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The Carboxypeptidase E Knockout Mouse Exhibits Endocrinological and Behavioral Deficits

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A carboxypeptidase E (CPE) knockout (KO) mouse was generated by deletion of exons 4 and 5 from the CPE gene, and its phenotype was characterized. KO mice became obese by 10–12 wk of age and reached 60–80 g by 40 wk. At this age, body fat content was more than double that in the wild-type (WT) controls. The null animals consumed more food overall, were less physically active during the light phase of the light-dark cycle, and burned fewer calories as fat than WT littermates. Fasting levels of glucose and insulin-like immunoreactivity in plasma were elevated in both male and female KO mice at approximately 20 wk; males recovered fully and females partially from this state by 32 wk. At this time, insulin-like immunoreactivity in the plasma, identified as proinsulin, was 50–100 times higher than that of the WT animals. The KO mice showed impaired glucose clearance and were insulin resistant. High levels of leptin and no circulating fully processed cocaine- and amphetamine-related transcript, a peptide that is responsive to leptin-induced feedback inhibition of feeding, were found in serum. The KO mice were subfertile and showed deficits in GnRH processing in the hypothalamus. Behavioral analyses revealed that KO animals showed diminished reactivity to stimuli and had reduced muscle strength and coordination, as well as visual placing and toe-pinch reflexes. These data demonstrate that CPE KO mice display a wide range of neural and endocrine abnormalities and suggest that CPE may have additional physiological roles beyond those ascribed to peptide processing and sorting of prohormones in cells. (*Endocrinology* 145: 5807–5819, 2004)

CARBOXYPEPTIDASE E (CPE) is a prohormone processing exopeptidase and a prohormone sorting receptor for the regulated secretory pathway (RSP) in endocrine and neuroendocrine cells (1–4). Peptide hormones and neuropeptides are synthesized as larger precursors that are first cleaved by prohormone convertases, either in between or on the carboxyl side of paired basic residues to yield basic residue-extended peptides (5–7). Within secretory granules of the RSP, CPE then cleaves the basic residues from these hormone and neuropeptide intermediates to generate bioactive peptides (1, 8). Aside from soluble CPE, the membrane form has also been shown to act as a receptor for sorting prohormones away from other non-RSP proteins at the *trans*-Golgi network (TGN), and into secretory granules in the RSP of pituitary (3, 9), and pancreatic β-cells (10), as well as in neuronal cell lines (11). The transmembrane form of CPE can be anchored to cholesterol/sphingolipid-rich microdomains known as lipid rafts or detergent-resistant membranes at the TGN (12–14). It acts as a sorting receptor at the TGN by binding to a specific sorting motif on the prohormone and bringing it into the immature granule as it buds off the TGN membrane. In pancreatic β-cells where sorting of proinsulin from other non-RSP proteins continues in the immature granule, CPE can act as a retention receptor to retain insulin within this organelle and prevent its removal via the constitutive-like pathway (15). Upon exocytosis, membrane CPE in secretory granules is internalized by a novel mechanism requiring the physical interaction of its cytoplasmic tail with ADP-ribosylation factor 6, a GTPase (16). CPE is then recycled back to the TGN for reuse as a sorting receptor or processing enzyme. These properties suggest that the C-terminal tail of CPE may perform some role in endocytosis, a suggestion recently supported by data showing the requirement for CPE in the endocytosis of eosinophil cationic protein in neuroendocrine cells (17).

CPE mis-sense polymorphisms have been found in patients with type 2 diabetes mellitus (18). In four different families, the R238W mutation altered CPE enzymatic activity and the patients showed early onset of type 2 diabetes. Another CPE mutation (*e.g.* S202P) was found in mice. These Cpe^{fat}/Cpe^{fat} mice were obese, infertile, and developed hyperproinsulinemia (19, 20). Mutant CPE in these mice was primarily in the precursor form and was differentially degraded in various tissues (20, 21). In pituitary cells, mutant CPE molecules were essentially all degraded, whereas in pancreas, they were partially degraded, and the undegraded molecules were trafficked from the endoplasmic reticulum to the TGN and packaged into secretory granules (21). Thus, because of the presence of residual mutant CPE in various tissues, this mouse may represent an inadequate null model for studies of CPE function. In *Caenorhabditis elegans*, a CPE

Abbreviations: CART, Cocaine- and amphetamine-related transcript; CPE, carboxypeptidase E; ES, embryonic stem; HET, heterozygous; IR, immunoreactivity; KO, knockout; RSP, regulated secretory pathway; TGN, *trans*-Golgi network; WT, wild-type.

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null mutant has been reported to exhibit deficient acetylcholine release at the neuromuscular junction implicating, perhaps, yet another nonenzymatic role of CPE in neurotransmission (22).

In view of the evidence that CPE may have additional roles besides that of a prohormone processing enzyme, it was important to generate a true CPE null or knockout (KO) mouse to study the loss-of-function phenotype of CPE. Such a mouse would be a valuable model for the study of type 2 diabetes and other diseases involving defective peptide hormone processing and trafficking. In this study, we generated a CPE KO mouse and characterized its phenotype with respect to its endocrine and behavioral functions in the whole animal.

Materials and Methods

Generation of the CPE knockout mouse

A targeting vector was generated from a 129Sv genomic mouse bacteria artificial chromosome clone (Research Genetics, Huntsville, AL) containing the *Cpe* gene. Exons 4 and 5 (encoding the enzymatic active site and the sorting signal binding domain of CPE) were floxed and flanked with a 2.2-kb *Eco*RV-*Eco*RI 5′arm followed by a loxP-neo-tk cassette and a 7.1-kb *Xho*I-*Bam*HI 3′ arm (Fig. 1, *left*). The *Not*I linearized construct was electroporated into W9.5 embryonic stem (ES) cells. The correctly targeted ES clone (normal karyotype) was electroporated with *Cre* recombinase to generate ES clones devoid of exons 4, 5, and the neo-tk cassette. The null CPE ES clones (normal karyotype) were microinjected into C57BL6 blastocysts. Chimeras from the mixed background (C57BL6/129Sv) were obtained and gave germline transmission. Heterozygous (HET) mutant mice were bred and mated to generate homozygous mutants. All mice studied here were from first and second generation backcross with C57BL6 mice and KO animals were compared with their wild-type (WT) littermates or age matched mice from breeding pairs with equivalent genetic backgrounds. All experiments were conducted with approved animal protocols at the National Institutes of Health and Duke University Medical Center.

Genotyping

Genomic DNA was extracted from tail snippets by standard procedures and subjected to PCR using a mixture of three primers (primer 1: 5′-GTAGCCTGGGTTGGAATCCTCGGCTATGTA-3′, primer 2: 5′-AGGAAAGATCACAAGACTCAGAAGCTGACAATCGC-3′, primer 3: 5′-CAGTAATGGGCCTGGACTTGTAATCCTTGTAGC-3′). The HotStarTaq PCR kit was used (QIAGEN, Germantown, MD) and the PCR run with the following program: 94 C for 5 min followed by 35 cycles at 94 C for 30 sec, 68 C for 30 sec, and 72 C for 3 min. The KO allele generated an approximately 1.6-kb band from primers 1 and 3, whereas the WT allele produces a band at approximately 1.0 kb from primers 1 and 2 (data not shown). The location of the primers that generates these bands is shown in Fig. 1, *left panel*.

Western blot

Whole pituitaries were dissected from WT, HET, and KO mice and immediately frozen on dry ice until analyzed. After thawing, by the addition of 1× SDS-PAGE sample buffer with reducing agent, a standard Western blotting procedure was used for the analysis of CPE from equivalent volumes of the homogenates. Rabbit anti-CPE antibody 7–8, generated in our laboratory (3) was used.

Food, water, and O_2/CO_2 consumption

At approximately 12 and 30 wk of age, food and water consumption were monitored over 3 d for WT and KO mice in a Columbus Instruments system (Columbus, OH) that measures moment-to-moment food intake in grams and water consumption as licks or volume. Because older KO mice were too large to traverse the chute to the food tray, food and water intake were measured in the home cages at the beginning and end of each light-dark cycle (lights on 0600–2000 h) for both WT and KO animals. O_2/CO_2 consumption was measured from the same 12- and 30-wk old WT and KO mice by the Oxymax system (Columbus Instruments) over 6 h during the light period. The respiratory quotient was calculated for each individual animal as the ratio of CO_2 production to O_2 consumption during periods of low activity (<10 activity events in a 10-min period over the last 2 h of testing). Energy expenditure was calculated as $[(3.815 + 1.232) \times VCO_2/VO_2) \times VO_2]$ where VCO_2 is the expired CO_2 volume at ml/kg·h and VO_2 is the expired O_2 volume in the same units (see Ref. 23).

