Visceral Fat: Higher Responsiveness of Fat Mass and Gene Expression to Calorie Restriction than Subcutaneous Fat

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Visceral fat accumulation is accompanied by several metabolic disorders. Here, we investigate the improvement of visceral fat accumulation in the early phase of diet. Hyperlipidemic obese patients received a low-calorie diet (1000 kcal/day) for 14 days. Visceral and subcutaneous fat accumulation was analyzed using ultrasonography. After 14 days of the diet, the average visceral fat of obese patients obviously decreased ($P < 0.05$), as well as the visceral fat-related secreted proteins, whereas subcutaneous fat did not decrease in these patients. These results show that visceral fat is reduced significantly in the early phase of diet therapy in humans. Therefore, to clarify its mechanism, we analyzed the expression of lipid metabolism-related genes in visceral and subcutaneous fat using obese rats. The Long-Evans Tokushima Otsuka (LETO) rats, as an obese model, were divided into two groups: fasting and non-fasting. The gene expressions in visceral and subcutaneous fat were measured by reverse transcriptase–polymerase chain reaction (RT-PCR). The expression of β_3-adrenergic receptor (AR), hormone sensitive lipase (HSL), peroxisome proliferator–activated receptor (PPAR)-γ, and uncoupling protein (UCP)-2 genes increased by 3.2-, 2.3-, 2.2-, and 2-fold in visceral fat ($P < 0.01$), but remained almost unchanged in subcutaneous fat. Taken together, the responsiveness of lipid metabolism-related genes to fasting is more sensitive in visceral fat than in subcutaneous fat in rats, suggesting that the different responsiveness to calorie restriction in fat tissues is due to the different induction of metabolism-related gene expression. Exp Biol Med 228:1118–1123, 2003

Key words: visceral fat accumulation; calorie restriction; gene expression; rat

Visceral fat accumulation is accompanied by several metabolic disorders, such as hyperlipidemia, hypertension, and glucose intolerance, leading to advanced atherosclerosis (1). A measurement using computerized tomography (CT) has been established to examine the accumulations of visceral and subcutaneous fat (2). We have established a different method using ultrasonography to evaluate the fat distributions by the measurements of pre-peritoneal (PPF) and subcutaneous (SF) fat thickness (3). The ultrasonographic measurements revealed that the thickness of PPF is positively correlated with visceral fat accumulation, and highly associated with the frequencies of metabolic disorders accompanied by visceral obesity (3, 4).

Calorie restriction is one of the main methods for reducing the body fat mass and is also known to be effective for reducing the complication of obesity. It has been reported using animals that visceral fat decreased by calorie restriction to about one-third of that of *ad libitum*-fed rats, whereas lean body mass was unchanged (5). Therefore, calorie restriction possibly means the reduction of visceral fat. However, whether this also applies to humans, particularly in the early phase of calorie restriction, is still unknown.

The analysis of the effect of calorie restriction on the lipid metabolism-related genes such as the adrenaline-response gene seems to be important to know the different response of genes in visceral fat and subcutaneous fat. Using mice with the implantation of adipocytes, we have recently identified that the functional significance of visceral fat accumulation for tumor necrosis factor (TNF)-α–induced insulin resistance is caused by the interaction of adipocytes with surrounding conditions in mesenteric area (6). During calorie restriction, lipid mobilization is induced; a series of physiologic changes happen and several lipid metabolism genes take part in these changes.

In this investigation, we first analyzed the early changes of fat distribution by the ultrasonographic measurements and clinical markers related with fat tissues through the way of calorie restriction in obese patients. Then, to

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know the mechanism of different responses of fat tissues for the calorie restriction, we used obese rats to analyze the gene expressions in fat tissues from different locations of the body during calorie restriction (7).

Materials and Methods

Subjects. Ten hyperlipidemic obese patients (6 males and 4 females, aged 49 ± 9 years, body mass index (BMI) 36.0 ± 3.1 kg/m^2) received a low-calorie diet (4186 KJ/d (i.e., 1000 kcal/day)) for 14 days. During the diet, none of the subjects were receiving any medication known to affect lipoprotein metabolism. The body weight was measured every day.

