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Physiologic and Endocrinologic Characterization of Male Sex-Biased Diabetes in C57BLKS/J Mice Congenic for the *fat* Mutation at the Carboxypeptidase E Locus

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The *fat* gene in mice represents a recessive mutation at the carboxypeptidase E (*Cpe*) locus. The mutant allele (*Cpe^{fat}*) encodes a highly unstable enzyme and produces an obesity phenotype characterized by attenuated processing of prohormones such as proinsulin that require this exopeptidase for full maturation. This article presents a preliminary physiologic and endocrinologic characterization of the stock of C57BLKS/LtJ-*Cpe^{fat}/Cpe^{fat}* mice at the backcross generation (N10) currently distributed by The Jackson Laboratory. Although previously reported not to be diabetogenic at N5, an additional five backcrosses to the C57BLKS/J background resulted in a male-biased development of both obesity and diabetes. Major differences distinguishing this mutant stock from the phenotypes produced by either the diabetes (*Lepr^{db}*) or obese (*Lep^{ob}*) mutations on the same inbred strain background are lack of hyperphagia and hypercorticism, sensitivity of diabetic males to exogenous insulin, and a milder and male-biased diabetes syndrome that is not associated with widespread β-cell necrosis and islet atrophy, and that often remits with age.

Key Words: Mice; carboxypeptidase E; obesity; diabetes.

Introduction

Noninsulin dependent diabetes mellitus (NIDDM) is a complex, multigenic disorder, which leads to development of overt hyperglycemia late in life. NIDDM is often presaged by obesity and insulin resistance. Several mouse models of hereditary obesity have been developed to study the genetics of susceptibility to NIDDM. These models have proven useful in elucidating particular pathways of the disease process *(1)*. For example, two unlinked autosomal recessive obesity mutations, diabetes (*db*, Chromosome 4) and obese (*ob*, Chromosome 6), produce comparable syndromes of severe insulin-resistant diabetes on the diabetes-susceptible C57BLKS (BKS) background *(2)*. That both mutations produce an identical phenotype in BKS mice suggests that each mutation might exert obesity-induced diabetogenic stress ("diabesity") via a common pathway. This was supported by the recent molecular findings that *db* is a mutation in the leptin receptor (and is now designated *Leprdb*) *(3,4)* and that *ob* is a mutation in the leptin ligand (now designated *Lepob*) *(5)*.

A more recently discovered mutation, fat (*fat*, Chromosome 8), was initially found not to be diabetogenic after five cycles of backcrossing from the HRS/J strain of origin onto the BKS background *(6)*. This apparent lack of diabetogenicity indicated that the mutation and the obesity it produced were not associated with leptin signaling through its receptor. Indeed, initial analysis of the mutation on the BKS background showed it to be distinct from the "diabesity" syndrome elicited by either *Leprdb*, or *Lepob*. Obesity was not detected until after puberty and virilization of hepatic sex steroid metabolism, assumed to be diabetogenic in BKS-*Leprdb* and BKS-*Lepob* females, was not found in the BKS females homozygous for the *fat* mutation at backcross five *(7,8)*. Indeed, molecular analysis has shown these mutant mice express a variant allele (designated *Cpefat*) in the structural gene for carboxypeptidase E, an enzyme required for normal processing of a variety of neuroendocrine and endocrine proproteins, including proinsulins I and II *(9)*. As a consequence of a missense mutation, in which a proline has been substituted for a serine at position 202, the enzyme is incorrectly folded such that it fails to transit from the rough endoplasmic reticulum into the regulated secretory pathway, and is rapidly degraded *(9–11)*. Although HRS-*Cpefat*/*Cpefat* mutant mice were assumed to be insulin resistant because of their early-developing and extreme "hyperinsulinemia" *(6)*, the molecular nature of the genetic defect indicated that what was originally measured as serum insulin was,

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in fact, predominantly proinsulins I and II along with incompletely processed conversion intermediates *(9)*. Proinsulins have only a fraction of the biologic potency of insulin. Therefore, a disturbed ratio of inactive to active insulins in pancreatic islets cells and in circulation characterizes these mice *(9)*. Thus, it seemed likely that development of obesity in these mutant mice occurrs via metabolic perturbations quite distinct from those occurring in response to the *Leprdb* and *Lepob* mutations.

The present report presents a physiologic and endocrinologic characterization of the 10th generation backcross stock of BKS/Lt-*Cpefat* mice. This updated characterization is important for several reasons. First, the diabetes status of the current stock differs substantially from the 5th backcross stock analyzed previously *(6–8)*. Specifically, further backcrossing of the *Cpefat* mutation onto the BKS background has eliminated HRS/J-derived genes protecting males against diabetes development that apparently were still present after only five backcrosses. This loss of as yet unidentified alleles from HRS was evidenced between the 5th and 9th backcrosses by the increasing severity and frequency of hyperglycemia primarily in *Cpefat*/*Cpefat* males, and occasionally in mutant females. Since the 10th backcross stock was sent from our research colony to the Animal Resources Unit of The Jackson Laboratory for distribution, it is important to define the characteristics of the current BKS-*Cpefat*/*Cpefat* mutant stock, and to contrast these characteristics associated with pathogenesis mediated by the two other diabetogenic mutations producing severe insulin resistance on the same background, *Leprdb* and *Lepob*.

Materials and Methods

Mice

The mutant *Cpefat* allele was originally transferred from the HRS/J inbred strain onto the BKS inbred strain background by outcross and four backcross cycles *(6)*. The current BKS-*Cpefat* congenic stock represents five additional backcross generations (N10) to the BKS/JLt, a substrain separated from the BKS/J production colony in the Animal Resources Unit of The Jackson Laboratory prior to 1988. The sex of heterozygous BKS-*Cpefat*/+ mice used for backcrossing was alternated to insure that both sex chromosomes were derived from BKS. Since *Cpefat* homozygotes do not breed, heterozygotes were identified by PCR typing (from tail snip DNA prepared at 3 wk of age) for two simple sequence repeats (*D8Mit69* and *D8Mit131*, Research Genetics, Huntsville, AL) distinguishing HRS genome proximal and distal to the *Cpefat* locus from the respective BKS alleles flanking the wild-type *Cpe* locus *(9)*. More recently, the genotypes were determined by allele specific PCR which distinguishes the *Cpefat* from the wild-type allele on agarose gel electrophoresis *(12)*.