Spontaneous activity

To assess activity, animals at approximately 12 and 30 wk of age were first adapted to the room for 2 h. Horizontal, vertical, and stereotypical activities in the open field (OF; 21 × 21 × 30 cm) were monitored by photobeams for 24 h (lights on 0600–2000 h at 340 lux) and food and

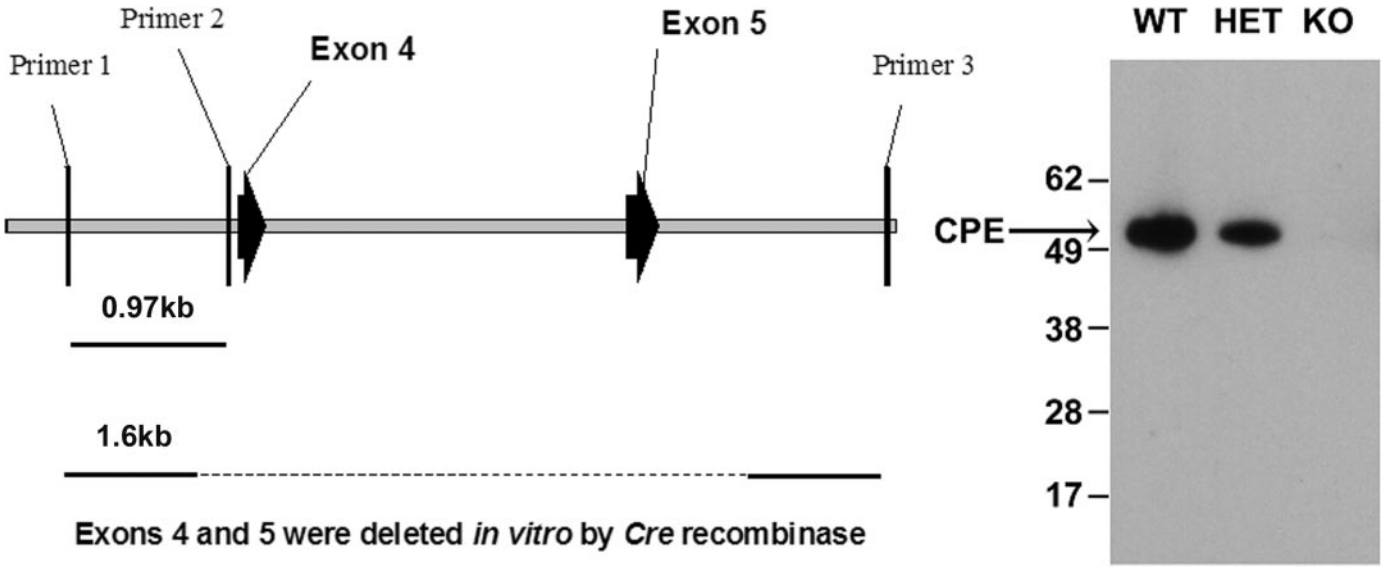

FIG. 1. Diagram showing deletion of murine *Cpe* and Western blot. *Left*, CPE construct showing exon 4 and exon 5 which were deleted *in vitro* by *Cre* recombinase. Primers for genotyping are depicted. *Right*, Western blot analysis of equivalent volumes (~25%) of whole pituitary homogenates showing the presence of CPE in WT and HET animals, and its absence in the null (KO) animals.

water were available *ad libitum*. Locomotor (horizontal distance in centimeters), vertical (counts), and stereotypical (repetitive beam breaks <1 sec apart) activities were analyzed with the VersaMax program (AccuScan Instruments, Columbus, OH). To assess motor abilities of 15-wk-old mice in a new environment, animals were placed in the center of a similar OF (40 × 40 × 35 cm) and given free exploration for 15 min. Horizontal and vertical movements were again counted as beam breaks.

Magnetic resonance imaging

Male and female WT and CPE KO mice (47–52 wk of age) were weighed and placed into the chamber of a MiniSpec NMR Analyzer (Bruker Optics, Inc., Billerica, MA), which determines total fat content. The weight of total fat for each mouse was converted to percent total body weight and averaged for each group.

ProteinChip profiling analysis of cocaine- and amphetamine-related transcript (CART) processing

The ProteinChip antibody capture kit (Ciphergen Biosystems, Fremont, CA) was used for the detection of CART proteins directly from the sera of WT and CPE KO mice. Briefly, according to the manufacturer's instructions, 1 μl of polyclonal CART (55–102) antibody (Phoenix Pharmaceuticals, Belmont, CA) was bound to recombinant Protein G that was precoupled to spots on the chips. After this, 2 μl of 100× diluted serum and 2 μl of binding buffer were added to individual spots on the chips and incubated for 1 h. After the incubation, the chips were washed twice with binding buffer, twice with PBS, and finally rinsed with water and air dried. The bound proteins were coated with the energy-absorbing matrix (cyno-4-hydroxy cinnapinic acid) and analyzed by surface-enhanced laser desorption/ionization-time of flight mass spectrometry using the Ciphergen ProteinChip reader. Negative control experiments (without antibodies or without serum) were run concurrently to control for experimental variations. All steps were performed at room temperature.

Serum leptin contents

Leptin levels were determined in nonfasted WT and CPE KO mice. Blood samples were collected in serum separator tubes, centrifuged immediately at 600 × *g* at 4 C for 10 min, and the serum collected, aliquoted, and stored at −20 C. Leptin was assayed directly from serum using a mouse leptin RIA kit (Linco Research Inc., St. Charles, MO).

Determination of fasting plasma glucose and insulin-like immunoreactivity (IR)

CPE KO mice and their WT and HET littermates were removed from their cages in the early morning and placed in fresh cages with water but without food for 6–8 h. After this period, blood was collected from the orbital sinus, plasma was separated from cells and frozen. Plasma glucose levels were determined using a hand-held Accu-Chek Compact glucometer (Roche Diagnostics Corp., Indianapolis, IN) or a Hemocue β-Glucose Analyzer (Hartman Medical Hemocue, Piano, TX). Fasting levels of insulin-like IR were determined directly from plasma by RIA using the Linco sensitive rat insulin kit that cross-reacts with mouse insulin 100% and with human, bovine, and porcine proinsulin 70–75%.

HPLC analysis of serum (pro)insulin

Fifty microliters of plasma from 27-wk-old WT and CPE KO females were acidified by addition of glacial acetic acid to 1 M. Samples were centrifuged at 13,000 rpm for 10 min to remove particulates. Forty microliters of supernatant from each sample were separated on a 4.6 × 250 mm 5 μm reversed-phase Jupiter C18 column (Phenomenex, Torrance, CA) as described by Neerman-Arbez and colleagues (24) with minor modifications (see Fig. 7 legend). Fractions were collected, lyophilized, and reconstituted in RIA buffer and assayed by RIA for insulin-like IR. Under these conditions, authentic human insulin (Phoenix Pharmaceuticals) eluted in fraction 8 and porcine proinsulin (a gift from Dr. Knud Vad, Novo Nordisk, Bagsvaerd, Denmark) eluted in fraction 23.

Glucose and insulin tolerance tests

Male and female CPE KO and WT animals (~12 and 30 wk old) were fasted overnight. After determination of baseline plasma glucose concentrations (see above), the animals were weighed and then administered a single injection of 25% D-glucose (ip; 11.1 mmol/kg body weight). Approximately 20 μl of blood were collected from the orbital sinus at 15, 30, 60, 120, and 180 min after glucose injection. Plasma was separated from red blood cells and used in the assay.

A separate group of nonfasted male and female CPE KO and WT mice at approximately 12 and 30 wk of age were weighed, and an initial plasma glucose determination was made. The mice were then given 1 U/kg (ip) recombinant human insulin (Novo Nordisk, Clayton, NJ). Clearance of plasma glucose was subsequently monitored at 15, 30, and 60 min post injection.