Animals and Tissues. Tokushima Institute Otsuka Pharmaceutical (Tokushima, Japan) kindly provided 4-week-old male LETO rats. The animals were fed a standard laboratory chow until 35 weeks of age. The LETO rats were divided into two groups: fasting and non-fasting (5 in each group). During experiment, the fasting groups were not given any chow for 48 hours whereas the non-fasting groups were maintained on food satiation. Then, after fasting for 12 hours, all the animals were euthanized under anesthesia. Blood samples were taken and serum were separated and stored at −20°C. Adipose tissues from mesentery and abdominal subcutis were removed and stored at −80°C.

Evaluation of Fat Accumulation. Visceral and subcutaneous fat accumulation of obese patients was analyzed every day as PPF (pre-peritoneal fat) and SF (subcutaneous fat) by Suzuki's method using ultrasonography (3). In the supine position, the probe was kept perpendicular to the skin at the point of the xiphoid process. Then, the thickness of PPF and SF were measured directly from the screen with electronic calipers.

Measurements of Plasma Lipids and Glucose. Triglycerides (TG) and cholesterol contents in plasma and in each lipoprotein fraction were measured by enzymatic colorimetric methods using commercial kits from Japan Commercial Company (Osaka). Plasma glucose content was measured by the enzyme-electrode method using the Advantage system (Roche Diagnostics, Tokyo).

Measurements of TNF-α, Insulin, and Leptin. TNF-α, insulin, and leptin were measured by enzyme-linked immunoassay using commercial kits. TNF-α was assayed with human TNF-α US kit from BioSource International, Inc. (California). Insulin was assayed with ELISA insulin kit from Morinaga Bioscience Institute (Yokohama, Japan). Leptin was assayed with human leptin assay kit from Immuno-Biological Laboratories (Gunma, Japan).

Reverse Transcriptase–Polymerase Chain Reaction. Total RNA was extracted from rat adipose tissues using RNeasy Mini Kit (Qiagen, Hilen, Germany). For reverse transcription (RT) –polymerase chain reaction (PCR), single-stranded cDNA was synthesized from 1 μg of Total RNA using SuperScript reverse transcriptase (Life Technologies, Tokyo, Japan) and random hexamer primers. One-tenth of the cDNA was subjected to PCR with sense and antisense primers as shown in Table I. The reaction mixture (50 μl) containing the cDNA, 50 pmoles of each of the primers, and 2.5 mM dNTP was heated to 95°C for 10 minutes, and then immediately cooled on ice. One unit of *Taq* DNA polymerase was added followed by 20, 25, or 30 cycles of re-annealing at 65°C for 1 minute, elongation at 72°C for 2 minutes, and denaturation at 94°C for 1 minute. PCR was performed with GeneAmp PCR system 2400 (Perkin Elmer, Wellesley, MA). PCR products were separated by electrophoresis (2% agarose gel) and visualized by ethidium bromide staining. Gels were photographed with LAS 1000 PLUS and analyzed with Image Gauge 3.2 software (Fujifilm, Japan). Amounts of LR11 mRNA were normalized using the amounts of cyclophilin B mRNA as reference (data not shown).

Table I. List and Sequence of Primers

Primer	Position	Sequence (5′–3′)	Size (bp)
β_3-AR			
Sense	327–353	ACCTTGGCGCTGACTGG	233
Antisense	543–559	ATGGGCGCAAACGACAC	
HSL			
Sense	2772–2791	TGCCCAGGAGTGTGTCTGAG	313
Antisense	3075–3084	AGGACACCTTGGCTTGAGCG	
PPAR-γ			
Sense	824–843	GCAAAGAGGTGGCCATCCGC	337
Antisense	1141–1160	ATGGCCAAGTCACTGTCATC	
UCP-2			
Sense	313–336	CAGTTCTACACCAAGGGCTCAGAG	323
Antisense	613–635	TCTGTCATGAGGTTGGCTTTCAG	
FAS			
Sense	3061–3080	GAGCTGCGGCTACGTGGCTA	340
Antisense	3381–3400	GCCGCCGTGAGGTTGCTGTT	