At N10, +/*Cpefat* heterozygotes were intercrossed to produce both homozygous mutants as well as lean control mice, heterozygous and homozygous for the wild-type allele. This stock has been sent to the Animal Resources Unit of The Jackson Laboratory for distribution. BKS-*Leprdb* and C57BL/6 (B6)-*Lepob* homozygotes were obtained from the Animal Resources Unit of The Jackson Laboratory. All strains were provided autoclaved chow (Diet 96WA, 6% fat, Emory Morse Co., Guilford, CT) and acidified water *ad libitum*. Temperature (23 ± 2°C), humidity (55 ± 5%), and light cycle (12 h light to 12 h dark) were strictly controlled.

Serum Parameters

Mutant mice and controls (both +/*Cpefat* and +/+) were weighed and plasma glucose (PG) monitored using a glucose analyzer (Beckman Instruments, Palo Alto, CA) biweekly beginning at 4 wk of age. Blood samples for PG, thyroid hormone (T4), insulin, corticosterone and glucagon testing were obtained from the retro-orbital sinus. Samples for PG, insulin, and glucagon were drawn from the same cohort of mice between 7 and 9 AM. Samples for thyroid testing were drawn from separate cohorts between 9 and 12 PM, whereas samples for corticosterone testing were also drawn from separate cohorts and collected under quiet conditions between 6 and 7 AM. Plasma proinsulins/insulins (not separated) and glucagon were determined by radioimmunoassay (RIA) using kits from Linco Research (St. Charles,MO). Rat insulin was used as a standard for insulin RIA. Corticosterone and and thyroxine (T4) were also determined using RIA kits (ICN Biomedicals Inc., Costa Mesa, CA). Insulin-like Growth Factor-1 (IGF-1) levels were determined in neutralized aliquots of acid-ethanol extracted serum samples. Anti-IGF-1 (BB#-189) was obtained through the National Hormone and Pituitary Program; rHIGF-1 (Bachem, Torrance, CA) was used as standard, and 125I-labeled IGF-1 was obtained from Amersham (Arlington Heights, IL).

Leptin Sensitivity

Leptin sensitivity of 12-wk-old *Cpefat* /*Cpefat* females was tested by treatment with recombinant leptin (kindly provided by PeproTech, Inc (Rocky Hill, NJ]. Ten mg of leptin was reconstituted in 0.9% saline, pH 9.0, to a concentration of 1 mg/mL. The pH was then lowered to 7.5 by addition of 1N HCl. A group of five 20-wk-old females were dosed with 5 μg leptin/g body weight by intraperitoneal injection once daily for 14 d. Five 8-wk-old *Cpefat*/*Cpefat* females (younger and therefore smaller than the leptin treated group) were treated daily with the saline vehicle. These younger mice were in the phase of rapid weight gain, thereby serving as a negative control for handling/injection stress. As positive controls, leptin deficient B6-*Lepob*/*Lepob* and leptin-sufficient B6- +/*Lepob* lean controls received the same treatment. Body weight and food consumption were monitored daily, whereas PG was determined once weekly.

Dexamethasone Treatment

BKS females homozygous for *Cpefat* and BKS-+/+ controls were treated with either 0.5 mg dexamethasone (dex) pellets or a placebo pellet (Innovative Research of America, Toledo, OH). Pellets were surgically implanted subcutaneously at the base of the neck. Body weight and PG were then obtained at d 0,3,7,10, and 14. To assess dex effects on induction of hepatic estrogen sulfotransferase (EST) activity, crude activities were assayed in liver homogenates from BKS-*Cpefat* /*Cpefat* and +/+ controls, as described previously, at both limiting (0.2 μ*M*) and saturating (10 μ*M*) concentrations of estrone *(8)*.

Insulin Sensitivity

Purified porcine insulin (Velosulin, Novo Nordisk Pharmaceuticals, Princeton, NJ), or normal saline as control was delivered continuously via subcutaneous implants of ALZET minipumps (model 2001, ALZA Scientific Products, Palo Alto, CA). Insulin was delivered at a rate of 0.5–1 U insulin/d for 7 d to *Cpefat* /*Cpefat* and +/*Cpefat* control males. Body weight and PG were determined at d 0, 3, and 7 as described above.

Histology

Upon necropsy, pancreases were harvested and placed in Bouin's fixative for 24 h. The tissue was then embedded in paraffin, cut in 5-μm sections, and granulated beta cells enumerated by staining with aldehyde fuchsin with hematoxylin and eosin counterstaining. For differential immunocytochemical staining of proinsulin, a proinsulin-specific monoclonal mouse antibody (GS9A8) *(13)* was kindly provided by Dr. Lelio Orci (Univ. of Geneva) and used at a dilution of 1/5,000 on paraffin embedded sections. Methods for immunocytochemical detection of the mouse monoclonal antibody using a biotinylated goat antimouse reagent were as described previously *(14)*.

Statistical Analysis

Significant main effects between gender and genotype were determined by two-way ANOVA using a computer program (Statview 4.5 for MacIntosh, Abacus Concepts, Berkeley, CA).

Results

Male Sex-Limited Diabetes Development

At weaning BKS/JLt-*Cpefat* /*Cpefat* mice were significantly lighter ($p < 0.0005$) than heterozygous or wild type littermates (Fig. 1A). At weaning (4 wk), average weight for lean (+/+ and +/*Cpefat* genotype) males was 16.4 ± 0.4 g ($n = 30$) compared to 12.9 ± 0.4 g ($n = 58$) for mutant males. Average lean female weaning weight was 14.5 ±0.2 g (n =29) vs 12.0± 0.3 g (n =48) for mutant females. However, body weights of both female and male mutant mice became significantly elevated above lean controls between 6 and 8 wk of age and attained weights of 45–55 g by 18 wk of age (Fig. 1A). As adults, the *Cpefat* mutants weighed 2–3 times that of lean littermates. The observed weight gain in mutants of either sex was not directly associated with food intake since *Cpefat* mutants consumed no more than lean controls (Table 1). Also, unlike *Leprdb* or *Lepob* mutants on the C57BLKS background, *Cpefat* mutant mice did not undergo rapid weight loss at six months of age but rather maintained a constant weight (Fig. 1A).

