Gene expression of lipid storage-related enzymes in adipose tissue of the genetically obese Zucker rat

Co-ordinated increase in transcriptional activity and potentiation by hyperinsulinaemia

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The genetically obese Zucker rat displays excessive fat storage capacity which is due to a tissue-specific increase in the activities of a number of lipid storage-related enzymes in adipose tissue. The aim of this study was to investigate the molecular mechanism responsible for this phenomenon. Lean (*Fa/fa*) and obese (*fa/fa*) Zucker rats were studied during the early stages of adipose tissue overdevelopment, both before (at 16 days of age) and after (at 30 days of age) the emergence of hyperinsulinaemia, in order to delineate the effects of the fatty genotype independently of those of hyperinsulinaemia. Lipoprotein lipase (LPL), glycerophosphate dehydrogenase (GPDH), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and malic enzyme (ME) mRNA levels in the adipose tissue of lean and obese rats were assessed by Northern blot analysis, and the relative transcription rates of the corresponding genes were compared in the two genotypes by a nuclear run-on assay. In normoinsulinaemic 16-day-old pre-obese rats, mRNA levels were increased over control values (LPL, 5-fold; ME, 2-fold; GAPDH, 3-fold), in close correlation with genotype-mediated differences in enzyme activities. Stimulation of the transcription rates of the ME and GAPDH genes was observed in obese rats, which could fully account for differences in steady-state mRNA levels. At this age, GPDH activity, mRNA level and transcription rate were similar in the two genotypes. In hyperinsulinaemic 30-day-old obese rats, a 6–7-fold increase in both mRNA and the transcription rate of GPDH emerged, together with an amplification of the genotype-mediated differences observed in younger animals (GAPDH, 6-fold; ME, 7.9-fold; LPL, 10-fold). These results demonstrate that the obese genotype exerts a co-ordinated control on the expression of these genes in adipose tissue, mainly at the transcriptional level. This genotype effect is greatly amplified by the development of hyperinsulinaemia.

INTRODUCTION

The genetically obese *fa/fa* rat, first described by Zucker & Zucker [1], bears an autosomal recessive mutation, the *fa* gene, the nature of which remains totally unknown. The phenotypic emergence of this genetic defect begins very early in life and profoundly alters the development of the adipose tissue towards excessive fat storage [2–4]. In an attempt to delineate the biochemical basis of this complex syndrome, investigations conducted to establish the chronology of metabolic alterations in adipose tissue have led to the identification of at least two distinct developmental stages taking place before the full expression of massive obesity. The first one corresponds to the suckling period, when a moderate increase in fat storage capacity can be detected, leading to adipose cell hypertrophy in obese pups [2,5]. The second takes place around weaning, and is characterized by the emergence of hormonal alterations such as hyperinsulinaemia [4,6] and hyper-responsiveness of adipocytes to insulin [7], triggering a burst in fat accretion in *fa/fa* rats [8]. In young pre-obese pups, the increased lipogenic capacity of adipose tissue is due to preferential channelling of nutrients into fatty acids, and a number of lipid storage-related enzyme activities are co-ordinately increased in a tissue-specific manner [3,5,9,10]. Among these are lipoprotein lipase (LPL; EC 3.1.1.34), which controls the entry of exogenous fatty acids into the adipose cell, fatty acid synthetase (FAS; EC 2.3.1.85) and malic enzyme (ME; EC 1.1.1.40), involved in the *de novo* synthesis of fatty acids from acetyl-CoA, glycerophosphate dehydrogenase (GPDH; EC 1.1.1.8), which provides the glycerophosphate moiety for

the esterification of fatty acids, and glycolytic enzymes such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH; EC 1.2.1.12), a key enzyme in adipose tissue for supplying lipogenic substrates from glucose.

