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Adult Carboxypeptidase E-Deficient *fat/fat* Mice Have a Near-Total Depletion of Brain CCK 8 Accompanied by a Massive Accumulation of Glycine and Arginine Extended CCK

Identification of CCK 8 Gly as the Immediate Precursor of CCK 8 in Rodent Brain

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Cholecystokinin (CCK) amide concentrations were reduced over 85% in all the major brain regions of carboxypeptidase E (Cpe)^{fat}/Cpe^{fat} mice in comparison to control mice. Using an radioimmunoassay (RIA) specific for glycine-extended CCK (CCK Gly), low levels of CCK Gly were detected in control (0.65 ng/g tissue) and were even lower in Cpe^{fat}/Cpe^{fat} (0.246 ng/g) mice brain extracts. After treatment with carboxypeptidase B, the level of CCK Gly in Cpe^{fat}/Cpe^{fat} in these brain extracts was elevated to 33.5 ng/g, about 51-fold higher than in control. On gel-filtration chromatography and high-performance liquid chromatography (HPLC), this material coeluted with CCK 8 Gly. These results demonstrate that CPE is required for the correct processing of arginine- and glycine-extended CCK in all major regions of the mouse brain. These results support the hypothesis that CCK 8 Gly is the immediate precursor of CCK 8 amide in mouse brain, not larger amidated forms like CCK 22 or CCK 33.

Key Words: CCK; *fat/fat*; carboxypeptidase E/B/D; obesity; leptin; paraventricular nucleus of the hypothalamus.

Introduction

Cholecystokinin (CCK) was first discovered as a gastrointestinal hormone that is released after the ingestion of food, and causes the contraction of the gallbladder and the release of enzymes from the pancreas. CCK is very abundant in the brain, where it serves as a neurotransmitter or neuromodulator, and may play a role in anxiety, memory, analgesia and satiety *(1)*. The satiety effect of CCK in the control of food intake has been demonstrated in several species *(2)*, including humans *(3)*.

During the biosynthesis of CCK 8, pro-CCK is tyrosine-sulfated and cleaved at a number of single and paired basic residues *(4)*. The further participation of a carboxypeptidase is likely. Carboxypeptidase E (Cpe), an enzyme found in the brain and various neuroendocrine tissues *(5)*, removes basic residues remaining at the carboxyl-terminus of some peptides prior to conversion of the carboxyl-terminal glycine to an amide by peptidylglycine-α-amidating monooxygenase (PAM) *(6)*.

Cpe^{fat}/Cpe^{fat} mice homozygous for the fat mutation possess a point mutation in the Cpe gene, which converts Ser^{202} to a Pro rendering it inactive *(7)*. At a biochemical level, the mutant protein when expressed in endocrine tumor cells, is unstable and is degraded in the endoplasmic reticulum *(5)*. Lack of this enzyme causes the animals to become obese at 8–12 wk of age. They are hyperglycemic, but they remain very insulin-sensitive. Pro-insulin is produced in abundance, but in the absence of Cpe, little of it is active *(7)*. Cpe^{fat}/Cpe^{fat} mice also have impaired processing of enkephalins, neurotensin, substance P, luteinizing hormone releasing hormone (LHRH), and gastrin *(8–10)*.

Our previous study on whole Cpe^{fat}/Cpe^{fat} mouse brains demonstrated that active CCK 8 amide levels were depleted by at least 80% compared to control brains *(11)*, whereas their duodenums were much less affected. In this present study, regional brain differences in this depletion were examined. Using freshly extracted tissues and a new RIA directed against CCK Gly, the glycine-extended CCK peptide, which accumulates in the brains of these mice, was identified.

Results

The levels of CCK in the major brain regions of control and Cpe^{fat}/Cpe^{fat} mice at 6–8 wk of age are compared in Fig. 1. The active form of CCK was detected by CCK RIA specific for amidated CCK, and the level was expressed as pg CCK immunoreactivity/μg protein. In the brains of con-

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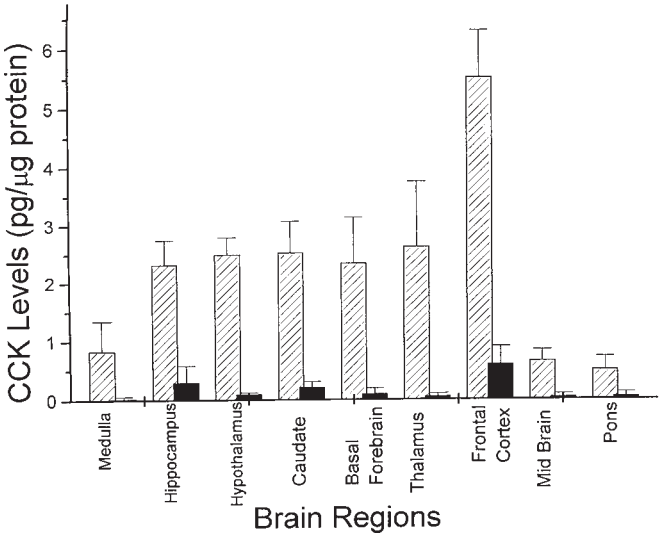

Fig. 1. Distribution of immunoreactive CCK in brain regions of control (shaded bars) and Cpe^{fat}/Cpe^{fat} (black bars) mice. Data are presented as means ± SEM, $n = 5$. Bars represent mean and vertical line SEM. Data were analyzed by two-way t-test, and $p < 0.5$ was considered significant.