Development of severe hyperglycemia after the development of gross obesity was a major feature distinguishing BKS/JLt-*Cpefat* /*Cpefat* males at the 10th backcross (Fig. 1B) from either HRS-*Cpefat* males or BKS-*Cpefat* males previously studied at earlier backcross generations *(6,7)*. Mean nonfasting PG levels were elevated by 12 wk (249 ±16 mg/dl) and attained an average value of 382 ± 26 mg/dL ($n = 28$) by 20 wk. One of the most unusual aspects of the diabetes in these males was maintenance of a stable body weight without increased food consumption during the period of chronic hyperglycemia between 12 and 30 wk of age. The diabetes syndrome in these BKS/JLt-*Cpefat* /*Cpefat* males was further distinguished from that observed either in BKS mice homozygous for the *Lepob* or *Leprdb* mutations. The latter two mutations on the BKS background produce a progressively more severe hyperglycemia in mice of both sexes, leading to weight loss and death of the mice. In the BKS/JLt-*Cpefat* /*Cpefat* males, hyperglycemia did not rise continuously. Instead, PG levels for individual males tended to plateau around 400–600 mg/dL, and, after 30 wk of age, most males remaining in the longitudinal study went into spontaneous remission from hyperglycemia. At 34 wk, only 5 of 16 mutant males exhibited PG values above 300 mg/dL. Body weights remained elevated as PG levels declined rapidly to levels below 300 mg/dL after 30 wk.

In contrast to the transient diabetes developing in males, the majority of BKS/JLt-*Cpefat* /*Cpefat* females exhibited resistance to the post-pubertal development of severe hyperglycemia occurring in most mutant males (Fig. 1B). Only 1 of 20 females at 20 wk showed a plasma glucose above 300 mg/dL, compared to 19 of 28 males in this category. Nevertheless, the plasma glucose levels of BKS/JLt-*Cpefat* /*Cpefat* females between 15 and 30 wk of age was significantly higher than in lean littermate controls.

Proinsulin Accumulation in Pancreatic Beta Cells

As previously observed in HRS/J mice homozygous for the *Cpefat* mutation *(6)*, BKS/JLt-*Cpefat* /*Cpefat* mice of both sexes also exhibited markedly elevated immunoreactive proinsulin/insulin level. Data in Table 1 show this elevation for virgin mice sampled in the postpubertal period. Significant increases were detected as early as weaning (4 wk of age), as was previously observed for this mutation on the HRS/J background. The reduced hyperglycemic stress on BKS/JLt-*Cpefat* /*Cpefat* females compared to mutant males was correlated with a significantly lower hyper(pro)insulinemia in the former (Table 1), a gender

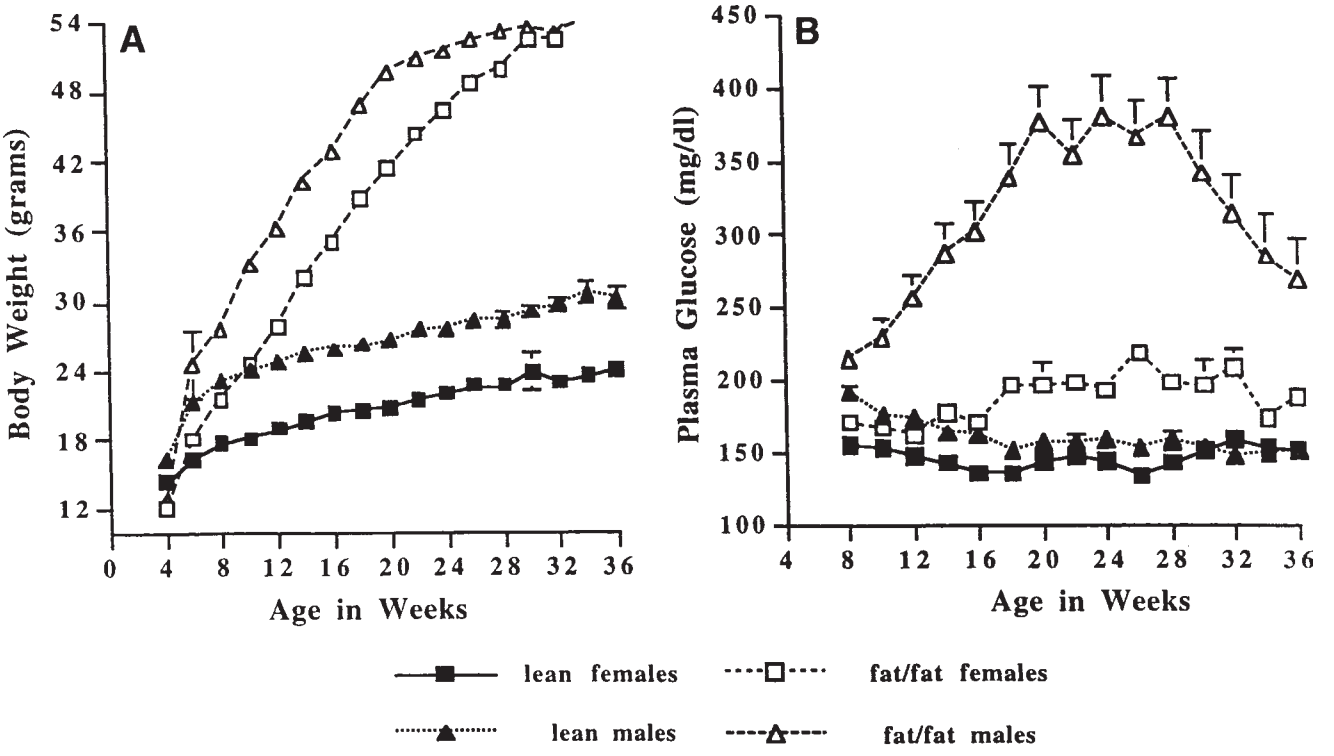

Fig. 1. Time course of body weight gain **(A)** and gender-dimorphic diabetes **(B)** in BKS/Lt-*Cpefat* mice. Triangles and squares indicate means (+SEM) for male and female mice, respectively. Open symbols represent *Cpefat*/*Cpefat* mice and darkened symbols denote +/+ or +/*Cpefat* (lean control) mice. Variable numbers of mice were sampled longitudinally, with the largest numbers sampled prior to 32 wk (lean males, 14–30; *Cpefat* males, 26–57; lean females, 7–25; *Cpefat* females, 11–39). Standard error bars of <3 g for body weight do not show in A.