Note. β_3-AR, β_3-adrenoreceptor (GenBank code: S56152); HSL, hormone-sensitive lipase (GenBank code: U4001); PPAR-γ, peroxisome proliferator-activated receptor-γ (GenBank code: AB611365); UCP-2, uncoupled protein-2 (GenBank code: AB006613); FAS, fatty acid synthase (GenBank code: NM017332).

Statistics. The results were shown as mean ± SD for each index respectively. Comparison of data between fasting and non-fasting groups was performed using the Student's t test for paired samples. A value of $P < 0.05$ was considered significant.

Results

Measurements of Fat Accumulations during Early Phase of Calorie Restriction. Table II shows the averaged changes of 10 subjects in body weight, BMI, fat accumulations in different location by ultrasonography, and plasma levels of insulin, TNF-α, leptin, and lipids before and after calorie restriction in this study. After calorie restriction for 14 days, the body weight and BMI decreased by 7.7% ($P < 0.05$) and 7.2% ($P < 0.05$), respectively. PPF, which is the index of visceral fat accumulation, decreased by 21% ($P < 0.05$), whereas SF, which is the index of subcutaneous fat accumulation, did not show significant change by the diet protocol. Figure 1 illustrates the averaged sequential changes in BMI and fat accumulation. The BMI decreased sharply in the first 6 days, and slowly but steady in the following days (Fig. 1A). Ultrasonographic measurements of fat accumulation revealed that the PPF decreased almost constantly day by day (Fig. 1B), whereas the SF did not change obviously for the 14 days (Fig. 1C).

Lipid Profiles and Clinical Markers Associated with Fat Tissues of Obese Patients before and after Diet. We measured the plasma levels of insulin, TNF-α, leptin, and lipids before and after the treatment (Table II). Plasma leptin concentration decreased by 22% ($P < 0.05$) after the diet compared with that before it in agreement with decreased fat deposition for 14 days as described previously. Plasma insulin levels decreased obviously by 20% ($P < 0.05$) after the treatment. The plasma concentrations of PAI-1, which is secreted largely by visceral fat and positively associated with the visceral fat accumulation (5), significantly decreased after 14 days. The lipid profile of the patients before and after the calorie restriction shows that serum levels of total and HDL-cholesterol did not change

Table II. Changes of Body Weight, Fat Accumulation, and Plasma Levels of Insulin, TNF-α, Leptin, and Lipids during Calorie Restriction

	Pretreatment	Posttreatment	P value
Body weight (kg)	98.4 ± 8.6	90.8 ± 6.8	$P < 0.05$
BMI (kg/m^2)	36.0 ± 3.1	33.4 ± 2.1	$P < 0.05$
PPF (mm)	12.2 ± 2.6	9.6 ± 1.9	$P < 0.05$
SF (mm)	17.4 ± 3.7	16.4 ± 3.9	N.S.
Insulin (ng/ml)	1.42 ± 0.24	1.14 ± 0.24	$P < 005$
PAI-1 (mg/dl)	15.6 ± 9.2	10.9 ± 2.3	$P < 005$
Leptin (ng/ml)	9.8 ± 2.6	7.6 ± 2.6	$P < 005$
TG (mg/dl)	211 ± 86	142 ± 62	$P < 0.05$
TC (ng/dl)	219 ± 59	188 ± 31	N.S.
HDL-C (mg/dl)	34 ± 15	32 ± 15	N.S.

Note. Data are the means ± SD ($n = 10$). PPF and SF were measured using ultrasonography. TG, triglyceride; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol.