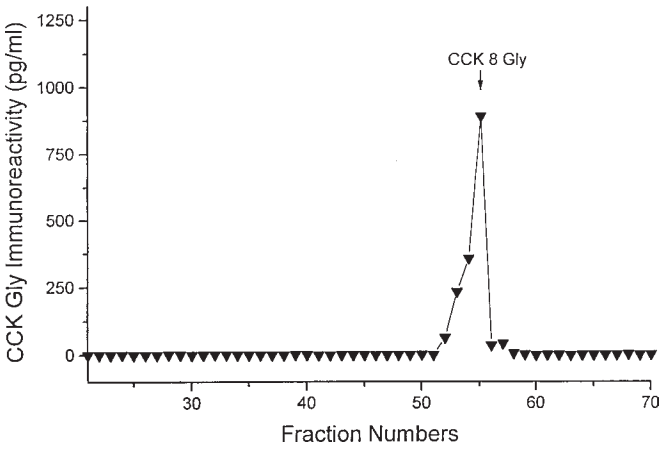

Fig. 2. Sephadex GCL-90 gel-filtration chromatography of Cpe^{fat}/Cpe^{fat} cerebral cortex treated with CpB. One-milliliter fractions were collected, and an aliquot assayed by the CCK Gly RIA. The arrow indicates the position of CCK 8 Gly standard.

trol mice, CCK concentration is similar to levels in rat brain *(12)*. In all the regions measured, the CCK concentrations were dramatically reduced (over 85%) in Cpe^{fat}/Cpe^{fat} mice when compared to control.

In our previous study *(11)*, the brains of Cpe^{fat}/Cpe^{fat} mice had 13-fold more immunoreactive glycine- and arginine-extended CCK peptide detected with an antiserum specific for gastrin Gly Arg Arg (which also crossreacted with CCK Gly Arg Arg). Based on size and antibody crossreactivity, this material appeared to be CCK 8 Gly Arg Arg. In the present study, we used a newly developed CCK Gly RIA that detects glycine extended CCK to identify this peptide further. This strategy was inspired by previous use of a gastrin Gly antiserum to detect pro-gastrin and pro-CCK in tumor extracts following trypsin and carboxypeptidase treatment *(13)*. Freshly prepared extracts from cortex of control and *fat*/*fat* mouse cortex ($n = 1$), which were sacrificed at 6 mo of age, were assayed with the CCK Gly RIA. Low levels of CCK Gly were detected in both control (0.65 ng/g) and even lower levels in Cpe^{fat}/Cpe^{fat} (0.246 ng/g) mice. This is an expected result, since CCK Gly immunoreactive peptides are presumed to be normal products of CCK biosynthesis, and reduced levels should be found in tissues lacking carboxypeptidase. After treatment with CpB, the levels of CCK Gly in Cpe^{fat}/Cpe^{fat} mice brain were elevated to 33.5 pg/g, about 25-fold higher than that in control mice, indicating a massive accumulation of glycine- and arginine-extended CCK peptide in the mutant mice.

In order to determine the molecular nature of this glycine-extended CCK form in Cpe^{fat}/Cpe^{fat} brain, extracts were subjected to a GCL-90 Sephadex column, and fractions were assayed for CCK Gly. The chromatography profile is shown in Fig. 2. Control mice brain extracts with or without CpB had very low levels of CCK Gly peptide, as did Cpe^{fat}/Cpe^{fat} mice without CpB (data not shown). Brain extracts from Cpe^{fat}/Cpe^{fat} mouse cerebral cortex after CpB treatment have a prominent peak, which coeluted with CCK 8 Gly.

To verify that the major peak observed on the Sephadex column from the Cpe^{fat}/Cpe^{fat} mice brain extracts after CpB treatment is CCK 8 Gly, the Cpe^{fat}/Cpe^{fat} brain sample was subjected to high-performance liquid chromatography (HPLC) and analyzed by CCK 8 Gly RIA. Since our synthetic CCK 8 Gly standard is not tyrosine-sulfated, brain extract samples were also desulfated by arysulfatase prior to HPLC chromatography. A single peak is observed in Cpe^{fat}/Cpe^{fat} samples at the same position as CCK 8 Gly standard (Fig. 3). Lesser amounts of the same peptide are also found in control mouse brain samples (data not shown). This supports the hypothesis that the glycine-extended form of CCK found in the brain of control and Cpe^{fat}/Cpe^{fat} mice is CCK Gly. It is likely that the form that accumulates in mutant mice is a glycine- and arginine-extended CCK 8, probably CCK 8 Gly Arg Arg.