difference not observed when the mutation was first described on the HRS/J inbred strain *(6)*. Previous analysis of plasma insulin showed that the proinsulin:insulin ratio was altered in the *Cpefat* mutants, such that proinsulin comprised an estimated 77% of the total immunoreactive insulin *(9)*. This altered ratio was reflected in a marked inbalance of the proinsulin to insulin levels in the pancreas of *Cpefat* mutants as well, such that an abnormal preponderance of proinsulin staining in the secretory granules of β-cells is observed (Fig. 2). The accumulation of proinsulin relative to the more biologically active form of insulin can be attributed to a lack of functional CPE in the pancreatic beta cells *(11)*.

The elevated plasma and pancreatic insulin levels would suggest that *Cpefat* mutant mice are insulin resistant. However, when *Cpefat* mutant males at N8-N9 were treated with exogenous insulin either by IP injection or by ALZET minipump implantation, PG levels rapidly returned to normal (Fig. 3). A morphologic correlate of the more normal glucose homeostasis was the finding that beta cells showed increased aldehyde fuchsin stainability (Fig. 2). This staining reflects the degree of beta granule content of the cell, and thus was indicative of a reduction of the hypersecretory stress as plasma glucose levels were reduced. Even without insulin therapy, islets of hyperglycemic BKS/JLt-*Cpefat* / *Cpefat* males do not undergo the widespread age-related β-cell necrosis and islet atrophy characteristic of the *Lepob* or *Leprdb* mutations on this same inbred background.

Other Endocrine Parameters

In contrast to the hypercorticism elicited by homozygous expression of either the *Leprdb* or *Lepob* mutations on the C57BLKS background *(15,16)*, corticosterone levels in BKS/JLt-*Cpefat* /*Cpefat* mice were within normal range (Table 1). BKS/JLt-*Cpefat* /*Cpefat* mutants remained normocorticoidemic even after they become obese (Table 1). Plasma glucagon levels were approx two- to threefold elevated in *Cpefat* mutants compared to sex-matched controls (sex differences not significantly different, but genotypic differences significant by ANOVA at $p = 0.001$). Mean T4 level was modestly decreased in adolescent mutants compared to lean controls (Table 1). However, because some mutant values fell within the littermate normal range, and because sampling of older mutants showed no significant difference between littermate control and mutant T4 means (data not shown), it is unlikely that the *Cpefat* mutants exhibit hypothyroidism. Despite disrupted processing of prohormones in the anterior pituitary of BKS/JLt-*Cpefat* /*Cpefat* mice, which reportedly resulted in increased serum levels of growth hormone *(17)*, normal serum IGF-1 levels were observed (Table 1). Serum levels

Table 1

Physiological Parameters for C57BKS/Lt-*Cpefat* N_{10} Males and Females*a*

Genotype gender	Body wt, in grams*b*	Food consumption*b*, g/mouse/d	Insulins*c*, ng/mL	Glucagon, pg/mL	IGF-1, ng/mL	Corticosterone, ng/mL	T4, µg/dL
fat/*fat* males	32.8 ± 3.7*g* (n = 5)	3.4 ± 0.4 (n = 5)	29.6 ± 2.8*g* (n = 5)	76 ± 5*f* (n = 5)	206 ± 3 (n = 10)	14.3 ± 5.1 (n = 9)	4.0 ± 0.2 (n = 5)*e*
Lean males	22.7 ± 1.1 (n = 5)	3.3 ± 0.3 (n = 5)	1.2 ± 0.2 (n = 4)	27 ± 13 (n = 5)	208 ± 11 (n = 5)	18.2 ± 13.7 (n = 4)	4.6 ± 0.7 (n = 5)
fat/*fat* females	21.5 ± 2.8*g* (n = 5)	3.0 ± 0.7 (n = 5)	12.9 ± 4.1*g* (n = 5)	94 ± 12*f* (n = 5)	nd*d*	7.7 ± 1.4 (n = 8)	4.2 ± 0.2*e* (n = 5)
Lean females	16.5 ± 0.7 (n + 5)	3.0 ± 0.2 (n = 5)	1.0 ± 0.2 (n = 4)	55 ± 21 (n = 5)	nd	4.6 ± 0.6 (n = 3)	5.8 ± 0.5 (n = 6)

*a*Data are mean ± SEM for 8–12-wk-old mice unless otherwise indicated.
*b*Food Consumption represents grams of food consumed per mouse per day over a 6-d period. Mice were 8 wk old.
*c*The RIA does not distinguish between immunoreactive proinsulins I and II from insulins I and II (the former present at high levels in serum of mutant mice).
*d*nd = not determined.
*e*Significant effect of genotype at $p = 0.025$.
*f*Significant effect of genotype at $p < 0.001$.
*g*Significant effect of sex and genotype at $p < 0.0005$.