The underlying mechanism as well as the nature of the triggering factors responsible for the stimulation of these lipid storage-related enzyme activities in the adipose tissue of genetically obese rats remain obscure. Although the stimulatory effect of insulin on the lipogenic pathway is well documented in normal rats, hyperinsulinaemia does not emerge in obese pups until 17 days of age [4], ruling out a possible role for this hormone in the early onset of obesity. Moreover, investigations examining the hormonal changes which might explain the stimulation of the fat storage pathway in the adipose tissue of very young *fa/fa* rats have not produced conclusive evidence. The aim of this study was to delineate the molecular mechanism of this co-ordinated rise in lipid storage-related enzyme activities in the mutant rat. We have previously demonstrated that stimulation of gene expression at the pre-translational level could be identified as the altered step to explain increased GAPDH activity in the adipose tissue of pre-obese pups [10]. Here we have addressed the question of the effect of the *fa/fa* genotype on gene transcription, and we have extended the study to the expression of several other lipid storage-related enzymes in adipose tissue. In order to establish the effect of the obese genotype independently of hyperinsulinaemia, rats have been studied both before and after weaning. By hybridization with specific cDNA probes, we demonstrate that LPL, GAPDH, GPDH and ME mRNA contents are higher in adipose tissue from obese rats.

Abbreviations used: LPL, lipoprotein lipase; FAS, fatty acid synthetase; ME, malic enzyme; GPDH, glycerophosphate dehydrogenase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PEPCK, phosphoenolpyruvate carboxykinase; SSC, 0.15 M-NaCl/0.015 M-sodium citrate.

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This essentially results from increased transcriptional activity in the mutant rat. In addition, our study suggests that the hyperinsulinaemic state of the obese rats further amplifies the overtranscription of these genes.

MATERIALS AND METHODS

Animals and tissues

Obese *fa/fa* rats and their lean *Fa/fa* littermates were bred in our laboratory from pairs originally provided by the Harriet G. Bird Memorial Hospital, Stow, MA, U.S.A. Heterozygous *Fa/fa* lean females and obese *fa/fa* males were mated, and pups were routinely kept with their mothers until 28 days of age, in rooms exposed to light from 07:00 h to 19:00 h. Weaning was performed on to a standard laboratory diet (AO4; UAR, Epinay sur Orge, France). Rats were studied in the fed state, either during suckling (16 days of age) or after weaning (30 days of age). They were killed by decapitation, and inguinal adipose tissue was quickly removed for further determinations. Before the obesity was frankly evident (16-day-old pups), the identification of the genotypes was performed by plotting fat pad weight versus body weight, as described previously [11].

Enzyme activity determinations

ME, GPDH and GAPDH activities were determined spectrophotometrically on cytosolic fractions (100 000 g, 1 h, 4 °C) of adipose tissue as previously described [4,10,12]. Adipose tissue homogenates in $\text{NH}_4\text{Cl}/\text{NH}_4\text{OH}$ (50 mM)/heparin (4 units/ml), pH 8.1, were centrifuged at 4 °C for 15 min at 5000 g, and used for measurement of LPL activity as described by Nilsson-Ehle & Schotz [13].

cDNA and plasmids

The LPL cDNA probe (pLPL35) encoding for human adipose tissue LPL [14] was provided by Dr. R. M. Lawn (Genentech Inc.). A full-length cDNA clone (pRGAPDH13) encoding for rat muscle GAPDH [15] was provided by Dr. J. M. Blanchard (INSERM, Montpellier, France). The cDNA probe for rat liver ME (pRME1) previously described [16] was a gift from V. M. Nikodem (NIH, Bethesda, MD, U.S.A.), pGPD1 and pAD20, encoding for 3T3-L1 GPDH and adipin respectively [17], were provided by Dr. H. Green (Harvard Medical School, Boston, MA, U.S.A.), and clone pPCK10, encoding for rat liver phosphoenolpyruvate carboxykinase (PEPCK) [18], was from R. W. Hanson, Cleveland, OH, U.S.A.

