Attenuated processing of proglucagon and glucagon-like peptide-1 in carboxypeptidase E-deficient mice

L Friis-Hansen, K A Lacourse¹, L C Samuelson¹ and J J Holst²

Department of Clinical Biochemistry, Rigshospitalet, DK-2100 Copenhagen, Denmark

¹Department of Physiology, University of Michigan, Ann Arbor, Michigan 48109-0622, USA

²Department of Physiology, The Panum Institute, DK-2200 Copenhagen, Denmark

(Requests for offprints should be addressed to L Friis-Hansen, Department of Clinical Biochemistry, KB-3014 Rigshospitalet, 9 Blegdamsvej, DK-2100 Copenhagen, Denmark; Email: lfh@rh.dk)

Abstract

The maturation of many peptide hormones is attenuated in carboxypeptidase E (CPE)-deficient *fat/fat* mice, leading to a slowly developing, adult-onset obesity with mild diabetes. To determine the contribution of the hormones generated from the proglucagon precursor to this phenotype, we studied the tissue-specific processing of glucagon and glucagon-like peptide-1 (GLP-1) in these mice. In all tissues examined there was a great reduction in mature

amidated GLP-1. Furthermore, a lack of CPE attenuates prohormone convertase processing of proglucagon in both the pancreas and the intestine. These findings suggest that defects in proglucagon processing together with other endocrine malfunctions could contribute to the diabetic and obesity phenotype in *fat/fat* mice.

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Introduction

The *CPE^{fat}* mouse strain (*fat*) exhibits a slowly developing adult-onset obesity with mild diabetes due to a spontaneous genetic mutation (Ser202Pro) in carboxypeptidase E (CPE) (Naggert *et al.* 1995), which abolishes enzyme activity and impair routing of CPE to the secretory granules (Varlamov *et al.* 1997). CPE removes C-terminal basic residues exposed after endoproteolytic processing of the prohormone precursor by the prohormone convertases (PCs). In all of the endocrine tissues examined so far, removal of C-terminal basic residues of peptide hormones has been attenuated in the *fat/fat* mice, but the overall response to the defect varies. Some endocrine systems respond by increasing the concentration of prohormone to maintain normal concentrations of the mature hormone, thus compensating for the attenuation in peptide hormone processing. Increased prohormone synthesis has been shown for insulin (Naggert *et al.* 1995), gastrin, (Lacourse *et al.* 1997, Udupi *et al.* 1997) and endocrine cholecystokinin (CCK) peptides (Cain *et al.* 1997, Lacourse *et al.* 1998). In other endocrine systems, there are no compensatory increases in hormone synthesis resulting in decreased concentrations of mature peptide hormone, as seen for dynorphin, neurotensin, melanin-concentrating hormone (Rovere *et al.* 1996) pro-opiomelanocortin (POMC) (Cool *et al.* 1997), substance P (Perloff *et al.* 1998) and neuronal CCK (Cain *et al.* 1997, Lacourse *et al.* 1998). Furthermore, Loh and co-workers

have suggested that CPE could also act as a sorting receptor for insulin and POMC, and that loss of CPE is followed by a shift from regulated to constitutive hormone secretion (Cool *et al.* 1997, Normant & Loh 1998, Rindler 1998).

Proglucagon is a neuroendocrine peptide hormone precursor with a very tissue-specific processing pattern (for a review, see Holst 1997). In the pancreatic α -cells, proglucagon is processed to yield glucagon and the major proglucagon fragment (MPGF) (see Fig. 1). In intestinal L cells and neurons, proglucagon processing results in the liberation of glicentin, glucagon-related polypeptide (GRPP), oxyntomodulin, glucagon-like peptide-1 (GLP-1), intervening peptide-2 (IP-2) and glucagon-like peptide-2 (GLP-2) (see Fig. 1). Glucagon is an important regulator of glucose metabolism, having effects grossly opposing those of insulin. Gut GLP-1 is one of the incretin hormones and lowers blood glucose by several mechanisms: it stimulates insulin secretion in a glucose-dependent manner; it confers glucose sensitivity to β -cells; it inhibits glucagon secretion; and it slows gastric emptying. GLP-1 in the brain has been shown to be involved in the regulation of satiety (Tang-Christensen *et al.* 1996, Turton *et al.* 1996). Recent research points to GLP-2 as being one of the regulators of small-intestinal growth (Drucker *et al.* 1996).