Discussion

By 6–8 wk of age when the *fat*/*fat* mice can first be distinguished from their littermates, active CCK levels were depleted by over 85% in their brains relative to controls. All brain regions examined were equally affected. Although no developmental study with a sufficient number of subjects for statistics was performed, it appears that the extent of CCK depletion increases with the age of the animals in parallel with the development of their obesity. By 6 mo of age, when the Cpe^{fat}/Cpe^{fat} mice are at least two times the size of their littermates, the levels of CCK in cerebral cortex were reduced by 90%. The degree of accumulation of

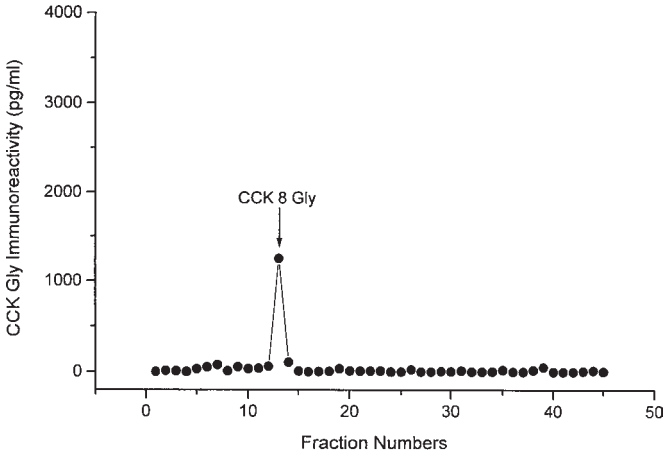

Fig. 3. HPLC profile of Cpe^{fat}/Cpe^{fat} mouse midbrain extracts treated with CpB and sulfatase. The samples were eluted with a gradient from 30% buffer B to 70% buffer B in 40 min at a flow rate of 1 mL/min. Forty-five 1.5-mL fractions were collected, and an aliquot assayed with CCK Gly RIA. The elution position of unsulfated CCK 8 Gly is indicated by the arrow.

the arginine and glycine extended form appears to increase also (13-fold vs 25-fold) between 6 and 8 wk and 6 mo. In contrast, the CCK levels of the duodenum of the oldest animals were not different in Cpe^{fat}/Cpe^{fat} and control mice, indicating that this is purely a central nervous system (CNS) deficiency.

These results prove that Cpe is required for normal processing of CCK 8 Gly Arg Arg to CCK 8 Gly. The presence of small amounts of CCK 8 amide in Cpe^{fat}/Cpe^{fat} mouse brain is probably being produced by other enzymes, such as carboxypeptidase D *(14)*. The absence of Cpe in the duodenum appears to have little effect on the production of active CCK peptide there. Perhaps it is also carboxypeptidase D that is active in this region.

The chromatography and HPLC results clearly demonstrated the accumulation of a glycine- and arginine-extended peptide in Cpe^{fat}/Cpe^{fat} mouse brain extracts, which can be converted to a CCK Gly-immunoreactive peptide with carboxypeptidase B (CpB) treatment. This provides further support for our previous observation that amidated CCK 8 originates from CCK 8 Gly Arg Arg instead of larger peptides, such as amidated CCK 33, CCK 22, or CCK 12. Immunoreactive glycine-extended CCK and gastrin peptides have been observed before *(15–17)*, but have never been chemically characterized. Glycine-extended CCK peptides are particularly abundant in CCK-expressing endocrine cells in culture where the amidation reaction appears to limit the production of amidated peptides *(18)*.

The physiological regulation of feeding is proving to be very complex and may involve interactions among CCK, leptin, NPY, bombesin, melanocortins, insulin, and glucagon at both central and peripheral sites. The observation that the Cpe^{fat}/Cpe^{fat} mouse has greatly decreased brain CCK but the duodenum is largely unaffected is something of a surprise, but it can perhaps be understood in the context of our current understanding of the possible role of CCK in the regulation of feeding. The recent reports that CCK and leptin act synergistically to reduce short-term food intake *(19)*, activate c-*fos* staining in oxytocin and vasopressin neurons in the paraventricular nucleus (PVN) of the hypothalamus *(19)*, as well as to activate a subtype of gastric vagal afferent terminals *(20)* may help explain why this CNS CCK deficiency produces profound obesity in these mice. It is known that CCK released peripherally in response to a meal activates capsaicin-sensitive afferent vagal fibers that transmit signals to various brain sites, including the PVN. In the PVN, signals from the periphery are integrated to regulate feeding behaviors. CCK is extensively colocalized with oxytocin in the PVN *(21)*, so if CCK is one of the neurotransmitters used by the PVN to regulate feeding, this part of the pathway may be interrupted in the *fat*/*fat* mouse, causing it to become obese.