RNA preparation and Northern blot analysis

Total RNA from frozen inguinal adipose tissues of lean and obese Zucker rats were extracted using the guanidinium isothiocyanate/LiCl method as described by Cathala *et al.* [19]. RNA was analysed by Northern blotting after electrophoresis on 1.5% agarose/0.66 M-formaldehyde gels and transfer on to nylon membranes (Hybond N, Amersham). For each blot, equal loading of lanes was confirmed by ethidium bromide fluorescence of ribosomal 18 S and 28 S RNA. Hybridization with ^{32}P -labelled cDNAs were performed as described previously [9]. Membranes were washed under stringent conditions ($0.1 \times \text{SSC}/0.1\%$ SDS, 30 min, 65 °C) and exposed to Fuji film at -70 °C with intensifying screens.

Nuclear run-on transcription assay

Nuclei were isolated from adipose tissues of lean and obese Zucker rats aged either 16 or 30 days. Briefly, tissues excised from at least 10 animals of each genotype were homogenized (Homogenizer 43; Virtis, New York, NY, U.S.A.) in ice-cold lysis buffer (5 ml/g) containing 10 mM-Tris/HCl, pH 7.4, 10 mM-

NaCl, 3 mM-MgCl₂ and 0.5% Nonidet P40 (v/v). The crude homogenate was filtered through a 20 μm nylon mesh and nuclei were pelleted twice by centrifugation (500 g, 5 min). The nuclear pellet was resuspended in 5 ml of lysis buffer and was layered on to a 5 ml cushion of glycerol storage buffer containing 50 mM-Tris/HCl, pH 8.3, 40% glycerol (v/v), 5 mM-MgCl₂ and 0.1 mM-EDTA. After centrifugation at 2000 g for 10 min at 4 °C, the purified nuclei were resuspended in 500 μl of glycerol storage buffer, checked under a light microscope and stored at -70 °C in small aliquots. One portion was assayed for DNA content according to Burton [20]. The protocol used for nuclear run-on transcription experiments was exactly as described by Zechner *et al.* [21]. Briefly, 50–100 μg of nuclear DNA was incubated with 100 μCi of [^{32}P]CTP in a total volume of 100 μl for 45 min at 32 °C. Under these conditions, CTP incorporation into labelled transcripts was linear with respect to time and amount of DNA added in the incubation medium. After Proteinase K and DNAase I treatment (1 $\mu\text{g}/\text{ml}$ each), phenol extraction and trichloroacetic acid precipitation, portions containing equal counts [$(1-2) \times 10^6$ d.p.m./ml] of labelled nuclear RNA from lean or obese rats were then used as probes, and were hybridized with plasmids treated for 30 min at 65 °C with 0.4 M-NaOH and immobilized on Zetaprobe membranes (5 μg of DNA per dot). The hybridization mix contained 45% deionized formamide, $4 \times \text{SSC}$, 0.1 M-sodium phosphate, pH 6.5, 0.1% SDS, $1 \times \text{Denhardt's}$ and 100 μg of yeast tRNA/ml. Hybridizations with ^{32}P -labelled nuclear RNA from lean or obese rats were carried out for 3 days at 42 °C in the presence of an equal amount of unlabelled nuclear RNA from the opposite genotype. The addition of unlabelled RNA ensured that the hybridization conditions in both reactions were identical, thus allowing direct comparison of the signals for lean and obese rats. After hybridization, the filters were washed twice in $2 \times \text{SSC}$ at 37 °C, treated with RNAase A (20 $\mu\text{g}/\text{ml}$) for 30 min at room temperature and washed twice in $2 \times \text{SSC}/0.5\%$ SDS at 60 °C, followed by a 30 min wash at 65 °C in $0.1 \times \text{SSC}/0.1\%$ SDS. Filters were exposed to Fuji film at -70 °C, and quantification of autoradiograms was performed by densitometry (Cliniscan).