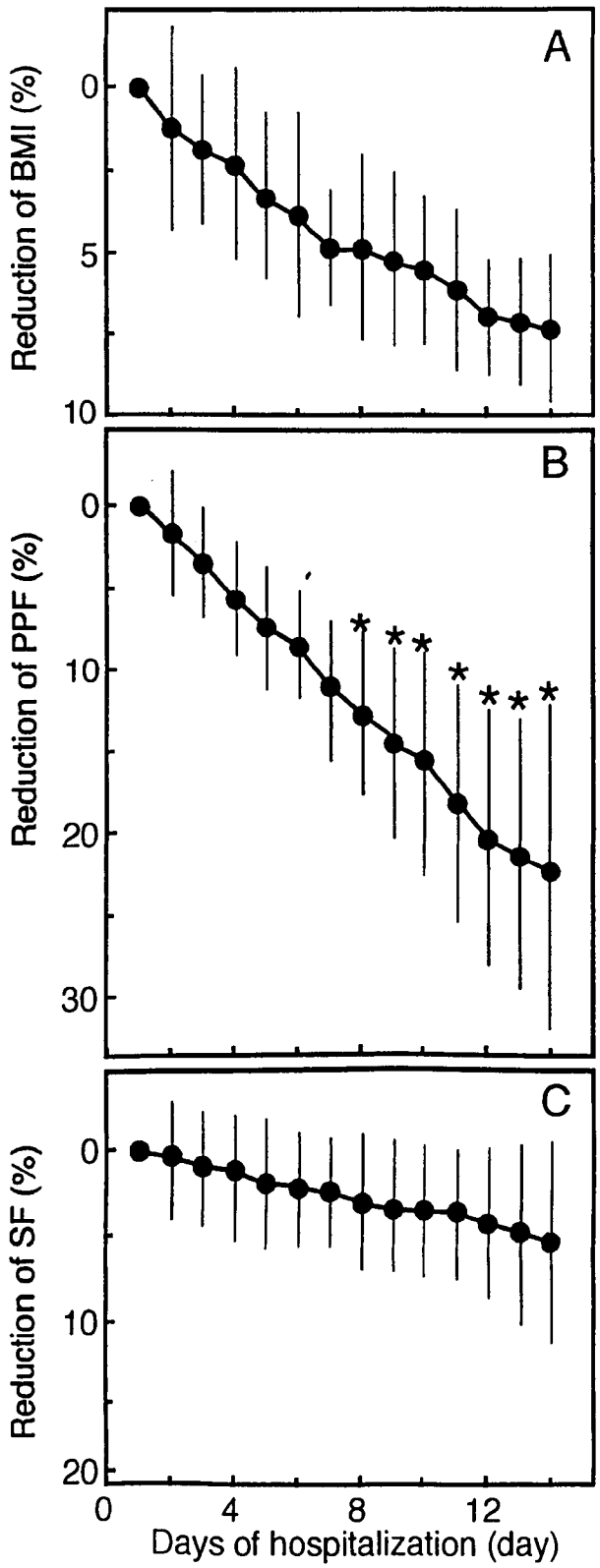

Figure 1. (A) Changes in BMI, (B) visceral fat, and (C) subcutaneous fat during hospitalization for 14 days. The changes were presented as percent reduction compared with the value of day 1. The fat thickness was measured by Suzuki's method using ultrasonography as described in the "Subjects and Methods" section. PPF, preperitoneal fat; SF, subcutaneous fat. Values are the means ± SD ($n = 10$). *$P < 0.05$ versus subcutaneous fat at the same day.

significantly by the diet for 14 days (Table II). However, Serum TG levels decreased drastically by 33% ($P < 0.05$) after the treatment compared with that before it. Ultracentrifugational separation of lipoproteins revealed that TG levels in intermediate density lipoprotein (IDL) fraction, which are believed to be atherogenic (8), decreased drastically (15.6 ± 9.2 mg/dl to 10.9 ± 2.3 mg/dl, $P < 005$) (data not shown). These results indicate that the decreased visceral fat accumulation evaluated using ultrasonography in the patients is accompanied by decreased levels of plasma leptin, insulin, PAI-1, and TG, particularly in IDL fraction, which are believed to be accompanied by insulin sensitivities in the patients.