Reproductive status and GnRH analysis

Fertility was assessed by pairing naive WT males and females or naive KO males and females (~9 wk age) for 30 d or until the female showed detectable signs of pregnancy, whichever occurred first. Pregnancy was monitored daily by visual inspection, palpation, and weight gain. Hypothalamic GnRH-like IR was measured by RIA with two different antisera after extraction procedures described previously (25–27). The A772 antiserum recognizes [Gln^1]-GnRH and mammalian GnRH but not intermediates with amino acid extensions at the C terminus (28), whereas the B9 antiserum binds mammalian GnRH and the intermediates, as well as very low recognition for the pro-GnRH (26). All samples were run in one assay and the intraassay variabilities were approximately 5%.

Assessment of general health and neurobehavioral status

Mice were housed under standard laboratory conditions with food and water provided *ad libitum*. At 15 wk of age, CPE KO mice and their HET and WT littermates were examined with the SHIRPA phenotypic behavioral screen as described (29). Observations were scored on gradient scales and are reported in Table 1, where in most cases an increasing number indicates enhanced vigor, strength or excitement except where noted (see Table 1 legend). See details at: http://www.mgu.har.mrc.ac.uk/mutabase/shirpa_summary.html.

Briefly, after an observational period in which body position, spontaneous activity, respiratory rate, and unusual behaviors (*e.g.* tremor or stereotypy) were assessed, each animal was evaluated for fur condition, eye opening, piloerection, body and limb tone, posture, and gait. Additional assessments (see Table 1) involved increased handling of the animals, during which indications of fear or aggression were noted. The same experimenter in the same room under the same conditions tested all animals, except that one or two additional experimenters assisted in all studies.

Statistics

The data are presented as means and SEM. The statistical analyses were performed with the Statistical Package for the Social Sciences, version 11.0 (Chicago, IL) or with StatView software by Abacus Concepts (Berkeley, CA). The body weight data were analyzed by univariate ANOVA across the 48 wk. Fasting levels of plasma glucose and insulin, and hypothalamic GnRH contents were evaluated with univariate ANOVA using a split-plot design with genotype and sex as the fixed factors. *A priori* tests for repeated-planned comparisons and reverse-Helmert contrasts were conducted to identify specific age-dependent changes in weight, whereas fasting glucose and insulin were examined as a function of genotype and sex. Glucose and insulin tolerance tests, feeding and drinking behavior, and spontaneous activity in the open field were analyzed with repeated measures ANOVA with fixed factors for genotype, sex, and age. To control for possible effects because of body weight, the data were also reanalyzed with analysis of covariance using weight as a covariate. *A priori* tests for repeated planned comparisons were used to determine whether changes in glucose occurred at specific time points between the fixed factors. Measures taken for energy expenditure and respiratory quotients were analyzed with univariate ANOVAs with fixed factors for genotype, sex, and age. Fertility of WT

TABLE 1. Results of the SHIRPA protocol

	+/+ (n = 12)	+/− (n = 6)	−/− (n = 20)
General health			
Eyelid closure (0–2)[a]	0	0	0.28 ± 0.16
Piloerection (0–1)	0	0	0
Abdominal tone (0–2)	1.0 ± 0	1.0 ± 0	0.75 ± 0.1[b]
Body tone (0–2)	0.92 ± 0.08	1.0 ± 0	0.65 ± 0.1
Limb tone (0–4)	1.2 ± 0.2	1.3 ± 0.2	1.0 ± 0.2
Tremor (0–2)	0	0	0.45 ± 0.1[b]
Reflexes			
Visual placing (0–4)	2.1 ± 0.1	2.0 ± 0	1.7 ± 0.1[c]
Auditory startle (0–1)	1.0 ± 0	1.0 ± 0	0.95 ± 0.05
Corneal reflex (0–2)	1.0 ± 0	1.0 ± 0	0.88 ± 0.1
Pinna reflex (0–2)	0.92 ± 0.08	0.83 ± 0.16	0.6 ± 0.1
Toe pinch (0–4)	2.5 ± 0.1	2.8 ± 0.2	1.5 ± 0.2[c]
Trunk curl (0–1)	0.87 ± 0.09	0.86 ± 0.09	0.95 ± 0.04
Reactivity			
Touch escape (1–3)	1.8 ± 0.3	2.5 ± 0.3	0.6 ± 0.1[d]
Transfer arousal (1–6)	4.5 ± 0.4	4.0 ± 0.4	2.8 ± 0.3[c]
Provoked dowel biting (0–1)	0.4 ± 0.1	0.6 ± 0.2	0.3 ± 0.1
Positional passivity (0–4)[a]	0.08 ± 0.08	0	1.0 ± 0.15[d]
Strength/coordination			
Grip strength (1–4)	2.4 ± 0.2	2.9 ± 0.3	1.2 ± 0.2[d]
Wire maneuver (1–4)[a]	0.8 ± 0.4	0.3 ± 0.3	3.1 ± 0.2[d]
Vertical pole (1–4)	2.6 ± 0.2	2.2 ± 0.4	1.4 ± 0.1[d]
Motor abilities			
Gait (0–3)[a]	0	0	0.85 ± 0.2[c]
Horizontal activity (movements)	503.8 ± 67	547.0 ± 108	260.5 ± 34[c]
Vertical activity (movements)	63.2 ± 14	55.8 ± 11	28.3 ± 7[d]
Time active	74.4 ± 2%	72.6 ± 4%	56.9 ± 3%

Assessment of general health, neurologic function and activity of 15-wk-old carbyoxypeptidase E knockout mice.
[a] Gradient scales in which increasing number indicates decreasing vigor, strength, or excitement. Different from +/+, [b] $P < 0.05$; [c] $P < 0.01$; [d] $P < 0.001$.

and KO mice were evaluated by χ^2 tests. The behavioral data were analyzed with ANOVA followed by comparisons between WT, HET, and CPE KO animals with Fisher's protected least significant difference. For all purposes, statistical significance was defined as $P < 0.05$.

Results

Body weight, body length, and obesity

Mice were studied between 1 and 48 wk of age (Fig. 2). Weights were recorded daily from postnatal d 1 until postnatal d 18 (data not shown). At 1–18 d after birth, both male and female CPE KO mice were generally lighter in weight than WT mice, but the difference was not statistically significant. At 4–7 wk, the weights of the KO mice increased and approached those of WT animals (Fig. 2). By 8 wk, both male and female KO mice were heavier than WT mice. For WT mice, the most significant weight gains occurred between 2 and 6 wk for males and between 1 and 8 wk for females. By comparison, significant weight gains for KO males were delayed and occurred between 6 and 8 wk with marginal increments in body weight occurring after this time. Weight gains for KO females was also delayed but week-to-week gains were slow and consistent over time and were not characterized by rapid increases as seen in the other groups of mice. By approximately 40 wk of age, the KO animals weighed 60–80 g and were two to three times heavier than their WT and HET littermates.

Body length (tip of nose to base of tail) was measured in mice at 12 and 30 wk of age. Older mice were significantly longer than younger animals with older KO males growing more in length than older WT males. No differences were discerned between WT and KO females.

Food and water consumption

Food and water intake were assessed in CPE mice at approximately 12 and 30 wk of age (Fig. 3). At 12 wk of age, KO females consumed more food during the dark phase of the cycle than the female WT controls (Fig. 3A), whereas WT and KO males ate similar amounts. By 30 wk, both male and female KO animals ate more food than their WT littermates and this was especially apparent during the dark phase of the cycle (Fig. 3B). Overall, the older mice consumed more food than the animals at 12 wk with greatest intakes occurring during the dark phase of the light-dark cycle (Fig. 3, A and B). Although KO females at 12 wk displayed the largest change in food intake between the light and dark cycles, the magnitude of this change was similar to that observed with older null females. Similar effects were also observed when the data were reanalyzed controlling for body weight (data not shown). Water intake was similar to that for food intake with respect to genotype, age, and sex, except for females where water intake by older WT and KO females was not different (data not shown).

