Differential polypeptide expression in adipose tissue of lean and obese Zucker rats

Evidence of specifically repressed peptides in 7-day-old pre-obese rats

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Using two-dimensional electrophoresis on total extracts of adipose tissue from young lean (*Fa/fa*) and obese (*fa/fa*) Zucker rats, we have investigated the existence of early events at the protein level, before obvious obesity. Our results indicate that the two genotypes do not differ at 3 days of age in terms of polypeptide pattern. By 7 days of age, two polypeptides are transiently repressed in the fatty genotype, leading us to suggest their potential involvement in the onset of obesity. However, most of the differences between the lean and obese rats are detected at 30 days of age, characterized by an increase in the accumulation of several peptides in the adipose tissue of obese rats, in good agreement with the multiple biochemical changes previously identified at this stage of the disease. These results present evidence of new peptides that may be of interest in the study of the obesity syndrome.

INTRODUCTION

The genetically obese Zucker rat (*fa/fa*), first described by Zucker & Zucker in 1961 [1], bears an autosomal recessive mutation, the *fa* gene, which is as yet unidentified. Adult animals homozygous for the *fa* gene exhibit massive obesity, sharing many traits in common with the human syndrome. This animal model can thus provide useful information for a better understanding of the etiology of the human disorder, in which the implication of genetic factors has become increasingly clear [2]. This genetic lesion has a considerable impact on adipose-tissue development in the mutant rat; however, it is not known whether a key regulatory pathway in this tissue is involved, or whether obesity results from an altered stimulation of the tissue by hormonal or growth factors. In rodents, the development of adipose tissue takes place during the first 2 days of postnatal life, concomitantly with an increase in circulating triacylglycerols provided by the mother's milk [3]. At that time, no difference between pups bearing the *fa/fa* genotype and their lean littermates can be detected with regard to either adipose-tissue storage capacity or circulating nutrients and hormones. The very first phenotypic expression of the mutation becomes manifest in adipose tissue after the first week of life. Then, although *fa/fa* pups are still visually indistinguishable from *Fa/fa* controls, a significant increase in activity of lipid-storage-related enzymes emerges in these pre-obese animals, leading to fat-cell hypertrophy [4]. These early genotype-mediated differences arise without any detectable change in food intake [4] or insulin levels [5] between the two genotypes, leading to the hypothesis that adipose tissue could constitute a primary site of expression of the genetic lesion in the mutant rat. Lastly, the full expression of the obese phenotype develops around weaning and is associated with the emergence of secondary aggravating disorders such as hyperphagia [6], hyperinsulinaemia and hyperlipogenesis in liver [5].

Although our present understanding of the role of these secondary alterations in increasing the severity of obesity has progressed significantly, reasons why metabolic activity of adi-

pose tissue is precociously altered towards fat storage in the mutant rat remain totally unknown. This prompted us to assess the genotype effect on the overall pattern of adipose-tissue proteins. The question was addressed by using two-dimensional gel electrophoresis, which could provide some insight into the magnitude of changes in adipose-tissue proteins between the two genotypes, and possibly lead to the identification of genotype markers. The present study was carried out on total adipose-tissue extracts of young *Fa/fa* and *fa/fa* rats, and the polypeptide maps were compared between the two genotypes at 3, 7 and 30 days of age, corresponding to different stages of the expression of the obesity syndrome. Several changes were observed in the accumulation of some peptides at 30 days of age in the obese rats; these might be correlated with the general metabolic dysfunctioning characteristic of the obese state. The absence of any strong difference between the two genotypes as early as 3 days of age led us to conclude that no abnormality of gene expression occurs in the adipose tissue at this early stage. An interesting feature is, however, pointed out in this study: the specific repression of two peptides in the 7-day-old pre-obese rats suggests the existence of transitory regulatory factors that might play a crucial role in the evolution of this genetic disorder.

