Impairment of Adipsin Expression Is Secondary to the Onset of Obesity in db/db Mice*

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The nature of the primary biochemical lesions in genetically obese mice, which might prove to be useful models for human obesity, remains totally obscure. The recent finding that the expression of adipsin was virtually suppressed in both db/db and ob/ob adult mice has opened new perspectives, suggesting a potential role for this defect in the pathogenesis of obesity. To be of etiological significance, adipsin deficiency must be present very early in life when excess fat storage starts to develop. We show here that at 10 days of age db/db pups exhibit significantly overdeveloped adipose tissue as compared with lean (+/db) pups but similar levels of both adipose tissue adipsin mRNA and serum adipsin. Adipsin expression was still normal in obese mice 15 days old but frankly deficient at 30 days of age, when hyperinsulinemia has developed. Thus the defect in adipsin expression in db/db mice is a secondary feature which cannot be ascribed a role in the onset of obesity.

Progress in the elucidation of the biochemical lesions of hereditary obesity in animal models might provide useful working hypotheses for the investigation of this disease in man where genetic factors play a determinant role (1-3). A provocative finding with potentially great implication for the understanding of this disorder was that the expression of adipsin mRNA was suppressed (>100-fold) in two strains of genetically obese mice, ob/ob and db/db (4). Adipsin is a recently discovered circulating glycoprotein of the serine protease family, which is predominantly synthesized and secreted by adipose tissue (5). This protein, the expression of which shows marked changes in pathophysiological states associated with variations in adipose tissue mass, presents the properties of a putative systemic regulator of energy balance (4). The severity of the defect in adipose tissue expression in obese mice suggests that this lesion could be of crucial importance in the obese phenotype expression. A major question to answer toward the elucidation of the role of adipsin impairment in the etiology of genetic obesity is whether the suppression of adipsin expression, which was discovered in adult mice (4), is primary or secondary to the onset of obesity. We therefore examined the levels of adipsin mRNA in adipose tissue and adipsin in serum from db/db mice at 10 days of age when obesity emerges and at 30 days of age when the syndrome has fully developed.

**EXPERIMENTAL PROCEDURES**

*Animals*—db/db obese mice and their lean littermates were bred in our laboratory from pairs of C57BL/KS +/+ mice, originally provided by Jackson Laboratory (Bar Harbor, ME). From this mating three genotypes (+/m+/m, homozygous lean; +/db+, heterozygous lean black pups; +/db+, homozygous obese black pups) were obtained in the progeny in the proportion of 1.4, 1.2, and 1.4, respectively. Only black lean (+/m/db+) or obese (+/db/db+) pups were used in this study. Animals were kept at 22°C in rooms exposed to a 12-h dark-light cycle. They were routinely weighed at 28 days of age and used in the fed state. At 10 days of age, pups were weighed, lightly anesthetized with diethyl ether, and used either for lipectomy of an entire inguinal fat pad (the only adipose tissue to be developed at this age) or for blood withdrawal from the jugular vein. Sera and tissues were kept at -80°C and processed 5 weeks later when obesity has developed, allowing identification retrospectively of the genotypes of the pups. Older (15- and 30-day-old) pups were killed by decapitation; blood was collected and inguinal adipose tissue removed. In 15-day-old pups, the genotype was diagnosed by plotting adipose tissue weight versus body weight according to the method previously described for 16-day-old Zucker rats (6). In 30-day-old pups, the genotype was diagnosed on the basis of fat pad weight.

*Insulin Determination*—Insulin was measured in duplicate by radioimmunoassay (Sorin CEA, France) on 50 μl of serum obtained by pooling sera from several pups of the same genotype, except for mice 30 days old where insulin determination was performed on individual mice.

*Northern Blot Analysis*—Adipose tissue total RNA was extracted as described previously (7) by using the guanidinium isothiocyanate/LiCl procedure (8) and was size-fractionated on a 1.5% agarose/formaldehyde gel (20 μg/lane). The amount of RNA/lane was identified as ethidium bromide staining of the 28 and 18 S ribosomal RNA after gel electrophoresis. After transfer to nylon membranes (Hybond N, Amersham Corp.), the blots were probed with the 32P-labeled cDNA insert (pAd20) encoding for adipsin (9), a gift from H. Green (Harvard Medical School, Boston, MA). Relative quantitation of the signals was assessed by densitometric scanning of autoradiograms.

*Western Blot Analysis*—Sera from individual mice were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10) electroblotted to nitrocellulose and probed using 1/100 dilution of an antibody raised against a 14-amino acid peptide from adiponectin (11). Detection was performed using [125I]-labeled donkey anti-rabbit IgG (Amersham Corp.). Quantitation was by counting of the appropriate bands excised from the blots and subtraction of background.

**RESULTS AND DISCUSSION**

The genotype identification was not possible in 10-day-old pups since there is no known db/db genotype marker. Therefore, adipose tissue and blood had to be obtained through surgery in order to keep the pups alive for later genotype identification. Table I shows that obesity in db/db mice is of very early onset since at 10 days of age, inguinal fat pad weights were already increased by 66% in db/db mice as compared with lean mice. A slight (7%) increase in body weight was also observed in obese mice, likely the result of a general overdevelopment of adipose stores. However, no change in insulinemia could be detected between lean and obese pups at this initial phase of the syndrome (Table I). By 30 days of age, obesity has markedly developed in the db/db mice, which have fat pads that are twice those of lean mice and display a severe hyperinsulinemia (Table I).