Activity and indirect calorimetry

Spontaneous activity was assessed in the OF over 24 h. At 12 wk of age, both male and female WT mice were more active during the light phase of the light-dark cycle than the homozygous mutants (Fig. 3C). However, during the dark phase, WT and KO males were equally active and the KO females displayed the highest rates of activity. Locomotion decreased with age, with the KO animals exhibiting lower levels during the light and dark phases of the cycle compared

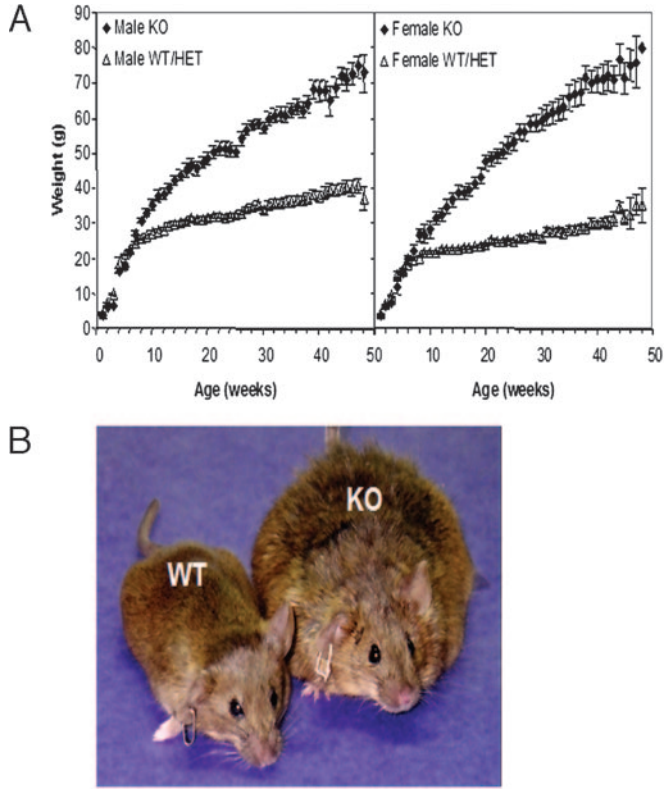

FIG. 2. Body weight as a function of age for CPE KO mice. A, Weights of male and female WT/HET and KO mice were recorded from 1–48 wk of age. n ≥ 50 animals from each group. B, Photograph of WT and CPE KO mice at 40 wk of age depicting the obesity and size of the null animal.

with WT controls (Fig. 3D). Although at 30 wk KO males and females had similar levels of locomotion during the dark phase, null females were less active than the males during the light phase at both 12 and 30 wk of age. Indeed, KO females demonstrated the greatest reduction in activity with age, whereas the decline seen with KO males was similar to that observed with WT controls. When rearing activity was considered, WT males and females displayed more vertical activity than KO mice regardless of age (data not shown). Rearing declined with age for all mice; however, it declined most precipitously for KO females who were less active than all other groups. By contrast, stereotypy scores were not distinguished by genotype and were reduced as the animals aged (data not shown).

Abnormalities in metabolism can contribute to obesity. We used indirect calorimetry to evaluate how the animals used their nutrients when activity levels were very low (Fig. 4, A and B). Although energy expenditure was not distinguished by genotype at 12 wk of age, older WT males and females expended more energy than the KO mice. These data show that basal metabolic rates are reduced in the older homozygous mutants. An examination of CO_2/O_2 exchange ratios reveals this respiratory quotient to be higher in null than WT animals at 12 and 30 wk of age (Fig. 4, C and D). This effect was significant for younger KO females and older null males.

Analysis of body fat content

Body fat content of 45- to 52-wk-old mice was analyzed by NMR. Male and female KO mice had body fat contents at 39.7% and 54.1% of their respective body weights, compared with 18.4% and 20.8% of weights from male and female WT animals.

Analysis of plasma leptin and CART from CPE KO mice

Leptin and CART, two peptides involved in feeding and energy expenditure (30, 31), were analyzed in plasma from WT and KO mice at 40–62 wk of age. KO animals (average weight 76.5 ± 3.9 g, n = 8) had plasma leptin levels at 131.7 ± 17.2 ng/ml. Age-matched WT mice (average weight 36.2 ± 1.6 g, n = 9) had leptin levels approximately four times lower (31.7 ± 6.6 ng/ml).

CART is synthesized as a precursor and processed to active CART I (5.1kD) and CART II (4.4 kDa) (32). To determine the molecular forms of CART in plasma of KO and WT mice, samples were analyzed by the Ciphergen protein chip assay (Fig. 5). Plasma from WT males contained primarily fully-processed CART II (4.4 kDa) and some CART I (5.1 kDa); no fully processed CART I or CART II were detected in plasma from KO mice. By contrast, concentrations of CART 6.2 kDa (Fig. 5) and 8.1 kDa intermediates (data not shown) were greatly enhanced in KO mice. Some pro-CART was also found in KO animals, but not in the WT controls (data no shown). Profiles from WT and KO females were similar to those for WT and KO males, respectively.

Development of diabetes in CPE KO mice

Fasting levels of glucose and insulin-like IR in plasma were monitored between 10 and 45 wk of age (Fig. 6). Because no genotype differences were noted between WT and HET animals, their data were collapsed into a single group. Although fasting plasma glucose was not distinguished by genotype at an early age, levels for both WT/HET and KO mice increased with age. With respect to males, fasting plasma glucose concentrations were significantly increased between 17 and 26 wk of age in KO males to a level approximately 2-fold higher than the WT/HET controls (Fig. 6, *top left*). Thereafter, no genotype differences were evident between males. For KO females, fasting plasma glucose levels became significantly augmented from 14 wk of age and persisted high throughout the remainder of the study (Fig. 6, *top right*). During this time, fasting plasma glucose contents in null females were increased by at least 2.5-fold over that of WT controls.

Analyses of fasting insulin contents in plasma revealed that insulin-like IR was low in WT/HET animals regardless of sex. By contrast, fasting plasma insulin levels significantly increased in male and female KO mice at 14–15 wk of age and remained elevated by 50- to 100-fold throughout life (Fig. 6, *bottom*).

HPLC analysis of (pro)insulin from plasma

Forms of insulin-like IR in plasma from 27-wk-old WT and KO animals were analyzed by HPLC. Greater than 90% of the insulin-like IR loaded onto the column from plasma of KO