EXPERIMENTAL

Animals and tissues

Obese (*fa/fa*) rats and their lean littermates were bred in our laboratory from pairs originally provided by the Harriet G. Bird Memorial Hospital, Stow, MA, U.S.A. Known heterozygous lean (*Fa/fa*) females and obese (*fa/fa*) males were mated. From this mating 50% of the litter is expected to be obese and 50% lean and of the heterozygous genotype. Since there is no rapid way to identify pup genotypes during the first 2 weeks of life, a biopsy of one whole fat-pad was removed under light diethyl ether anaesthesia at 3 or 7 days of age, immediately frozen in liquid N₂ and kept at -80 °C until pups reached 6 weeks of age and genotype identification could be performed. Adipose fat-

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Table 1. Adipose tissue and body weights of animals used in experiments

Values are means $\pm$ S.E.M. Differences between age-matched lean and fatty rats were assessed by Student's *t* test: * *P* < 0.05; ** *P* < 0.01; *** *P* < 0.001.

Age	Body wt. (g)		Adipose-tissue wt. (mg)	
	Lean	Fatty	Lean	Fatty
3	7.8 $\pm$ 0.16	8.3 $\pm$ 0.10	25.0 $\pm$ 1.40	29.0 $\pm$ 1.50
7	10.4 $\pm$ 0.19	11.0 $\pm$ 0.50	32.7 $\pm$ 2.11**	48.9 $\pm$ 3.68**
30	49.3 $\pm$ 1.34*	54.6 $\pm$ 1.68*	666 $\pm$ 78***	1884 $\pm$ 119***

pads were also removed from 30-day-old rats after decapitation, and the genotypes were immediately identified on the basis of adipose-tissue weight. Tissues were frozen and stored at -80°C . Storage at -80°C over these time periods did not affect the results obtained.

Two-dimensional gel electrophoresis

Frozen tissues from individual rats (30 days of age) or pooled from 3–5 pups of the same litter (3 and 7 days of age) were pulverized and homogenized in a boiling solution (100 mg/1.2 ml) of 0.3% SDS, 1% β -mercaptoethanol and 50 mM-Tris, pH 8, for 4 min. The homogenates were cooled on

ice for 5 min and centrifuged for 10 min at 10000 *g* to remove any tissue fragments and supernatant fat cake. The clear infranatants were then removed, and mixed with 0.1 vol. of a solution of 1 mg of DNAase/ml and 0.5 mg of RNAase/ml. The extracts were immediately frozen in liquid N_2 and freeze-dried. Dried samples were resuspended in 9.95 M-urea/4% Nonidet P40/2% Ampholines (LKB) pH 5–7/100 mM-dithiothreitol at a protein concentration of 10 mg/ml, as assessed by the Bradford technique [7], and stored at -80°C . A 30 μl portion of the tissue extract was loaded on to the isofocusing gel (pH gradient 5–7). Two-dimensional PAGE (12.5% acrylamide) was carried out as previously described [8,9]. Preliminary experiments led us to select these conditions (pI and M_r ranges) to detect the largest possible number of well-resolved polypeptides. The simultaneous migration of 12 extracts allowed a high degree of reproducibility from one gel to another. To increase the sensitivity of detection, the proteins were silver-stained as described by Morrissey [10]. M_r values of the observed proteins were determined by comparison with M_r markers applied to a slot on the same gel. This technique allowed us to detect about 500 spots for each adipose-tissue extract. Evaluation of differences between the two genotypes were performed by comparing the polypeptide maps of age-matched *Fa/fa* and *fa/fa* littermates, in three independent experiments for each age group. Differences between the two genotypes were taken into account when found reproducibly in the three experiments.

Fig 1. Two-dimensional PAGE of adipose-tissue extracts of 30-day-old lean (a) and fatty (b) rats

M_r standards are indicated on the right-hand side of the Figure. The position of actin is indicated for reference. Large arrowheads show genotype-mediated differences. Note that spot E is specific to the 7-day-old animals; its position is, however, indicated for completeness. Abbreviations: IEF, isoelectric focusing; SDS, SDS/PAGE.

Fig 2. Two-dimensional PAGE of adipose-tissue extracts of 7-day-old lean (a) and fatty (b) rats

Differences previously observed in older animals are indicated as described in Fig. 1. Note the presence of spot E, which was not expressed at 30 days of age either in lean or fatty rats. Several peptides other than those selected were differentially expressed in lean or obese pups by this age. These differences were not taken into account, since they were not consistently observed in all three experiments. Abbreviations as in Fig. 1.