Northern blot analysis of total adipose tissue RNA provided...
Unimpaired Adipsin Expression in Preobese db/db Mice

Table I

Body and inguinal fat pad weights, and insulinemia in lean (+/db) and obese (db/db) mice

Values are expressed as means ± S.E. with the number of independent determinations in parentheses. Statistical differences between age-matched lean and obese mice were estimated by Student's t test: *, p < 0.05; ***, p < 0.001. See "Experimental Procedures" for animal description.

|                | 10 days* | 15 days* | 30 days* |
|----------------|----------|----------|----------|
| Lean (g)       |          |          |          |
| Body weight    | 5.2 ± 0.06 | 5.6 ± 0.10 | 7.1 ± 0.30 |
| Inguinal fat pad weight (mg) | 27.1 ± 0.93 | 44.3 ± 1.74 | 101 ± 12 |
| Insulin (microunits/ml) | 40.2 ± 6.2 | 40.1 ± 4.8b | 31.2 |
| Obese (g)      |          |          |          |
| Body weight    | 5.5 ± 0.08 | 5.8 ± 0.20 | 7.2 ± 0.40 |
| Inguinal fat pad weight (mg) | 37.8 ± 0.99 | 52.3 ± 1.78 | 107 ± 13 |
| Insulin (microunits/ml) | 50.2 ± 6.5 | 50.1 ± 4.8b | 31.2 |
| * Age.         |          |          |          |
| # Mean of 2 values ± one-half of the range. |
| $ Unique determination. |

Fig. 1. Expression of adipsin mRNA in adipose tissue from lean (+/db) and obese (db/db) mice at 10 or 30 days of age. Animals are the same as those described in Table I. Total RNA were extracted from inguinal fat pads pooled from 4 to 12 pups at 10 days of age or from individual mice at 30 days of age. 20 µg of total RNA were electrophoresed per lane. This autoradiogram is representative of three independent experiments.

Fig. 2. Western blot analysis of adipsin in sera from obese db/db mice (lanes db) and lean +/db mice (lanes +) at 10 or 15 days of age. See Table I for animal description. Each sample (10 µl of serum) represents a single mouse. Antiserum was used after prior absorption with excess peptide 2 (6) in lanes "immune + excess peptide." The migration of molecular weight markers is indicated on the left. This autoradiogram is representative of three independent experiments.

Fig. 3. Western blot analysis of adipsin in sera from obese db/db mice (lanes db) and lean +/db mice (lanes +) at 30 days of age. 5 µl of serum from individual mice were loaded in each lane. Animals were the same as those in Table I. This autoradiogram is representative of three independent experiments.

Evidence that, in 10-day-old mice, adipsin mRNA was readily detected in both lean and obese animals and expressed at similar levels in the two genotypes (Fig. 1). In sharp contrast, there was a dramatic (88%) reduction in adipsin mRNA in obese as compared with lean mice at 30 days of age (Fig. 1). However, due to the higher (2.3-fold) RNA content per fat pad in obese than in lean mice at this age, adipsin mRNA abundance per fat pad was reduced by only 71% in obese as compared with lean mice. Adipsin mRNA was virtually absent in 2-month-old obese mice (data not shown). Fig. 1 also demonstrates that there is a differential regulation of adipsin expression with age in the two genotypes. A severalfold increase in adipsin mRNA occurred between 10 and 30 days of age in lean mice whereas a decline was observed in obese mice. When normalized for the amount of total tissue RNA, the levels of adipsin mRNA would be increased by 25-fold in lean against 4-fold in obese mice between 10 and 30 days of age. The conclusion that the defect in adipsin expression in db/db mice developed secondarily to the onset of obesity was further supported by our data on the levels of circulating adipsin. As shown in Fig. 2 it is clear that serum adipsin concentrations were essentially identical in lean and obese pups at 10 days of age and still at 15 days of age in spite of a marked overdevelopment of adipose tissue in obese pups (Table I). In contrast, in postweaning 30-day-old mice, a large (65%) decrease in circulating adipsin levels was observed in obese as compared with lean mice (Fig. 3), in close quantitative agreement with the reduction in adipsin mRNA per total fat pad.

All together our data demonstrate that adipsin expression is not impaired in the young suckling db/db pup at the time when the obese phenotype is emerging. This argues against a role for adipsin deficiency in the induction of the array of metabolic abnormalities involved in the initial phase of adipose tissue overdevelopment. Adipsin’s impairment is rather a secondary feature brought about by the neuroendocrine factors that develop in the postweaning obese mice. One likely candidate is the severe hyperinsulinemia of these animals. This is supported by the recent finding that insulin exerted a...
negative effect on the levels of adipsin mRNA in 3T3-F442A adipocytes by decreasing both adipsin gene transcription and adipsin mRNA stability (11). Glucocorticoids might also be involved in the suppression of adipsin expression in these genetically obese mice. Adrenalectomy has been shown to increase adipsin gene expression in adult ob/ob mice, whereas corticosterone injections to lean mice did the opposite (12). However, if it has been well documented that plasma corticosterone concentrations are elevated in adult obese db/db mice (13, 14), there is no data in the literature to support that this abnormality is already present in young db/db mice 30 days old.

In conclusion, the present work demonstrates that adipsin deficiency is a distal effect of the db mutation that is not required for obesity to develop. Its interrelation with the numerous secondary complications of the full syndrome remains to be established.

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