RESULTS

Developmental changes in enzyme activities

Table 1 shows the effects of the *fa/fa* genotype on LPL, ME, GAPDH and GPDH activities in adipose tissue of both suckling 16-day-old and weaned 30-day-old rats. In 16-day-old suckling obese rats as compared with lean littermates, significantly higher activities can be observed for all of the enzymes except GPDH. At this age, the genotype-mediated differences in enzyme activities, when expressed per mg of protein, reached 2-, 1.8- and 2.8-fold for LPL, ME and GAPDH respectively. In 30-day-old weaned obese rats, a large increase in GPDH specific activity emerged (5.5-fold over the lean values), whereas genotype-mediated differences for ME and GAPDH were dramatically amplified (ME, 16-fold; GAPDH, 5-fold). In contrast, although LPL activity was still higher in 30-day-old obese rats than in lean animals, the difference between the two genotypes remained constant (2-fold) from 16 to 30 days of age.

mRNA levels in adipose tissue of lean and obese Zucker rats

A previously published study [10] of GAPDH mRNA in the adipose tissue of Zucker rats demonstrated increased GAPDH mRNA levels in obese compared with lean rats ranging from 3-fold to 6-fold at 16 and 30 days of age respectively. In the present work, Northern blot analysis of adipose tissue mRNA encoding for LPL, ME and GPDH has been performed in lean and obese Zucker rats aged 16 and 30 days (Fig. 1). It is clear that for all

Table 1. Enzyme activities in adipose tissue of 16- and 30-day-old lean and obese Zucker rats

Results are means $\pm$ S.E.M., with the numbers of animals studied in parentheses. The differences between age-matched lean and obese animals were assessed by Student's *t* test: **P* < 0.05; ***P* < 0.01; ****P* < 0.001. 1 unit of enzyme activity represents 1 μ mol of product formed per min (ME, GAPDH and GPDH) or per h (LPL) at 37 °C. Protein content determinations were performed according to Bradford [31].

Enzyme	Enzyme activity (munits/mg of protein)			
	16 days		30 days	
	Lean	Obese	Lean	Obese
LPL	386 $\pm$ 43 (4)	793 $\pm$ 71 (5)*	547 $\pm$ 83 (6)	1145 $\pm$ 102 (5)*
ME	4.6 $\pm$ 0.5 (4)	7.7 $\pm$ 0.9 (5)**	38 $\pm$ 8 (5)	625 $\pm$ 74 (6)***
GAPDH	81 $\pm$ 17 (9)	224 $\pm$ 32 (17)*	222 $\pm$ 63 (9)	1017 $\pm$ 71 (11)***
GPDH	267 $\pm$ 107 (4)	251 $\pm$ 109 (3)	328 $\pm$ 100 (7)	1798 $\pm$ 581 (8)**

Fig. 1. Northern blot analysis of adipose tissue LPL, ME, and GPDH mRNAs in lean and obese Zucker rats

For 16-day-old rats (a), all the tissues from rats of the same litter were pooled according to the genotype before RNA preparation. For 30-day-old rats (b), individuals were used for RNA extraction. The RNA concentration was determined spectrometrically by measuring absorbance at 260 nm. Total RNA (40–60 μ g) from 16- or 30-day-old lean (lanes 1) and obese (lanes 2) Zucker rats was run, with the amount depending on the age of the animals and the probe used. Human LPL cDNA was labelled to a specific radioactivity of 2×10^9 c.p.m./ μ g of DNA using the multiprime DNA labelling kit from Amersham, and hybridization revealed the presence of a unique 3.5 kb mRNA for adipose tissue rat LPL. Two separate mRNA species (2.1 and 3.1 kb) were observed for ME. GPDH mRNA was 3.5 kb in length. Results are representative of three separate experiments. Hybridization signals obtained for adipsin mRNA (1.1 kb) are shown at the bottom for comparison.

the mRNA species studied, hybridization with specific cDNA probes did not reveal any difference between the two genotypes in the size of the mRNAs. However, important quantitative changes can be observed in the steady-state levels of these mRNAs in adipose tissue of obese compared with lean rats. Thus signals obtained in lean and obese rats for each mRNA were quantified by densitometry, and results were expressed as -fold changes in obese rats versus age-matched lean controls from the same litter taken as a reference. For each age group, mean values calculated from at least three independent experiments are shown in Table 2. During the suckling period, a 5-fold increase in LPL

Table 2. Comparison of genotype-dependent differences in content and relative rates of transcription of mRNAs encoding for ME, GAPDH, GPDH and LPL in adipose tissue

Values are means $\pm$ S.E.M. for genotype-mediated differences in hybridization signals, as determined by densitometric scanning of autoradiograms obtained from either Northern blots or run-on transcription assays. For each enzyme, differences between age-matched lean and obese rats from the same litter were expressed as the fold increase in obese above lean values. At least three independent experiments were considered. ND, not determined.