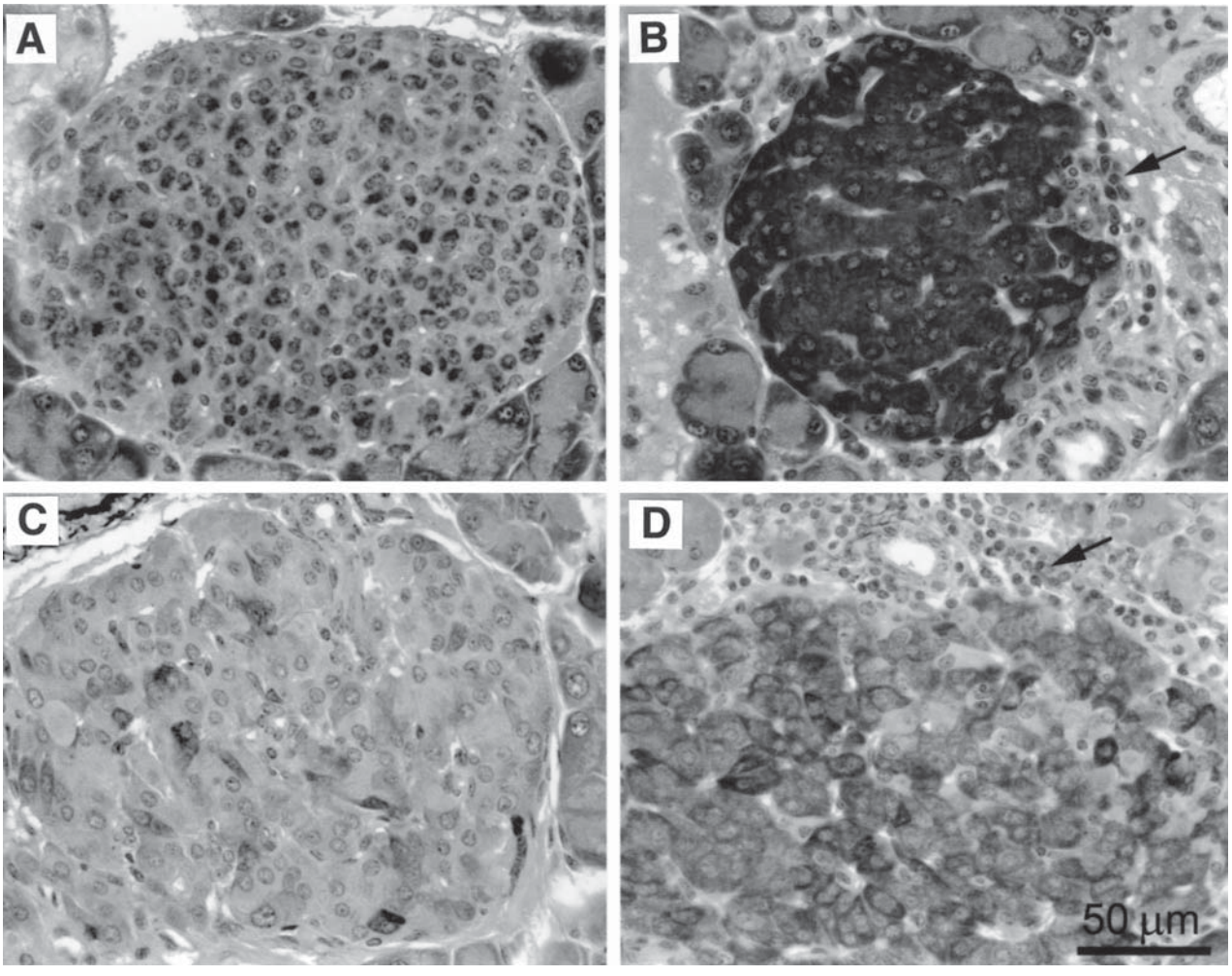

Fig. 2. Pancreatic islet histology in BKS/Lt-*Cpefat* males (N8). All magnifications are 400X. **(A)** Immunocytochemical staining of an islet from a 10 mo-old BKS-+/+ male with a monoclonal antiserum against proinsulin showing the typical peri-nuclear staining consistent with normal proinsulin localization primarily in the trans-Golgi. **(B)** Immunocytochemical staining for proinsulin in an islet from a 28-wk-old hyperglycemic BKS/Lt-*Cpefat* male. An anomalous diffuse stain indicative of widespread intracellular distribution is shown. The arrow denotes a peri-insular leukocytic infiltrate commonly seen in pancreata of mutant mice of both sexes. **(C)** Aldehyde fuchsin staining of insulin in the islet of a hyperglycemic 18 wk-old male implanted with a saline containing osmotic minipump for 1 wk prior to necropsy. Islet shows fibrotic changes and only a few beta cells are stained, indicative of β-degranulation. This correlated with high plasma glucose (548 mg/dL) and plasma (pro)insulin (26 ng/mL). **(D)** Recovery of aldehyde fuchsin staining in an islet of an initially hyperglycemic 18-wk-old male implanted with a osmotic minipump delivering 0.5 U/d of insulin for 1 wk prior to necropsy. Beta cells are staining more heavily, correlating with a reduction in levels of both plasma glucose (234 mg/dL) and plasma (pro)insulin (10 ng/mL). The arrow denotes a peri-insular leukocytic infiltrate.

of leptin in BKS/JLt-*Cpefat* /*Cpefat* mice have been reported to be elevated *(18)*. Under these circumstances it was not surprising that leptin administration daily for 7 d had no effect on body weight or food consumption, whereas the same dose regimen prevented weight gain and reduced food intake in young C57BL/6J-*Lepob*/*Lepob* mice used as positive controls (Table 2).