Comparison of Biochemical Profiles in Obese Rats under Non-Fasting and Fasting Conditions. These clear relationships of metabolic markers accompanied with visceral fat accumulation and PPF (and not SF) measured by the ultrasonography suggested different responsiveness of gene expression in fat tissues depending on the localization in the bodies. Therefore, the gene expressions, particularly lipid metabolism-related genes after fasting, were analyzed in fat tissues from different localization using LETO rats. Table III shows the averaged changes of all animals in blood glucose, TG, and FFA. The glucose concentration decreased by 43% ($P < 0.05$), TG decreased by 33%, but FFA increased to 2.2-fold ($P < 0.01$) in the fasting group compared with the non-fasting group.

Effect of Fasting on Expression of Lipid Metabolism-Related Genes in Obese Rats. Figure 2A and 2B show the expression of β_3-adrenergic receptor (AR) and hormone sensitive receptor (HSL) genes in visceral and subcutaneous adipose tissues. Compared with the non-fasting group, the expression of β_3-AR of the fasting group increased 3.2-fold in visceral fat ($P < 0.01$), but remained almost unchanged in subcutaneous fat (Fig. 2A). The expression of HSL gene showed the same trend. In the fasting group, expression of HSL increased 2.3-fold in visceral fat ($P < 0.01$), but only slightly increased in subcutaneous fat (Fig. 2B). Figure 2C, 2D, and 2E show the expression of peroxisome proliferator–activated receptor (PPAR)-γ, uncoupling protein (UCP)-2, and fatty acid synthase (FAS) genes in visceral and subcutaneous adipose tissues. Compared with the non-fasting group, the expression of PPAR-γ gene of the fasting group increased 2.2-fold in visceral fat ($P < 0.01$), but remained almost unchanged in subcutaneous fat (Fig. 2C). The expression of UCP-2 showed the same trend. Compared with the non-fasting group, the expression of UCP-2 in the fasting group increased 2-fold ($P < 0.05$) in visceral fat, but only slightly increased in subcutaneous fat (Fig. 2D). FAS showed different characteristics. In visceral fat, FAS could be detected only in very low levels in both the non-fasting and fasting groups. In subcutaneous fat,

Table III. Biochemistry Indexes of Obese Rats

	Nonfasting	Fasting	*P* value
Glucose (mg/dl)	179 ± 62	102 ± 21	$P < 0.05$
TG (mg/dl)	45 ± 22	30 ± 11	N.S.
FFA (uEq/l)	230 ± 66	497 ± 122	$P < 0.01$