Enzyme	Relative mRNA abundance (fold increase)		Relative run-on activity (fold increase)	
	16 days	30 days	16 days	30 days
ME	2.3 $\pm$ 0.4	7.9 $\pm$ 1.5	2.6 $\pm$ 0.6	9.2 $\pm$ 3.0
GAPDH	3.1 $\pm$ 0.6	6.0 $\pm$ 0.7	1.8 $\pm$ 0.1	4.7 $\pm$ 1.7
GPDH	1.6 $\pm$ 0.7	6.9 $\pm$ 0.7	1.0 $\pm$ 0.3	6.0 $\pm$ 2.0
LPL	5.0 $\pm$ 0.6	10.7 $\pm$ 3.5	ND	ND

mRNA can be observed in obese rats when compared with age-matched controls. Similarly, the ME mRNA level in adipose tissue from obese rats was 2.3-fold higher than that of lean animals. In contrast, no significant difference between the two genotypes was detected for GPDH mRNA. After weaning (30 days of age), an increase in the GPDH mRNA level emerged in obese rats (7-fold over lean values), and genotype-mediated differences were dramatically amplified for both LPL and ME mRNAs (10-fold and 7.9-fold respectively). These genotype-mediated differences were not due to an overall increase in mRNA in obese rats, since after hybridization of blots with an adipsin cDNA probe (pAD20) no difference between age-matched lean and obese rats was observed (Fig. 1), in agreement with previously published results [22].

Transcriptional activity in lean and obese Zucker rats

Transcription rates for ME, GAPDH and GPDH genes have been compared in lean and obese Zucker rats aged either 16 or 30 days by run-on nuclear assays, as illustrated in Fig. 2. From such experiments, a direct comparison of the hybridization signals between the two genotypes could be performed, since the hybridization efficiency of newly elongated labelled transcripts was equalized in the two genotypes by the addition of the same amount of unlabelled nuclear RNA isolated from the opposite genotype. No difference was observed between the two genotypes using a probe encoding for PEPCK as a positive control (Fig. 2).

Fig. 2. Nuclear run-on transcription assays of adipose tissue from lean and obese Zucker rats

Elongation of *in vivo* initiated RNA transcripts was performed as described in the Materials and methods section. A 5 µg portion of each plasmid was immobilized and hybridized with labelled RNA from lean (upper slots) or obese (lower slots) rats aged either 16 days (a) or 30 days (b). Lanes: 1, ME; 2, GAPDH; 3, GPDH; 4, pBR322; 5, PEPCK. Results are representative of three independent experiments.

Quantitative results obtained by plotting -fold increases in obese versus age-matched lean controls as a reference are presented in Table 2, clearly showing that, at both 16 and 30 days of age, increases in mRNA content for each of the enzymes studied could be fully accounted for by corresponding increases in the relative rate of gene transcription. For LPL, however, it was not possible to compare transcription rates between lean and obese rats, because hybridization signals never reached sufficiently high levels (similar to those obtained with the control pBR322 plasmid). A possible explanation for this is the relatively low identity between the LPL rat adipose tissue mRNA and the human cDNA insert used in this study, leading to a low hybridization efficiency of labelled transcripts despite an extensive incubation period. This possibility is further reinforced by the necessity of using an LPL cDNA probe labelled to a high specific radioactivity to obtain a signal by Northern blot analysis (see Fig. 1 legend).