Dexamethasone Implantation: Effects on Glucose Homeostasis

As described above, BKS/JLt-*Cpefat* /*Cpefat* mutant females are rarely hyperglycemic. However when treated with 0.5 mg dexamethasone by subcutaneous implantation for 7 d, mutant females expressed PG levels of 400–600mg/dL; levels similar to those spontaneously developing in mutant males (Fig. 4). A comparable dexamethasone induction of hepatic EST activity was observed in both BKS/JLt-*Cpefat* /*Cpefat* mutant and control females. This enzyme is not constutitively expressed in the liver of female mice since its effect would be to reduce receptor-active estrogen levels in hepatocytes. Consistent with previous studies using the yellow agouti (*Avy*) mutation *(19)*, glucocorticoid administration was only diabetogenic in obese BKS/JLt-*Cpefat* /*Cpefat* females, and not the lean +/? controls, demonstrating that virilization of hepatic metabolism was only diabetogenic in the context of other obesity-elic-

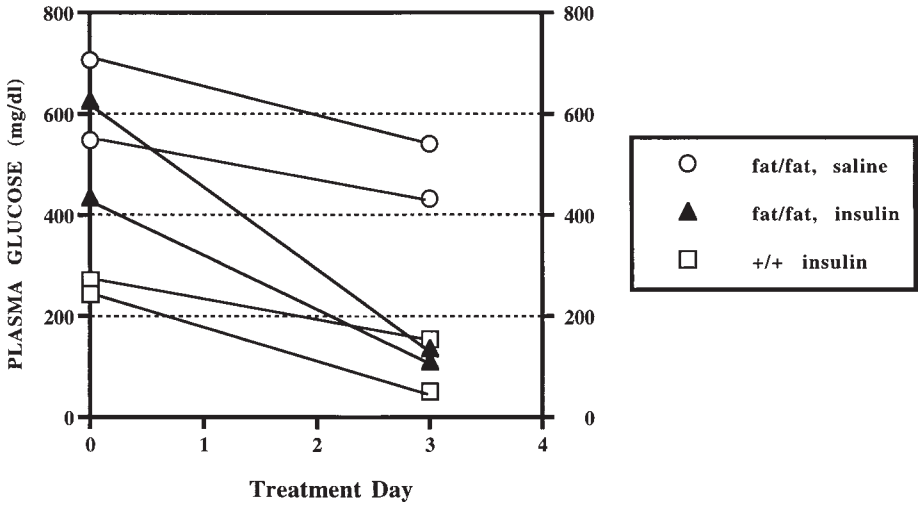

Fig. 3. Glycemic responses to insulin treatment. Data for pairs of males per genotype/treatment are plotted individually.

Table 2

No Effect of 1-wk Leptin Treatment (5 μg/g body wt) on BKS-*Cpefat* Females (mean ± sem)*a*

Genotype/treatment*b*	Body wt (d 0), g	Body wt (d 7), g	PG (d 0), mg/dL	PG (d 7), mg/dL	Food consumption, g/mouse/d
Cpefat saline	20.9 ± 0.9	29.2 ± 0.9	214 ± 7	203 ± 6	3.3 ± 0.3
Cpefat leptin	43.7 ± 3.5	43.5 ± 3.4	228 ± 12	222 ± 10	3.0 ± 0.2
Lepob saline	35.5 ± 1.5	41.5 ± 1.7	226 ± 22	240 ± 21	5.7 ± 0.4
Lepob leptin	38.7 ± 1.5	36.8 ± 1.3	290 ± 2	226 ± 18	2.5 ± 0.5

*a*B6-*Lepob* females are positive controls.

bN = 5 females/group. Different starting weights of *Cpefat* females reflect different ages of the two groups (saline controls, 8 wk old; leptin-treated mice, 20 wk old). B6-*Lepob* females were 6 wk old.

ited perturbations. These studies indicate that endogenous female sex steroids account in part for the diabetes resistance of obese BKS/JLt-*Cpefat* /*Cpefat* females.

Discussion

This study details numerous differences in the pathophysiology of the "diabesity" syndrome produced by the *Cpefat* mutation on the same C57BLKS/J inbred strain background previously used to study the diabetogenicity of the *Lepob* and *Leprdb* mutations *(2)*. A number of protein prohormones regulating nutrient intake and satiety are known to require CPE processing *(20)*. Yet unlike *Lepob* and *Leprdb* mutants, *Cpefat* mutants are not hyperphagic (Table 1). The absence of hyperphagia in these mice probably reflects a balance between incomplete processing of prohormones that stimulate feeding and those that inhibit feeding, as well as the presence of other carboxypeptidases that may partially compensate for CPE deficiency in the brain *(21)* and in endocrine organs *(11)*. *Lepob* and *Leprdb* mutants are hyperphagic at least in part due to a lack of leptin/ leptin receptor mediated neuropeptide Y suppression and elevated serum glucocorticoid levels. When treated with exogenous leptin, *Lepob* mutants dramatically reduce food intake *(22)*. However, *Cpefat* mutants, like certain other murine obesity models such as the New Zealand Obese (NZO) mouse, do not respond to peripherally administered leptin at similar doses *(23)*. This is not surprising in view of the finding that *Cpefat* mice are hyperleptinemic, a likely consequence of the increased fat mass in these mutants *(24)*.

Although balanced defects in the exopeptidase processing of protein prohormones that stimulate and that inhibit feeding may account for the absence of hyperphagia, defects in neuroendocrine regulation resulting from incomplete CPE processing must nonetheless explain the development of obesity. One of the salient differences between the effects of the *Cpefat* mutation versus *Lepob* and *Leprdb* is the failure of the former to elicit hypercorticism. Hence, we propose that *Cpefat* mutants are not obese due to hyperphagia, but perhaps as a result of imbalances caused by failure to correctly process or release proopiomelanocortin (POMC) and other hormone precursors from the pituitary. Indeed, a human family has been identified in whose offspring mutations in the POMC gene lead to morbid obesity *(25)*. POMC is the precursor for ACTH, β-endorphin and α-melanocyte stimulating hormone. Reduced levels of