RESULTS AND DISCUSSION

Since we were attempting to characterize polypeptide changes related to early expression of the genetic obesity of the Zucker rat, we studied adipose-tissue extracts obtained from the subcutaneous inguinal area, the first adipose site to develop in newborn rats. Some characteristics of the animals used in this study are shown in Table 1, pointing out that the *fa/fa* genotype was undetectable at 3 days of age, when no difference between *Fa/fa* and *fa/fa* pups could be observed either in inguinal adipose-tissue weight or in total body weight. In contrast, by 7 days of age, although the obese phenotype was not visually apparent, a significant increase (67%) in inguinal adipose-tissue weight can be detected in the *fa/fa* pre-obese pups as compared with the *Fa/fa* lean controls, with no change in total body weight. This higher fat accretion in *fa/fa* rats became markedly evident during the suckling-to-weaning transition, leading to visually detectable obesity at 30 days of age. At that time, dramatic alteration in body composition had developed, obese animals exhibiting an increase in inguinal adipose-tissue weight reaching 3-fold that of the lean animals.

Two-dimensional PAGE of adipose tissue extracts revealed, as expected, a great similarity between the two genotypes for each age studied. Indeed, since this technique allows the detection of hundreds of gene products, and since only one gene is primarily

affected in this mutation, the similarity found in this study between the two genotypes is not surprising.

No qualitative difference could be detected in the polypeptide composition of the adipose-tissue extracts from both genotypes. However, several polypeptides showed differences in their pattern of accumulation according to the age of the animals.

Major polypeptide changes in 30-day-old obese animals (Fig. 1)

Two-dimensional electrophoresis of total adipose tissue extracts of 30-day-old lean and obese Zucker rats revealed five major changes and three minor changes in the accumulation of several peptides between the two genotypes. Major changes occurred in polypeptides D, F, G, H and I, of M_r 24000, 23000, 19000, 18000 and 14500 respectively. The amounts of these proteins were dramatically increased in fatty rats compared with lean ones (Fig. 1), especially for peptides G, H and I. Minor changes affected polypeptides A, B and C, of M_r 41000, 40000 and 37000 respectively. The amounts of these proteins were indeed increased in the obese rats, but to a lesser extent than for those cited above.

At 30 days of age, the obese phenotype had emerged and numerous alterations have been reported in adipose tissue [11], which can be related to the higher fat-storage capacity of the fatty genotype. These mainly involve the expression of the genes encoding the enzymes of the lipogenic pathway (lipoprotein

Fig 3. Two-dimensional PAGE of adipose tissue extracts of 3-day-old lean (a) and fatty (b) rats

Arrowheads indicate peptides which were different in older animals. Note that no difference can be detected at this age.

lipase [12], fatty acid synthetase and malic enzyme [5], glycerophosphate dehydrogenase [11] and of glycolysis (glyceraldehyde-3-phosphate dehydrogenase [13]), and the insulin-sensitive glucose transporter [14], which is higher in the obese rats compared with controls. The obesity of the 30-day-old Zucker rat is complicated by secondary aggravating disorders, especially hyperinsulinaemia, all together contributing to the higher rate of fat accretion in hypertrophied adipose cells. In such an altered metabolic situation, our finding of the accumulation of at least eight polypeptides in adipose tissue of obese rats is thus not surprising. In terms of M_r , spot C (37000) might be tentatively identified as glycerophosphate dehydrogenase, which has been shown to be strongly induced in adipose tissue of obese Zucker rats [11]. In contrast, no speculative identification can be made for peptides A, B, D, F, G, H and I, since their M_r values do not correspond to those of any previously reported enzymes whose activity increased in obese rats. However, the two-dimensional gels presented here do not resolve proteins of M_r greater than 200000. They thus exclude several proteins known to be induced in obese Zucker rats, such as fatty acid synthetase and acetyl-CoA carboxylase [11]. In the same manner, all proteins whose pI values are lower than 5 or greater than 7 are also excluded in our study. This is the case for lipoprotein lipase, whose pI is 4.5, and protein S14, pI 7.3, which has been well characterized by two-dimensional PAGE as being induced co-ordinatedly with lipogenic enzymes in liver and adipose tissue [15]. To summarize, despite the underestimate of the total number of changes in proteins between lean and obese rats, our finding that major

differences occur at 30 days of age is compatible with the multiplicity of biochemical alterations that have been previously identified at this age.