DISCUSSION

The aim of this study was to elucidate the molecular mechanisms responsible for the high lipid storage-related enzyme activities in adipose tissue from genetically obese Zucker rats. Our data demonstrate that high activities of ME, GPDH and GAPDH in the adipose tissue of weaned obese rats can be fully accounted for by the stimulation of gene transcription. Since a frank hyperinsulinaemia has already developed at 30 days of age in the obese rat [4,10], it is very likely that some of the differences observed between the two genotypes at this age are due to high circulating insulin levels. In agreement with this hypothesis, it has been shown that GAPDH transcription can be stimulated by insulin, and a cis-acting insulin-responsive element has been recently characterized in this gene [23]. In addition, a positive effect of insulin on ME gene transcription, as assessed by nuclear run-on assay, has been reported in the liver of insulin-injected streptozotocin-induced diabetic rats [24]. For GPDH, however, although insulin can potentially stimulate enzyme activity in 3T3-F442A adipocytes [12], the effect of the hormone has been shown to be exerted primarily at the post-transcriptional level, by a stabilizing effect on GPDH mRNA [25,26]. This apparent discrepancy between previously reported effects of insulin on cellular models *in vitro* and our present data obtained *in vivo* on hyperinsulinaemic animals would suggest that, in the genetically obese rat, hyperinsulinaemia alone is not the only stimulus involved in the transcriptional stimulation of GPDH gene expression.

The effect of the fatty genotype was next examined independently of that of hyperinsulinaemia in 16-day-old normo-

insulinaemic *fa/fa* rats. Again, in these animals, the overexpression of GAPDH and ME can be related to increased transcriptional activity. However, the genotype-mediated differences at this age are much more discrete than those observed in weaned 30-day-old rats. This observation confirmed the amplifying effect of hyperinsulinaemia on the expression of these genes.

Our study shows for the first time that a higher content of LPL mRNA can be observed in the adipose tissue of both suckling and weaned obese rats compared with lean ones. However, unlike the other enzymes studied, the magnitude of the increase in enzyme activity in the obese rat is much smaller than the difference in LPL mRNA levels. Such a discrepancy has been previously reported for adipose tissue LPL in the guinea pig, where tumour necrosis factor induced a 55% decrease in LPL activity whereas the extent of its mRNA diminution was only 20% [27]. In the obese Zucker rat we have shown previously [3] that genotype-mediated differences in LPL activity could be related to proportional variations of immunotitrable LPL protein, ruling out the involvement of post-translational events in the stimulation of adipose tissue LPL activity in obese animals. The reason why the increase in LPL activity is lower than that of its mRNA cannot be explained from the present study, since we did not measure the synthesis rate of the enzyme. The question of the insulin effect on LPL expression has been addressed in many studies on different cellular systems and species, and still remains unclear. Increased LPL activities without any change in LPL mRNA have been observed in insulin-treated 3T3-L1 adipocytes, suggesting a post-translational effect of insulin on these cultured cells [28]. In the present work, we show that the genotype-mediated differences in LPL mRNA levels are greatly amplified after the emergence of hyperinsulinaemia, suggesting that insulin could stimulate LPL activity by increasing the steady-state level of its mRNA. This observation is in agreement with recently published data on LPL expression in rat adipocytes showing that insulin could induce changes in LPL mRNA stability [29].

Taken together, our results demonstrate for the first time that ME, GAPDH and LPL are part of a subset of enzymes whose expression is stimulated in the adipose tissue of the genetically obese Zucker rat. To these can be added FAS (results not shown) and the insulin-sensitive glucose transporter GLUT4 [30], which are both overexpressed in the adipose tissue of obese animals. The overexpression of this subset of genes is an early feature of the onset of obesity and is adipose-tissue-specific, since no difference between the two genotypes can be detected in liver [4,10]. In addition, although transcription rates of LPL and GLUT4 have not been measured, our study points out that transcriptional activity of all the other genes examined is the only altered step in the adipose tissue of obese rats. Thus these results argue for the existence of common trans-acting factor(s) in the adipose tissue of genetically obese rats which could trigger the high expression of this subset of genes. One could speculate that such putative factor(s) are under the direct or indirect control of the *fa* gene, since its expression appears to be independent of hormonal changes such as hyperinsulinaemia.

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