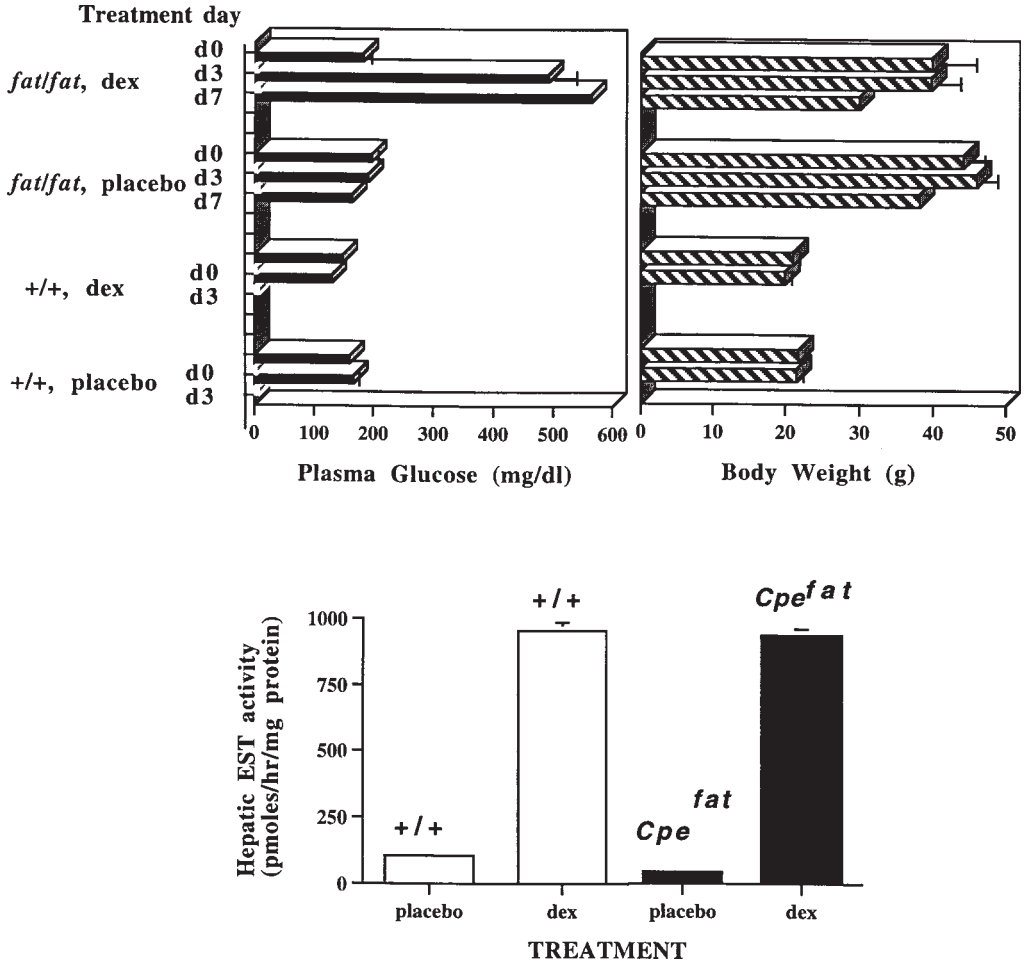

Fig. 4. Dexamethasone (dex) induces hepatic estrogen sulfotransferase (EST) activity and hyperglycemia in nominally diabetes-resistant *Cpefat* females but only EST without hyperglycemia in lean (+/+) control females. EST assays performed 3 d postimplantation of 0.5 mg dex using two donors/treatment group. Concentration of E1 substrate was 0.2 μ*M* .

mature ACTH in BKS/JLt-*Cpefat* /*Cpefat* females has been reported *(17)*, and would explain why hypercorticism is not observed in these mutant mice.

Varmalov et al. *(11)* have shown that CPE-deficient pancreatic beta cell lines are capable of secreting insulin and proinsulin via the regulated secretory pathway (RSP), proving that CPE is not obligatory for proper targeting of proinsulin in the pancreas. However, Cool et al. *(26)* have contended that, in the anterior and intermediate pituitary, CPE bound to the membrane of the trans-Golgi may serve as a receptor for sorting POMC into the RSP. This group reported constitutive rather than regulated release of POMC, along with proenkephalins, and chromogranin B by pituitary explants from *Cpefat* /*Cpefat* females. This group subsequently reported increased levels of circulating growth hormone (GH) and decreased levels of ACTH *(17)*. Our finding (Table 1) that IGF-1 levels are not different between mutants and controls is surprising, since an increase in GH would be anticipated to be reflected by an increase in serum IGF-1. Data in Table 1 demonstrate that mutant mice of both sexes weigh significantly less than controls at 4 wk of age. Between 6 and 8 wk of age, mutants undergo a rapid period of weight gain, until, by wk 8, they weigh 1–3 g more than controls. Maturation of IGF-2 requires C-terminal processing, although it has not been demonstrated that CPE is the enzyme actually involved. Conceivably, Cpe*fat* mutants may be deficient in fully-processed IGF-2 due to lack of functional CPE, thus delaying growth prior to puberty. At puberty, as the influence of IGF-1 supercedes that of IGF-2, *Cpefat* mutants rapidLy gain weight, with the imbalances of the mis-sorted and improperly processed pituitary prohormones perhaps contributing to the development of obesity.

Another major distinction between the *Cpefat* mutation versus the *Leprdb* and *Lepob* mutations expressing on the C57BLKS/J background is the differential effect of gender on "diabesity." BKS/JLt-*Cpefat* /*Cpefat* females, unlike either *Cpefat* mutant males or *Leprdb* and *Lepob* mutants of either sex, have retained the diabetes resistance originally observed in mice of both sexes when the mutation was first characterized on the HRS/J strain of origin *(6)*. BKS-*Cpefat* females are further distinguished from BKS-*Leprdb* and

Lepob females in that *Cpefat* females do not show the virilizing shifts in sex steroid sulfotransferase activities previously associated with diabetogenesis in the former two mutant stocks *(7)*. Hepatic sulfotransferases inactivate sex steroids and their precursors by sulfation. In rodents, estrogens potentiate and androgens antagonize glucose homeostasis. Estrogens increase the density of hepatocyte surface insulin receptors, while androgens counter these effects *(27)*. Thus, the balance of hepatic estrogens and androgens plays a crucial role in glucose regulation in mice *(1)*.