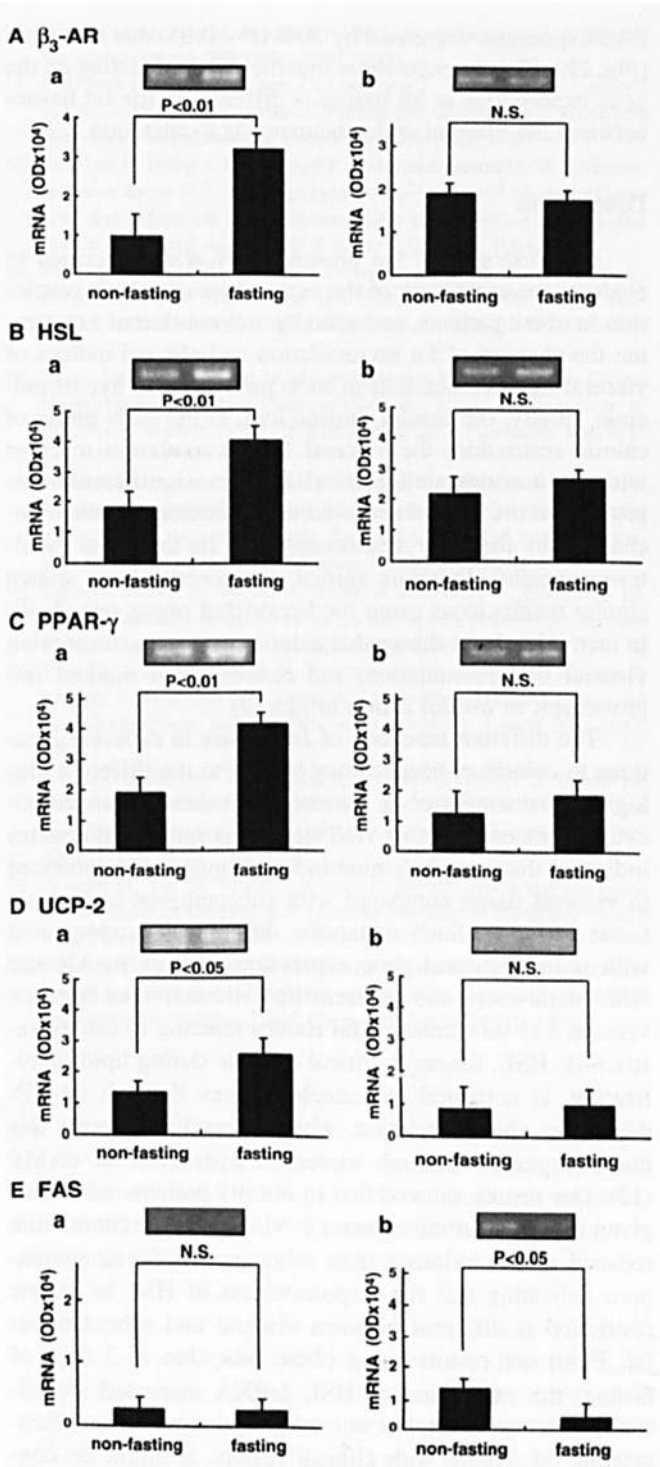

Figure 2. (A) β_3-AR, (B) HSL, (C) PPAR-γ, (D) UCP-2, and (E) FAS gene expression in visceral (a) and subcutaneous (b) fats of LETO rats under non-fasting or fasting condition. Total RNA from visceral and subcutaneous fats of LETO rats with fasting or non-fasting condition was prepared and used for cDNA synthesis as described in the "Subjects and Methods" section. RT-PCR for 30 cycles was performed for β_3-AR and HSL respectively. One-tenth of amplified fragments were used for electrophoresis on a 2.0% agarose gel. Values are the means ± SD ($n = 5$). The inset shows the results of a typical RT-PCR experiment.

FAS expression decreased by 30% ($P < 0.05$) due to fasting (Fig. 2E). These results show that the effect of fasting on the gene expressions in fat tissues is different in the fat tissues between the visceral and subcutaneous localization.

Discussion

The first part of the present study was performed to evaluate the usefulness of the early phase of calorie restriction in obese patients, and actually was conducted to examine the changes of fat accumulation and clinical indices of visceral fat accumulation in such patients with hyperlipidemia. Firstly, our results showed that, in the early phase of calorie restriction, the visceral fat accumulation together with its accompanied clinical indexes significantly improved but the subcutaneous fat accumulation remained unchanged by the daily measurements of fat thickness by ultrasonography. Previous animal experiments have shown similar results to us using food-restricted obese rats (5, 9). In fact, it has been shown that calorie restriction can prevent visceral fat accumulation, and resulted in a marked improvement in insulin action in rats (9).