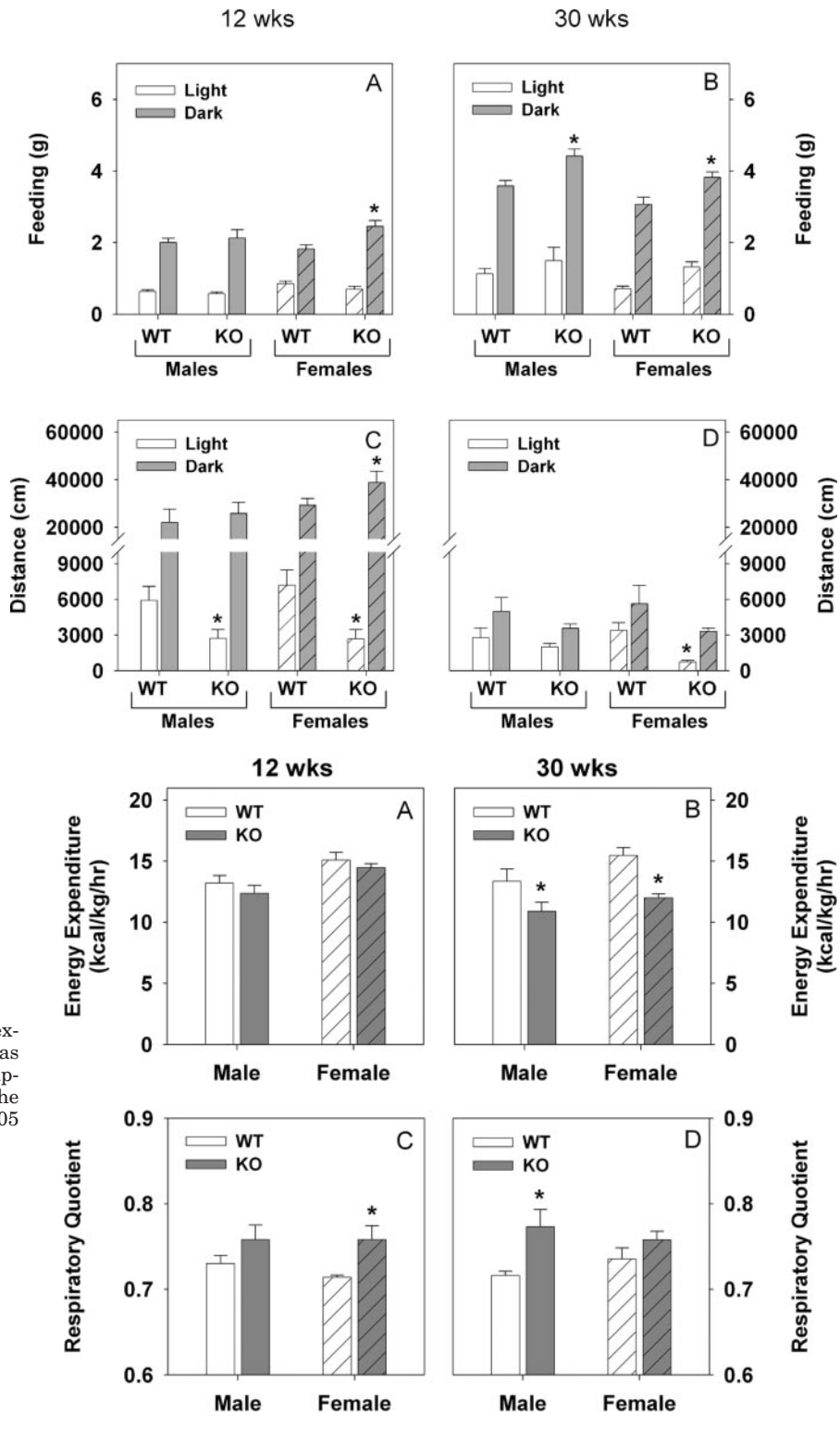

FIG. 3. Food intake and locomotor activity for CPE mice. Food consumption for male and female WT and KO mice at approximately 12 (A) and 30 wk of age (B) during the light and dark phases of the light-dark cycle. C and D, Spontaneous locomotor activity from the same WT and KO mice. *, $P < 0.05$ from WT controls during the light or dark phase of the light-dark cycle, n = 6–10 mice/group.

FIG. 4. Basal metabolic rates and respiratory exchange ratios in CPE mice. Energy expenditure was measured in resting WT and CPE KO mice at approximately 12 (A) and 30 wk of age (B). C and D, The respiratory quotient in the same mice. *, $P < 0.05$ from WT controls, n = 6–9 mice/group.

mice was recovered in fractions where authentic proinsulin elutes (Fig. 7). No mature insulin was detected, although small quantities cannot be discounted. No insulin-like IR material was detectable in WT plasma after HPLC because of the low levels in the original sample (data not shown). The elution profiles of the standards used are consistent with the previously described immunoreactive profiles of these molecules (33).

Glucose tolerance and insulin resistance

Glucose tolerance was examined in KO and WT mice at approximately 12 and 30 wk of age (Fig. 8). Following in-

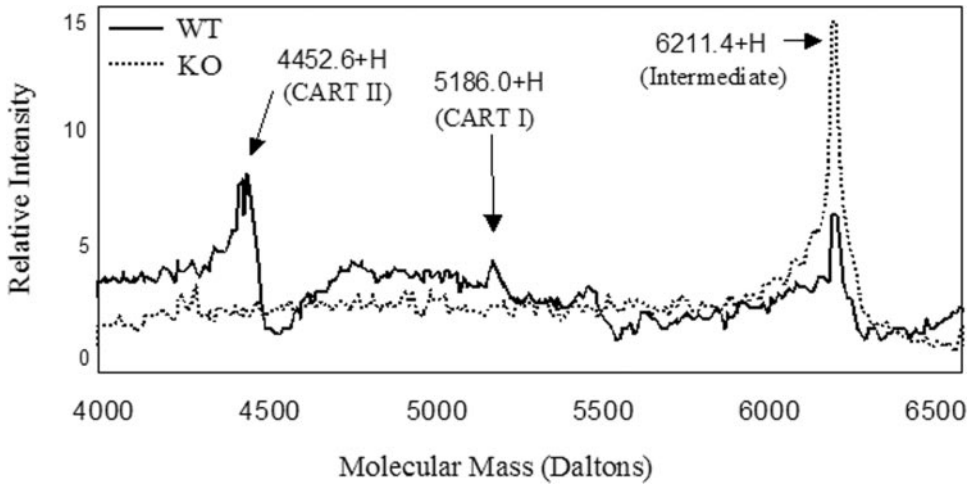

FIG. 5. Representative surface-enhanced laser desorption/ionization-time of flight mass spectrometry chromatographic profiles of CART-like IR in serum from male WT and CPE KO mice. The sera from WT and KO animals were analyzed using a PG20 ProteinChip, which was coupled with anti-CART (55–102). The relative intensity (percentage) in mass/charge is plotted for each peptide and shown for the region between 4000 and 6500 Daltons. Active forms of CART, CART I (4.4 kDa), and CART II (5.1 kDa) were present in serum from WT (*solid line*) mice, but not in CPE KO animals (*dashed line*). Intermediate forms of CART [either 6.2 kDa or 8.1 kDa (not shown)] were found in greater amounts in KO *vs.* WT animals. The profiles from sera of the female mice were similar to that for WT and KO males.

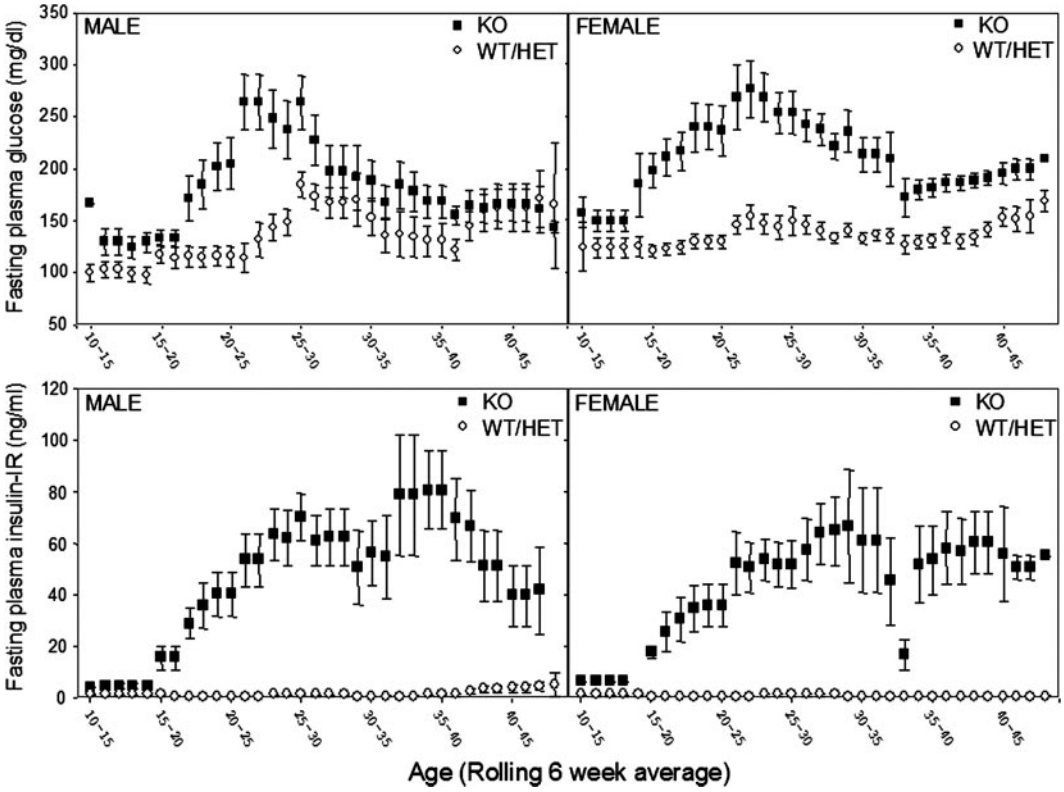

FIG. 6. Fasting plasma glucose and insulin levels in CPE mice. Animals were fasted for 6–8 h and blood was collected from mice at various ages. Because no significant differences were detected between WT and HET animals, their data were combined and compared with KO mice. *Top*, Fasting plasma glucose levels in WT/HET and KO males (*left*) and females (*right*). *Bottom*, Fasting plasma insulin-like IR in the same mice. n = 19–25 mice/group. Data are reported as a 6-wk rolling average.

jection of glucose, plasma glucose levels were significantly higher in KO mice at 12 and 30 wk compared with the WT controls. Although glucose levels decreased over time in the KO mice, the levels remained significantly elevated compared with WT animals. Levels of plasma glucose were higher for KO females than KO males at both age groups after glucose administration. Overall, male and female WT mice did not differ in their responses to glucose challenge at either age.