Hepatocytes of female mice constitutively express DHEA sulfotransferase activity to sulfurylate DHEA and androgens. They do not constitutively express estrogen sulfotransferase *(8,19)*, an estrogen-inactivating enzyme expressed constitutively in mouse testis *(28)*. C57BLKS/J-*Lepob* and *Leprdb* mutant females, both of which develop diabetes, exhibit anomalously high levels of hepatic estrogen sulfotransferase (EST) and low expression of dehydroepiandrosterone sulfotransferase (DST). In *Avy* (viable yellow) obese mice, where a female resistance to diabesity comparable to that observed in BKS/JLt-*Cpefat* /*Cpefat* females exists, dexamethasone administration also induces EST expression in the liver and triggers hyperglycemia development *(18)*. As demonstrated in this report, the *Cpefat* mutation is differentiated from *Leprdb* and *Lepob* by its inability to elicit hypercorticism, probably owing to the defects in ACTH processing in the pituitary *(17)*. When hypercorticism was induced by dexamethasone implants in both lean and *Cpefat* females, hepatic EST expression occurred in both genotypes, but hyperglycemia only developed in the obese mutant females. These data indicate that hepatic virilization alone in females is not sufficient for the development of hyperglycemia since lean females did not develop hyperglycemia. Clearly, the many CPE-dependent pathways associated with nutrient partitioning affected by the *Cpefat* mutation, including the defect in insulin processing, prime *Cpefat* females for glucocorticoid-induced hyperglycemia.

The basis for the age-associated *Cpefat* male remission from hyperglycemia is unknown. Unlike hyperglycemic C57BLKS/J-*Lepob* and *Leprdb* mutants, BKS/JLt-*Cpefat* / *Cpefat* males are not insulin resistant. This would explain why preliminary studies indicated no elevation in gluconeogenic enzymes in hyperglycemic BKS/JLt-*Cpefat* / *Cpefat* males, in contrast to what is observed in C57BLKS/J-*Lepob and Leprdb* mutants. Post-pubertal remission from hyperglycemia in B6-*Lepob*/*Lepob* males is well established. *(29)*. Interestingly, a parallel to the spontaneous remission from hyperglycemia in BKS/JLt-*Cpefat* /*Cpefat* males has been observed in group-housed C3H.SW virgin males, which undergo a spontaneous remission from hyperinsulinemia/glucose intolerance syndrome at approximately the same time as BKS/JLt-*Cpefat* /*Cpefat* males *(30)*. Since both the testes and adrenal glands were involved in eliciting the C3H.SW male syndrome, an age-related decrease in diabetogenic function(s) mediated via the hypothalamic/pituitary/adrenal axis was assumed. A low activity sex steroid sulfohydrolase (*Sts*) allele expressed on both the X and Y chromosome was associated with increased diabetic susceptibility *(30)*. The enzyme encoded by this gene frees sulfoconjugated estrogens to receptor-active forms, such that age-dependent changes in sex steroid metabolism may be involved. In normal BKS and B6 males, non-fasted plasma glucose levels are highest around puberty and decline with age *(31,32)*. The age-dependent decline in non-fasting blood glucose levels may be related to age-related decreases in expression of GH and prolactin genes in the pituitary *(33)*. If young BKS/JLt-*Cpefat*/*Cpefat* males are indeed hypersecreting GH via a constitutive pathway as reported *(17)*, then other compensatory carboxypeptidases, similar to the recently-discovered Carboxypeptidase D *(34)*, may accumulate in somatotropes with age and restore a more regulated secretion.

Age-dependent accumulation of a redundant carboxypeptidase (like Carboxypeptidase D) may account for age-associated changes in the endocrine pancreas of BKS/JLt-*Cpefat* /*Cpefat* males. Aldehyde fuchsin staining of pancreatic islets from older, normoglycemic mutants of both sexes showed well-granulated beta cells similar to that produced by insulin therapy of younger, hyperglycemic males (Fig. 2). Variable degrees of islet hyperplasia and beta cell hypertrophy also were observed as mutants of both sexes aged, similar to the report of islet size changes of the normoglycemic *Cpefat* mutants on the HRS/J background *(6)*. However, despite age-associated remission in males, a variety of pancreatic pathologies were noted, notably, leukocytic infiltrates, often associated with loss of the exocrine parenchyma and its replacement by fatty tissue. Tumor necrosis factor (TNFα) is reportedly a diabetogenic factor in obese hyperglycemic mice *(35)*. TNFα production by the lymphoid infiltrates or by adipocytes was not examined in this study; however, local TNFα secretion by leukocytes is unlikely to be the basis for the hyperglycemia since the infitrates were also present in female pancreas

In summary, this study has contrasted the expression of the *Cpefat* mutation on the same diabetes-susceptible BKS inbred strain background previously used to analyze the strong (and indistinguishable) diabetogenic effects of two other obesity-producing mutations, *Lepob* and *Leprdb*. The hallmark of the *Cpefat* mutation has been development of early hyper(pro)insulinemia followed by postpubertal obesity, but not necessarily diabetes. Sufficient numbers of backcrosses of the *Cpefat* mutation onto the BKS background presumably resulted in the loss of HRS/J donor strain genome that repressed diabetogenesis. It is certainly possible that further backcrossing to BKS will lead to development of diabetes in mutant females as well as males. It was initially assumed that the diabetes development in BKS/JLt-*Cpefat*/*Cpefat* males modeled for type II diabetes in humans, since insulin resistance was assumed. However,

the finding that the majority of "insulins" in circulation are actually proinsulins and C-terminally extended forms of insulin, led to the elucidation of the molecular basis for this genetic defect, a missense mutation in the *Cpe* gene. Therefore, sensitivity of diabetic *Cpefat* males to exogeneous insulin can be explained by the low biologic potency of the unprocessed proinsulins and their partially processed intermediates. The marked differences in response of the same inbred strain background genome to the different obesity-producing mutations discussed provide insight into the basis for both the genetic and clinical heterogeneity underlying glucose intolerance disorders in humans. The loss of BKS/JLt male resistance to the diabetogenic effects of the *Cpefat* mutation between the 6th and 10th backcross generations presumably reflected loss (through recombination) of residual HRS/J protective genes that were heterozygous in earlier backcross generations. Although the genome of a congenic stock statistically may be 99.9% recipient type after 10 backcrosses, 20 backcrosses are required to produce a true congenic stock in which donor strain genome is limited to a small segment around the introduced locus.

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