The different reactions of fat tissues in different locations to calorie restriction may be due to the different biological characteristics of visceral and subcutaneous adipocytes. Evidence from *ex vivo* studies on human adipocytes indicated that catecholamine-induced lipolysis is enhanced in visceral tissue compared with subcutaneous abdominal tissue (10, 11). Such metabolic differences accompanied with some regulated gene expression such as β_3-AR and HSL might cause the different lipolytic activities between visceral and subcutaneous fat tissues reacting to calorie restriction. HSL, the most critical enzyme during lipid mobilization, is activated by catecholamines through cAMP-dependent phosphorylation, whereas insulin prevents this phosphorylation through increased hydrolysis of cAMP (12). Our results showed that in obesity patients who were given energy restriction therapy, visceral fat accumulation reduced more obviously than subcutaneous fat accumulation, indicating that the responsiveness of HSL to calorie restriction is different between visceral and subcutaneous fat. From our results using obese rats, due to 2 days of fasting, the expression of HSL mRNA increased significantly in visceral fat, but only slightly increased in subcutaneous fat. Along with clinical results, it might be concluded that the lipid mobilization due to calorie restriction is more active in visceral fat than in subcutaneous fat due to the induction of HSL gene expression.

Among the β-adrenergic receptor family, the β_3-AR plays a central role in the regulation of lipolysis in rodent white and brown adipose tissue (13). β_3-AR mRNA levels were significantly higher in adipose tissue from lean mice compared with obese mice (14). In the present study using rats, in fasting state, the expression of β_3-AR was induced significantly in visceral fat, but almost remained unchanged in subcutaneous fat, indicating that the sensitivity to β-adrenergic stimulation is also different in a different adipose area. During calorie restriction, the responsiveness to increased β-adrenergic stimulation might also increase in visceral fat than in subcutaneous fat, thereby causing the increased lipid mobilization. The rise in plasma insulin levels is associated with a reduction in β_3-AR mRNA levels and β-adrenergic responsiveness in adipose tissue (15). Therefore, the induction of β_3-AR mRNA in visceral fat in our results is due to the fasting-induced insulin sharp-down secretion.

Uncoupling proteins have been suggested to be involved in obesity. It was reported that in obesity-resistant mice, UCP-2 expression in white fat was increased 2-fold in response to 2 weeks of a high-fat diet, but there was no effect of diet on UCP-2 levels in obesity-prone mice (16). In addition, fasting (48 hours) markedly increased UCP-2 mRNA expression in rat skeletal muscle (17). In the present study, we found that due to calorie restriction, the expression of UCP-2 was induced significantly in visceral fat of rats, but only slightly in subcutaneous fat. Together with the clinical data, it could be supposed that this difference in fat accumulation reduction is partially due to the different extent of energy expenditure between visceral and subcutaneous fat. The expression of PPAR-γ was induced a lot due to calorie restriction in visceral fat, but not in subcutaneous fat. Considering that FFAs are the natural ligand of PPAR-γ (18), it might be the different sensitivity of PPAR-γ gene responsiveness to FFAs in visceral and subcutaneous fat that causes the different expression of UCP-2, thereby causing the different energy expenditure in visceral and subcutaneous fat.

It has been reported that during calorie restriction and re-feeding, inhibition and induction of FAS expression are large in magnitude in liver and adipose tissue (19). Our results showed that during calorie restriction, the expression of the FAS gene in subcutaneous fat decreased by nearly 50%. This might be due to the effect of elevated circulating FFAs in fasting state. Although the molecular mechanisms underlying the suppression of FAS transcription by fatty acids are still unclear, it was reported that fatty acids in cultured chick embryo hepatocytes inhibited thyroid hormone-stimulated FAS gene transcription (20). One possible mechanism might be through the activation of PPARs by fatty acids (18). In the present experiment, the expression of FAS was not detected in visceral fat of both the non-fasting and fasting group. The possible reason might be that the lipid mobilization in visceral fat is already active, so the expression of the FAS gene is inhibited by the high level of FFAs even in a non-fasting state.

In summary, these results show that visceral fat is obviously reduced in the early phase of diet therapy. The responsiveness of lipid metabolism-related genes to calorie restriction is more sensitive in visceral fat than in subcutaneous fat. This might indicate that there is a difference of physiologic characteristics of adipose cells in different locations of the body.

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