Insulin resistance in nonfasted CPE KO and WT mice was

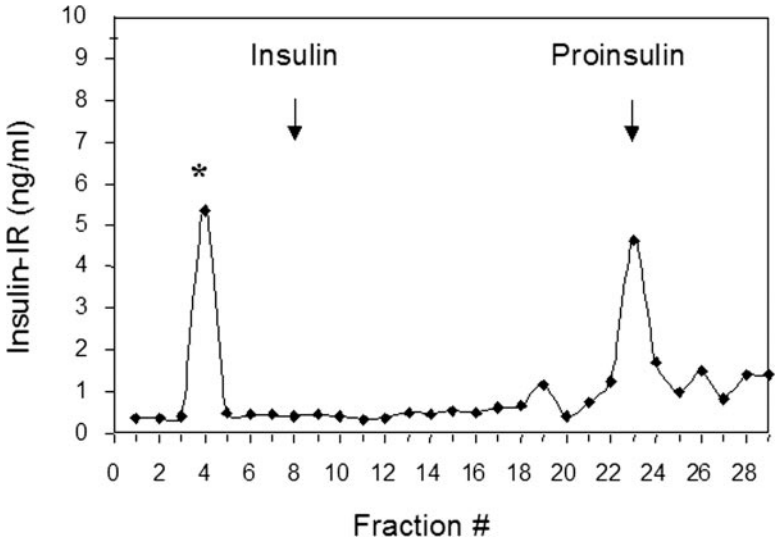

FIG. 7. Representative chromatographic profile of proinsulin and insulin in plasma from CPE null mice. Materials in plasma from KO females (27 wk old) were separated by reversed-phase HPLC. The column was equilibrated in 65% buffer A [50 mM phosphoric acid, 20 mM triethylamine, and 50 mM sodium perchlorate (pH 3.0)] and 35% buffer B (90% acetonitrile in water). After injection, buffer B was maintained at 35% for 5 min and then increased to 40% over the next 25 min. Fractions were assayed for insulin-IR by RIA. Approximately 90% of the injected insulin-IR was recovered in fractions 22–24. *Arrows* indicate the fractions where authentic insulin and proinsulin elute. *, A nonspecific peak present to the same extent in the WT sample (data not shown).

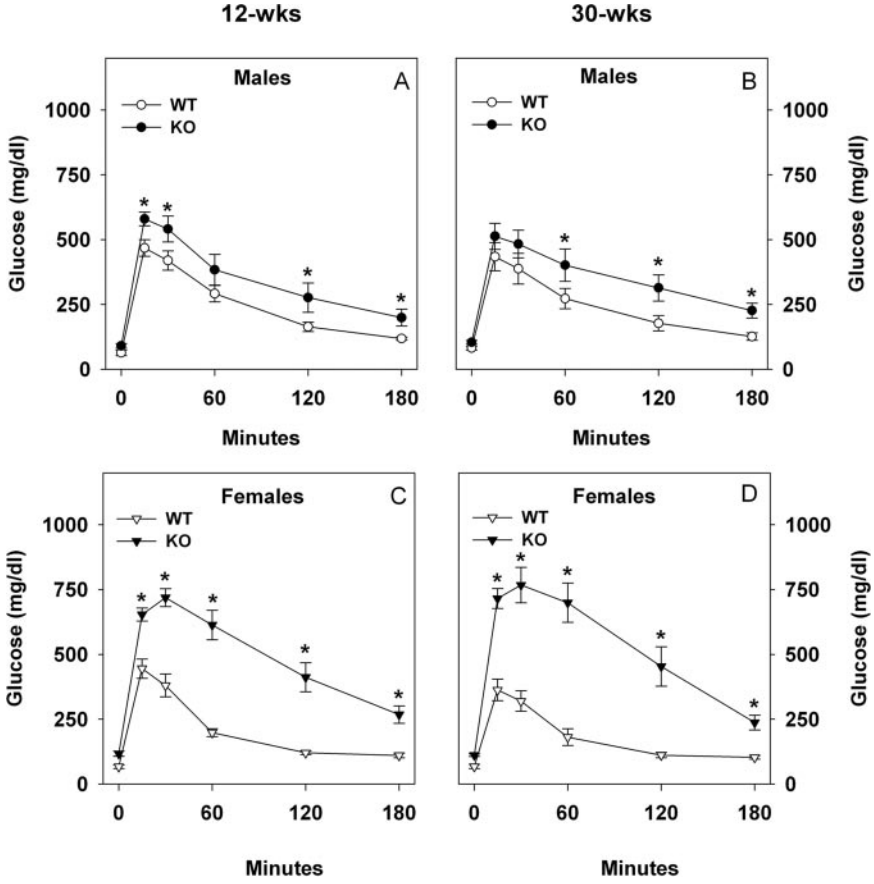

FIG. 8. Glucose tolerance in CPE mice at approximately 12 and 30 wk of age. Male (A and B) and female (C and D) WT and KO animals were fasted overnight, blood was collected the next morning (0 min), and animals were administered a solution of 25% D-glucose (ip, 11.1 mmol/kg body weight). Blood was collected 15, 30, 60, 120, and 180 min after injection and plasma glucose levels were determined. *, $P < 0.05$ from the WT control, n = 14–18 mice/group.

studied at approximately 12 and 30 wk of age (Fig. 9). At 12 wk of age, WT and KO females had higher baseline levels of glucose compared with values at 30 wk. After insulin administration, levels of plasma glucose were reduced in both WT and KO females; however, the response in KO females was blunted with respect to WT controls. Both WT and KO males at 12 wk of age showed similar responses to insulin over the 60 min. By 30 wk of age, however, plasma glucose levels in both male and female KO mice were suppressed significantly to lesser extents than male and female WT controls. Male and female WT mice at 30 wk were more responsive to insulin administration than the same groups at 12 wk of age.

To examine the basis of insulin tolerance in more detail, epididymal adipose cells from 50- to 60-wk-old animals were tested for insulin and proinsulin resistance. U-[^{14}C]-glucose uptake into isolated cells, a measure of glucose transport activity, was measured in the presence of supramaximal concentrations of insulin or proinsulin. In cells from WT male

