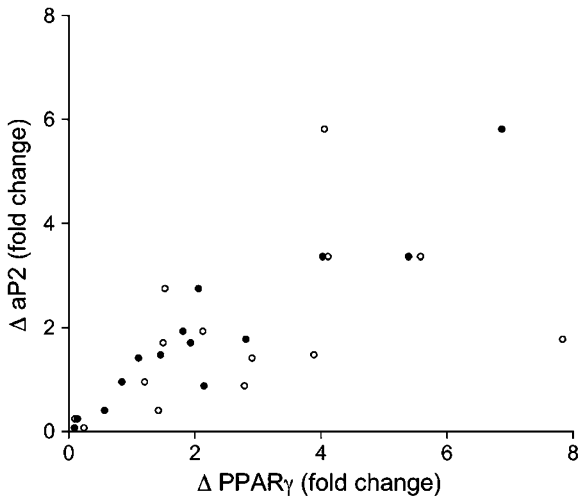

Figure 2 Relationship between changes in PPARγ1 (●) and PPARγ2 (○) mRNA levels and changes in aP2 mRNA levels.

Interestingly, high initial PPARγ1 and 2 mRNA levels tended to relate to smaller changes in expression of these genes ($r_S=-0.54$, $P=0.05$ for PPARγ1; $r_S=-0.48$, $P=0.06$ for PPARγ2). Nevertheless, PPARγ1 and 2 mRNA levels were related to aP2 mRNA levels at baseline and after overfeeding ($r_S=0.56$ to 0.85, $P<0.05$) and changes in the expression of PPARγ1 and 2 expression were positively related to changes in aP2 expression ($r_S=0.88$ and 0.74 respectively, $P<0.01$; Figure 2), indicating that PPARγ activity is in proportion to the PPARγ gene expression and to changes in gene expression.

Changes in plasma leptin concentrations were positively related to changes in mRNA levels of PPARγ1, PPARγ2 and aP2 ($r_S=0.57$ to 0.71, $P<0.02$), but these correlations disappeared after correction of changes in leptin for fat mass gain ($P>0.45$), which indicates that the changes in gene expression were directly associated with changes in fat mass.

There was a positive relation between changes in plasma insulin concentrations and PPARγ2 mRNA levels ($r_S=0.70$, $P=0.01$), but there was no statistically significant relation with PPARγ1, aP2 and UCP2 ($P>0.06$) mRNA levels.

Discussion

The present study aimed to investigate whether the initial PPARγ activity is related to fat mass generation during short-term overfeeding. The initial PPARγ gene expression was not significantly related to fat mass gain during overfeeding, but the change in PPARγ expression was positively correlated to fat mass gain. We measured the two PPARγ isoforms, PPARγ1 and PPARγ2, separately. Both isoforms can stimulate adipogenesis but there is evidence that at low ligand concentrations, PPARγ2 has a greater ability to stimulate adipogenesis.[5]

In this study, we did not see different activities of both isoforms: both PPARγ1 and 2 gene expressions were related to the expression of aP2 and the gene expressions were induced to the same extent. AP2 mRNA levels are primary regulated by PPARγ and are shown to be a quantitative measure of PPARγ activity,[7,8] therefore PPARγ mRNA levels did reflect PPARγ activity *in vivo*. This suggests that the ability to increase PPARγ activity might be involved in the susceptibility to gain weight during overfeeding.

In a population of Finnish men and women, the common Pro12Ala polymorphism in the PPARγ2 gene is associated with lower BMI.[16] The mutant PPARγ2 protein showed an impaired ability to mediate TZD-induced adipogenesis,[16,17] therefore the association with lower BMI is likely to be the result of the lower transcriptional capacity of the Ala variant of PPARγ2.[16] The mRNA expression of PPARγ2 target genes in the adipose tissue of morbid obese subjects did not seem to be influenced by the Pro12Ala polymorphism,[18] which suggests that particular environmental factors, like diet, influence the contribution of the genotype to the development of obesity.

The study included healthy nonobese and normoglycemic female subjects. Fasting plasma glucose was the same before and after the overfeeding period, and although there were large interindividual differences, mean fasting plasma insulin did not change significantly. An increased PPARγ activity, leading to adipocyte differentiation, results in an increase in insulin sensitivity.[19] Indeed, we found a positive relation between changes in plasma insulin and PPARγ2 mRNA, and a trend towards a relation with PPARγ1 mRNA. Changes in plasma insulin tended to be related to fat mass gain. This effect of short-term overfeeding was in accordance with the observed increase in PPARγ mRNA levels in subcutaneous abdominal fat tissue after a 3 h insulin infusion[20] and the induction of PPARγ mRNA in isolated human adipocytes by insulin.[3]

The weight gain period in this study was 2 weeks and the extent of induction of PPARγ expression was positively related to fat gain over this interval. We can only speculate on the relation in the longer term. Fat mass is determined by the number of fat cells and by the continuous uptake and release of energy by the fat cell. PPARγ is required for the formation of new fat cells and plays a major role in fat storage and metabolism. Therefore, both slow and fast adaptation of the PPARγ expression to a positive energy balance will lead to an increased fat mass but the time span may vary.

Another factor in determining fat mass gain in response to overfeeding is the thermogenic capacity of adipose tissue. UCP2 is homologue of the brown adipose tissue UCP1, which uncouples proton entry in the mitochondrial matrix from energy production, thereby dissipating energy as heat. The expression of UCP2 is reduced in adipose tissue of obese vs lean subjects.[12] In a population-based sample from the same geographic area, a common UCP2 polymorphism could account for 15% of the obesity prevalence,[12] and genetic variation at the UCP2 locus is associated with energy expenditure in Pima Indians.[21] Together, these observations suggest a role for UCP2 in obesity by influencing energy metabolism although a specific uncoupling function has not been confirmed in humans.[10] In addition, UCP2 is at least in part regulated through PPARγ as PPARγ agonists increases adipose UCP2 expression.[9–11] Direct evidence was provided by the observation that a 5 h infusion with nonesterified fatty acids upregulated UCP2 and PPARγ mRNA levels in subcutaneous adipose tissue.[22] However, in the present study, we found no effect of overfeeding on UCP2 mRNA and we found no statistically significant relation between changes in PPARγ and UCP2 mRNA levels. Changes in plasma insulin were not related to changes in UCP2 mRNA either. This suggests that the response of UCP2 to overfeeding is not a major determinant of fat mass gain, although again, the study period might have influenced these results.

In conclusion, these data suggest that the ability to increase PPARγ activity might be involved in the susceptibility to gain weight during a positive energy balance.

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