We next investigated whether some of the peptide changes observed in the fully developed obesity of 30-day-old mutant rats could be detected during the earlier stages of the onset of obesity. We thus examined polypeptide maps of adipose-tissue extracts obtained from younger animals (7 days of age).

Major polypeptide changes in 7-day-old obese pups (Fig. 2)

Among peptides which were over-expressed in the obese rats at 30 days of age, all except peptide G were expressed at 7 days. Polypeptides A, B, C, F, H and I were detected in equivalent amounts in both genotypes (Fig. 2), strongly suggesting that their accumulation in the obese rat occurs as a consequence of hormonal and nutritional alterations of the fully developed syndrome. The absence of any over-expressed peptides in the 7-day-old obese pups compared with lean ones does not contradict results of previous studies [12], which showed higher amounts of some lipid-storage-related proteins at this age. These changes are probably too faint to be detected under our conditions. Interestingly, two spots were found to be strongly repressed in the 7-day-old *fa/fa* pups (Fig. 2b) as compared with *Fa/fa* ones (Fig. 2a). These are peptides D and E, of M_r 24000 and 23000 respectively. The former was over-induced in the 30-day-old obese rats; conversely, the latter was not detectable at this age in either genotype (Fig. 1). These peptides could be good candidates for early targets of the expression of the *fa* gene in adipose tissue;

however, the possibility that the differential expression of these peptides reflects changes in the rate of development of adipose tissue between the two genotypes cannot be discounted. We have recently detected the presence of peptide E in an obese rat aged 10 days, while spot D was still repressed (results not shown). This would thus suggest a delay in the expression of the peptide E in the mutant rat, which is surprising, considering the overdevelopment of adipose tissue characteristic of the obese state. Nevertheless, the transitory repression of peptides D and E could drive further important changes in adipose tissue towards excessive lipid storage. Since we studied total adipose tissue rather than isolated adipocytes, the possibility that the differences observed could be due to non-adipocyte cells has to be mentioned. However, previous experiments have shown that, until 30 days of age, adipose tissue from both genotypes displays the same amount of adipocyte and non-adipocyte cells [16]. In order to determine whether these peptides were involved in prior events to the onset of obesity, we further investigated the adipose tissue of 3-day-old animals.

No polypeptide change in 3-day-old *Fa/fa* and *fa/fa* pups (Fig. 3)

At this age, polypeptide patterns in *Fa/fa* and *fa/fa* pups exhibited strikingly strong similarity; indeed, it was not possible to detect any difference between the two genotypes (Fig. 3). Moreover, none of the differences observed at 7 and/or 30 days of age were observed at this earlier stage. Peptides D and E were undetectable in both lean and pre-obese pups, as well as peptide G. The lack of polypeptide abnormality in adipose tissue of 3-day-old *fa/fa* rats raises the question of the adipose tissue as a primary site of expression of the genetic lesion in the Zucker rat. However, since 3 days of age corresponds to the very early stage of adipose-tissue development in the rat, it cannot be excluded that the expression of the *fa* gene could require a certain degree of tissue differentiation.

General conclusions

To our knowledge, two-dimensional electrophoresis has never been used to analyse adipose tissues from genetically obese animals. This original approach reveals many major changes at the protein level which can be observed once the obese syndrome

had developed (30 days of age). Conversely, it does not demonstrate any precocious change in the pattern of polypeptide accumulation during the first 3 days of tissue development.

Our finding concerning the two peptides which are strongly repressed in the mutant rats at 7 days of age, concomitant with the emergence of the increased fat-storage capacity, could be of interest. It suggests the occurrence of a specific defect in the young *fa/fa* rat; one could assume that these repressed peptides may be involved in the onset of obesity. Nevertheless, since these repressions are transient, there is no evidence of their direct relation to the primary genetic defect. Further investigations would be necessary to characterize these peptides and clearly establish their physiological importance in the development of the adipose tissue.

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