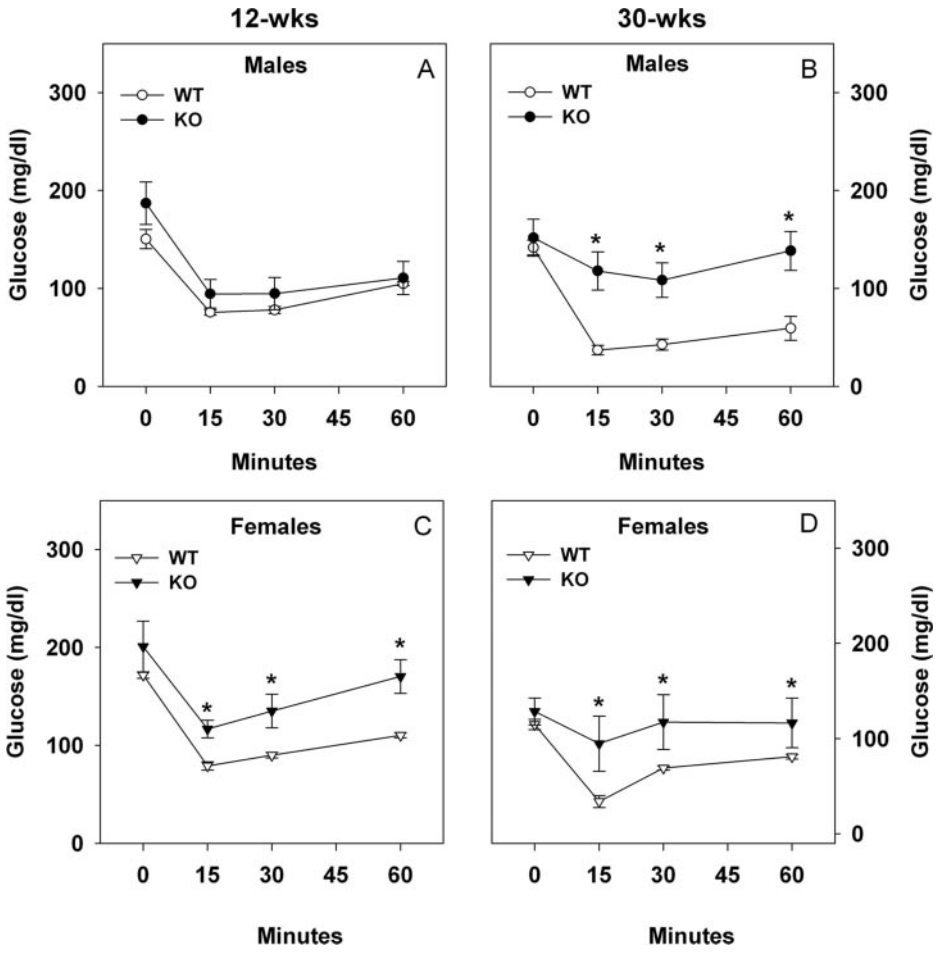

FIG. 9. Insulin tolerance test in CPE mice. At approximately 12 and 30 wk of age, nonfasted male and female CPE WT and KO mice were weighed, and an initial plasma glucose determination was made. The mice were given 1U/kg (ip) recombinant human insulin and glucose clearance was monitored at 15, 30, and 60 min post injection. *, $P < 0.05$ from the WT control, n = 6–12 mice/group.

and female animals, both insulin and proinsulin stimulated glucose transport activity by approximately 2.5-fold (data not shown). The corresponding responses in cells from KO males were reduced by 50%, whereas the cells from KO females were almost totally unresponsive (data not shown).

Reproductive status of CPE mice

Fertility of CPE mice was examined daily after pairing male and female WT or male and female KO animals for 30 d. Approximately 89% (17/19 animals) of the WT females became pregnant, whereas only 5% (1/20 animals) of the KO females showed evidence of pregnancy. The litter sizes were similar between WT (6.9 ± 0.3 pups) and KO dams (six pups).

Analysis of hypothalamic GnRH (a major regulator of reproduction in mammals) by A772 antiserum revealed that mature GnRH-like IR was reduced by approximately 75% in KO males and by 50% in null females compared with their respective WT controls (Fig. 10A). However, assay of the same samples with the B9 antiserum showed comparable levels of GnRH-like IR between WT and KO males, and at least 2.2-fold more GnRH-like IR in KO females compared with WT controls (Fig. 10B). To evaluate processing, the ratio of A772 IR (fully processed GnRH) to B9 IR (GnRH plus intermediates) was calculated. This ratio was reduced by more than 78% in both male and female KO mice (Fig. 10C).

Neurological and behavioral status

No main effects of sex or a sex by genotype interaction were observed for any test. Hence, results were collapsed across sex and presented as the total number of animals in each genotype. Behavioral performance of HET mice did not differ significantly from WT animals on any measure (Table 1). The condition of the fur (*e.g.* social interaction) was similar in all animals and all mice were well groomed. The KO mice at 15 wk of age exhibited less abdominal muscle tone than WT and HET controls and several KO animals displayed a mild tremor.

Although KO mice had reduced responses in all reflexes tested, only visual placing and toe pinch were significantly impaired. KO animals were also lethargic and significantly less responsive in measures of reactivity (*e.g.* touch escape, transfer arousal, and, positional passivity); however, they did not have significantly reduced responses to provoked dowel biting. Compared with WT and HET animals, the KO mice were significantly impaired in almost every test of strength and coordination (grip strength, wire maneuver, and vertical pole), and motor performance (*e.g.* gait, horizontal activity, and vertical activity; time active).

Morbidity

Under conditions of normal colony maintenance (*i.e.* without invasive experimentation), six of 12 KO females and five

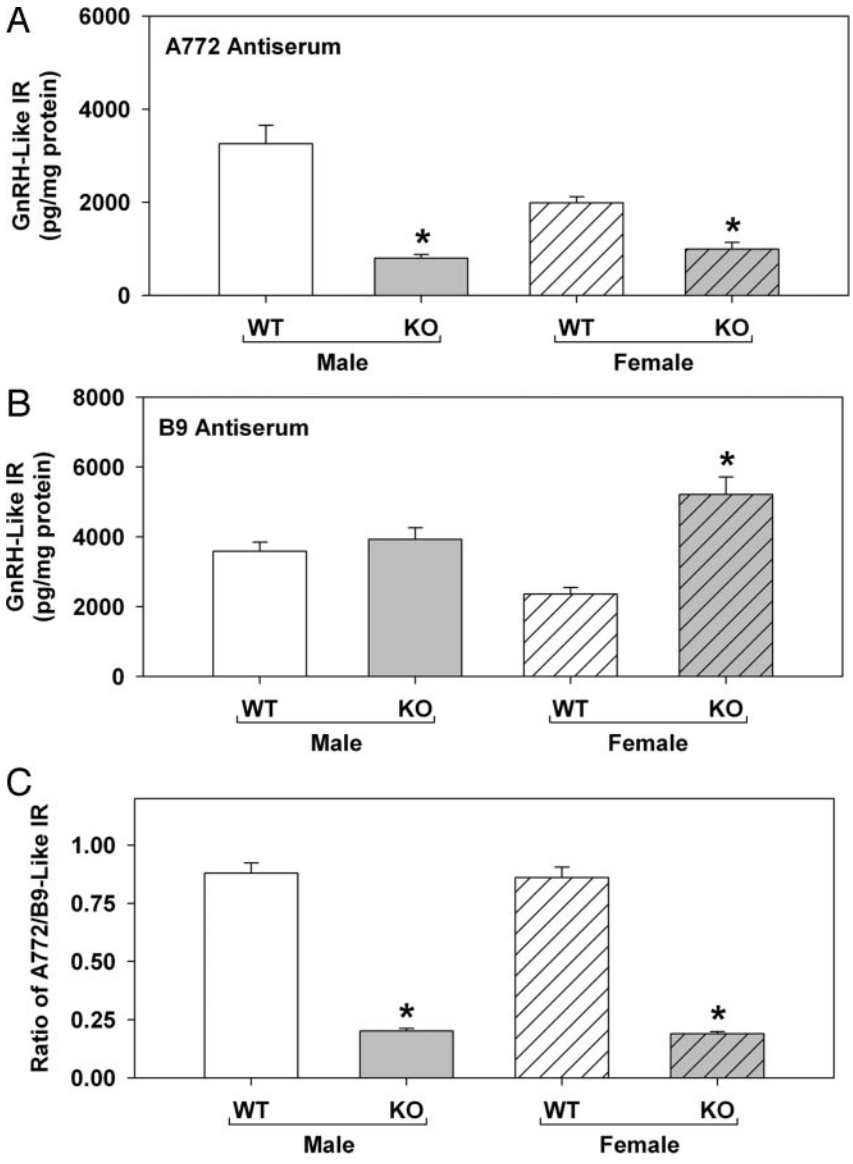

FIG. 10. Hypothalamic GnRH levels in CPE mice. A, GnRH-like IR as measured with the A772 antiserum in male and female WT and KO mice. The A772 recognizes the fully processed GnRH and does not bind any intermediates with extended amino acids at the C terminus. B, GnRH-like IR as measured with the B9 antiserum that recognizes the decapeptide and all GnRH intermediates. C, The ratio of GnRH-like IR as quantified with the A772 and B9 antiserum. *, $P < 0.05$ from the WT controls, n = 10 mice/group.

of 20 KO males have died for unknown reasons. Of these 11 mice, seven of them (four males and three females) died between the ages of 32 and 52 wk. In comparison, two of 44 WT animals died by 10 wk of age and three of 106 HET animals died at 23, 38, and 65 wk of age, respectively.