Although the *fat*/*fat* mouse represents a complex phenotype that is known to be defective in the processing of a number of amidated neuropeptides, it may provide an useful model to test the role of central CCK in satiety and in other behaviors associated with CCK.

Materials and Methods

Animals

Control (Cpe^{fat}/Cpe^{+}; Cpe^{+}/Cpe^{+}) and Cpe^{fat}/Cpe^{fat} mice were obtained from the Jackson Laboratories (Bar Harbor, ME). They were maintained and sacrificed as previously described *(10)*. For measurement of CCK levels in specific brain regions, the mice were sacrificed at 6–8 wk old, just as the *fat*/*fat* mice could be easily distinguished from the controls. The mice were decapitated. The brains were quickly removed and cut with a razor blade into slices. The major brain regions were free-hand dissected with a scalpel, frozen immediately on dry ice, and stored –80°C. For the chromatography, one pair of 6-mo-old mice were sacrificed, and cortex and the rest of the forebrain separated prior to extraction. Tissue samples were extracted by sonication in 0.1 *N* HCl.

Radioimmunoassay (RIA)

The CCK RIA was performed as previously described *(12)*. Antiserum R5 is highly specific for amidated CCK. It displays 0.001% crossreactivity with CCK 8 Gly, gastrin 13 Gly, CCK 8 Gly Arg, and CCK 8 Gly Arg Arg. The CCK Gly RIA utilized a CCK 8 Gly antiserum (#22) generated from unsulfated CCK 8 Gly conjugated in a 50:1 molar ratio to keyhole limpet hemocyanin with 0.1% glutaraldehyde. The tracer was ^{125}I-labeled Gastrin 13 Gly (Gly-Leu-Glu-Glu-Glu-Glu-Glu-Ala-Tyr-Met-Asp-Phe-Gly) separated by Sephadex G10 and DEAE A-25. With ^{125}I-labeled Gastrin 13 Gly as tracer, antiserum 22 crossreacts 40% with

CCK 8 Gly, 0.4% with sulfated CCK 8 amide, 1.25% with unsulfated CCK 8 Gly Arg, and 1.25% with unsulfated CCK 8 Gly Arg Arg. The assay detects about 1 pg G13Gly and has an ED_{50} of about 50 pg. It has an intra-assay variation of about 8% and an interassay variation of about 15%.

Sample Preparation for Chromatography

Tissue extracts were partially purified by Sep-Pak C18 Cartridges (Millipore, Bedford, MA), with an elution buffer of 90% acetonitrile/0.1% trifluoroacetic acid (TFA). Samples eluted from the Sep-Pak elution buffer were concentrated to 1 mL in a Speedvac concentrator, followed by centrifugation at 4°C for 15 min to clarify. For CpB treatment, Sep-Pak purified samples (1.0 mL) were treated with 100 μL (500 μg) CpB (Boehringer, Indianapolis, IN) at 20°C for 30 min, and the reaction stopped by boiling for 10 min. For HPLC analysis, samples were treated in the same manner, except that 65 m*M* dithiothreitol were added to the sample before Sep-Pak and to the Sep-Pak elution buffer to prevent the oxidation of the two methionines in CCK 8. Samples (1 mL) for HPLC were also desulfated by incubation with arylsulfatase (2.5 mg) (Sigma 8629) (in 500 mL of 0.2% NaCl) in 200 m*M* acetate buffer, pH 5.0, at 37°C for 2 h, as previously described *(22)*.

Gel-Filtration Chromatography

Brain extracts (1 mL/sample) prepared as described above were applied to a 2.5 × 45 cm GCL-90 (Isco, Inc., Lincoln, NE) gel-filtration column, which was equilibrated and run at 4°C in 50 m*M* Tris, pH 7.8, containing 200 m*M* NaCl, 0.02% sodium azide, and 0.1% BSA. Fractions of 1 mL were collected, and peptide elution determined by RIA.

High-Performance Liquid Chromatography (HPLC)

A Vydac reverse-phase C18 column was run at a flow rate of 1 mL/min with buffer A: 0.09% TFA and buffer B: 90% acetonitrile in 0.09% TFA. For the gradient conditions, see Fig. 3. All solvents were sparged with He gas prior to and throughout analysis. Fractions of 1.5 mL were collected. Aliquots were dried in a SpeedVac concentrator and assayed by CCK Gly RIA. Elution of synthetic peptide standards was also monitored spectroscopically at 214 nm.

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