Discussion

CPE KO mice develop obesity

Body weight measurements revealed that the CPE KO mice were initially lighter in weight than the WT animals but became heavier by 8 wk of age. This enhancement in body weight continued, so that, by 40 wk of age KO weights exceeded those of the WT controls by 2- to 3-fold. KO females appeared to be more affected than the KO males because the weight difference attributed to gender was overcome earlier, such that male and female KO mice had overlapping progressions in weight gain. This is in contrast to the $CPE^{fat/fat}$ mouse where weight differences between male and female mutants was maintained until adulthood (34). Hence, the CPE KO mice present a more severe obese phenotype. Indeed, $CPE^{fat/fat}$ mice did not appear to get as heavy as the KO mice, although this may be because of mouse strain differences. Analyses of body composition by NMR confirmed that the weight gain in the KO mice was primarily because of an accumulation of body fat, accounting for approximately 40% and more than 50% of the body weight of male and female mice, respectively. Part of the male KO weight gain could be explained by increased body length, but this does not appear to be the case for KO and WT females whose body lengths were similar. Analysis of food consumption, showed that KO females consumed more food by 12 wk of age than WT controls. Again, this is in contrast to $CPE^{fat/fat}$ mice whose food intake at similar ages were not different from WT animals (34). Whereas KO and WT males showed no difference in food or water consumption at 12 wk, similar to the male $CPE^{fat/fat}$ mice at that age, the KO males did display an enhanced food consumption at 30 wk.

This increased consumption is likely a result of improper

signaling in the control of eating behavior that involves the balance of orexigenic and anorectic neuropeptides in the hypothalamus (35). Proper maturation of these neuropeptides requires correct trafficking and processing within these peptidergic neurons, of which CPE plays an important role. One of these neuropeptides, CART, functions as an anorectic peptide by inhibiting eating behavior and is secreted from the hypothalamus in response to leptin (30, 31). However, it is only active when processed to its mature form. Because fully processed levels of CART are absent in KO mice, these mutants should have less control over their eating behavior compared with WT animals. This processing deficit may contribute to their obesity by augmented eating.

In addition to consuming more food, we also observed that the KO animals exhibited reduced spontaneous activity during the light phase of the light-dark cycle, they had reduced energy expenditures, and they appeared to use less of their fat as calories than the WT controls. These findings indicate that for CPE KO animals, physical inactivity, reduced basal metabolism, and reduction in fat expenditure may contribute to enhanced lipid stores and obesity of the CPE KO animals.

CPE KO mice become diabetic

Both male and female CPE KO mice became hyperglycemic by approximately 17 wk of age. However, in glucose tolerance tests, glucose handling was abnormal even in the 12 wk group of KO animals. As observed with the obesity and feeding behavior of the KO females at this age, glucose clearance was significantly more impaired in KO females than males, suggesting that impaired glucose clearance in the KO females is an additional factor contributing to the severity of their obesity phenotype. Indeed, although KO males recovered from the diabetes later in life, high fasting levels of glucose persisted in the null females, although some diminution was observed after approximately 35 wk. This reversal in diabetes was previously observed in the $CPE^{fat/fat}$ males; however, in contrast to the CPE KO females, the $CPE^{fat/fat}$ females exhibited only mild hyperglycemia (34).

The rise in fasting plasma glucose was accompanied by an augmentation in the fasting plasma insulin-like IR, which was identified by HPLC as proinsulin. This rise in proinsulin began at 15–20 wk, and it remained elevated by 50- to 100-fold throughout life compared with WT controls. For KO males, the high levels of proinsulin were maintained through 40–45 wk; 10 wk after fasting glucose levels had returned to near WT levels. These data suggest that, for the KO males, the recovery from diabetes may be because of the very high levels of circulating proinsulin because proinsulin is known to have biological activity, but at 1% of the efficacy of normal insulin (36). However, this does not appear to be the case for KO females whose levels of proinsulin remain as high as for KO males yet they continue to be hyperglycemic. As a possible explanation, this dichotomy may occur early in life as young KO males (12 wk) respond relatively normally to insulin challenge, whereas it is blunted in KO females. Additionally, in older animals (50–60 wk of age), glucose uptake activity in adipose cells is completely resistant in females, whereas in males it is reduced by 50%.

Maintenance of normal plasma glucose levels beyond 37 wk in the KO males could also be because of the high levels of leptin, which is reported to increase hepatic insulin sensitivity (37). Enhanced glucose uptake into the liver could account for the reversal of the hyperglycemia if this effect was specific for males only. Enlarged livers with abundant fat deposits were observed in a majority of these CPE KO mice (Cawley, N. X., and Y. P. Loh, unpublished observations).

CPE KO mice are lethargic and exhibit defective muscle physiology

To explore the nature of the physical inactivity that may contribute to the obesity, neurobehavioral evaluations of the CPE KO mice were performed. The observations showed that the KO animals had reduced performance in all tests that required muscle strength or coordination. For example, deficits occurred in reflexes of visual placing and toe pinch; the reactivity measures of touch escape, transfer arousal, and positional passivity; and the measures of strength and coordination such as grip strength, wire maneuver and vertical pole climbing. In addition, all measures of motor abilities in the open field were reduced in the CPE KO animals. Although the greater body weight of the CPE KO animals may contribute to their inactivity, the reduction of abdominal tone as early as 15 wk of age suggests that a defect in muscle physiology could contribute to the lethargy of the CPE KO animals. In fact, metabolic abnormalities in muscle because of the obesity, such as reduced oxidative enzyme activity (38), could account for some of these deficits. Despite these points, KO performance on reflexes that do not require extensive movements of the body such as the corneal reflex, pinna reflex, trunk curl, or provoked dowel biting were also somewhat reduced, suggesting that CPE KO mice may also possess some sensory/neural defects.

Reproductive deficits in CPE KO mice

The results of the mating studies showed that although the KO animals are not completely infertile, reproduction is severely impaired. Successful reproduction requires an intact hypothalamic-pituitary-gonadal system. Within this feedback system, GnRH is the only major reproductive peptide. Because CPE is an exopeptidase responsible for removing basic amino acids from the C terminus of peptides, we postulated some time ago that this enzyme was critical in processing the pro-GnRH to the bioactive decapeptide (25, 26). Analyses of GnRH-like IR with the A772 antiserum suggests that fully-processed GnRH levels in hypothalamus are significantly depressed in KO animals. By contrast, when concentrations are measured with the B9 antiserum, levels of GnRH and its intermediates appear to be similar to or even enhanced above values for the WT controls. Given the antigenic determinants of the two antisera (26, 28), our results suggest that processing of C-terminal extended GnRH intermediates in hypothalamus is severely impaired in the CPE KO mouse and that this abnormality likely contributes to the sub-fertility in these animals. Similar findings have been reported in the $CPE^{fat/fat}$ mouse (27).

Role of CPE in nervous system function

Our studies on the CPE KO mouse indicate that CPE is important at many different physiological levels. Most intriguing are the neurological deficits. Because CPE plays a critical role in the processing (39, 40) and sorting (3, 9, 10) of peptide hormones and neuropeptides in the RSP, it is not surprising that lack of regulated secretion of mature neuropeptides in the central nervous system can exert effects on neurotransmission and, perhaps, neuronal development of these mice. Indeed, deficits in neurotransmission have been reported at the neuromuscular junction in a mutant CPE null *C. elegans* (22) and at photoreceptors in the retina of CPE KO mice (41). A role of CPE in neurodevelopment is highly plausible because CPE is expressed in the developing nervous system during the time of synapotgenesis (42) before expression of the endocrine system in the embryo. Furthermore, our recent studies indicate that sorting and activity-dependent secretion of brain-derived neurotrophic factor from embryonic central nervous system neurons of the CPE KO mouse is obliterated (Lou, H., S.-K. Kim, E. Zaitsev, C. R. Snell, B. Lu, and Y. P. Loh, submitted for publication). This deficit could have a profound effect on neural development, synaptic plasticity, and memory (43).

In summary, studies on the CPE KO mouse have provided new insights into the possible roles of CPE, particularly within the nervous system that heretofore have not been recognized. Additionally, many challenges lie ahead in understanding the mechanisms underlying the onset of obesity and the reversal of hyperglycemia in CPE KO mice. Continued studies on this mouse model will provide exciting new insights into the important roles CPE play, beyond that of a prohormone processing enzyme (1) and sorting receptor (3, 10) in endocrine and neuroendocrine cells.

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