The tissue-specific processing of glucagon is achieved by tissue-specific expression of prohormone convertases. Prohormone convertase (PC) 2 is important for the

Figure 1 The upper part of the figure shows the structural organisation of mouse proglucagon, with indications of the amino acid numbers (after the signal peptide has been cleaved off). The binding sites for antibodies (Ab) 390, 2135, 4304 and 4305 are also shown (see Materials and Methods). Shown below are the two different processing patterns of proglucagon. In the pancreas, processing of proglucagon primarily results in the liberation of glucagon and the major proglucagon fragment (MPGF). The aberrant pancreatic processing pattern, leading to the formation of glicentin 1–61, IP-1, GLP-1, IP-2 and GLP-2, is shown inside the dotted lines Proglucagon processing in the brain and gut mainly produces glicentin, oxyntomodulin, GLP-1, GLP-2 and IP-2. Glicentin always includes the glucagon and GRPP sequence but is found both with (glicentin 1–69) and without the IP-1 sequence (glicentin 1–61).

maturation of glucagon, supported by the attenuated glucagon processing in mice lacking PC2 or 7B2 (Furuta *et al.* 1997, Westphal *et al.* 1999). (7B2 is a neuroendocrine protein required for activation of PC2; a lack of 7B2 renders PC2 inactive (Westphal *et al.* 1999).) PC1/3 alone may be the enzyme responsible for liberating GLP-1 and -2 (Rouille *et al.* 1995, 1997). Cleavage by PC1/3 or PC2 leaves one or two basic residues at the C-terminus of the

newly formed peptide, and these are subsequently removed by a carboxypeptidase. In the case of GLP-1, this removal is a prerequisite for carboxyamidation (Fig. 2). To date, the importance of amidation of GLP-1 is not fully understood, but it is believed that it might slow degradation of the hormone. Prohormone convertase cleavage of proglucagon also leaves basic amino acids at the C-terminus of glucagon, oxyntomodulin, glicentin,

Figure 2 The figure shows the post-translational processing steps leading to the formation of amidated GLP-1. After endopeptidolytic cleavage by the prohormone convertases, carboxypeptidases such as CPE cleave the C-terminal arginine from the arginyl-glycine extended GLP-1. This then allows the glycine to be used as the amide donor in the amidation process. The numbers refer to the amino acid numbers in proGLP-1. The binding sites for Ab 390 and Ab 2135 are also shown (see Materials and Methods).

intervening peptide-1 (IP-1) and IP-2, all of which are subject to removal by a carboxypeptidase.

Given the involvement of glucagon and GLP-1 in both glucose metabolism and food intake, and their possible contribution to the obesity phenotype, we studied the processing and maturation of these hormones in *fat/fat* mice to establish the importance of CPE *in vivo* in proglucagon processing.

Materials and Methods

Mice and genotyping

Heterozygous *fat/+* mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Mice were kept with a ratio of 12 h light:12 h darkness and were allowed to feed *ad libitum* on Purina 5008 chow (Purina Mills Inc., Richmond, IN, USA). Homozygous *fat/fat* and wild-type (+/+) mice were generated by intercrossing heterozygotes and were subsequently identified by genotyping the offspring by using an allele-specific PCR assay (Lacourse *et al.* 1997) on genomic DNA prepared from tail biopsies (Miller *et al.* 1988).

Tissue isolation and extraction

Adult mice 2–6 months old were anaesthetised, and the brains, pancreases, stomachs, small intestines and colons were rapidly dissected, washed gently in ice-cold PBS and frozen in liquid nitrogen (Danish Animal Ethical Committee approval no. 1998–56–84). To extract the peptides, the tissues were boiled in 5–10 volumes distilled water, homogenised using a Polytron homogeniser (Kinematica, Lucerne, Switzerland) and centrifuged at 10 000 *g*. The supernatants were collected and the pellets were re-extracted in 750 µl 1 M CH₃COOH and left at 4 °C for 1 h, after which the extracts were centrifuged again at 10 000 *g*. The acidic supernatants were collected, pooled with the neutral supernatant and then stored at –20 °C. Before further analysis, the extracts were loaded onto SepPak C18 cartridges (Waters, Milford, MA, USA), which were activated according to the manufacturer's instructions and eluted with 70% (v/v) ethanol containing 0.1% trifluoroacetic acid. After evaporation in a vacuum centrifuge, the eluates were reconstituted in assay buffer (PBS supplemented with 0.1% human serum albumin and 0.6 mmol/l thiomethyl). Half of the reconstituted extract for each tissue was subjected to RIA as described below, and the remaining extracts were pooled and subjected to chromatography.

Chromatography

Pools (*n* = 4–6) of pancreatic or small-intestinal extracts were applied to Sephadex G-50 (Pharmacia, Uppsala,

Sweden) superfine columns (16 × 1000 mm), which were eluted at 4 °C with sodium phosphate buffer (pH 7.4) containing 0.1% human serum albumin. Fractions of 1.0 ml were collected at a rate of 4.0 ml/h.

The columns were calibrated with ¹²⁵I-labelled albumin, to determine the void volume (*v*₀), and with ²²NaCl, to determine the total available volume (*v*_t). Subsequently, the elution constants (*k*_d) were calculated as *k*_d = (fraction no. × volume of the fractions – *v*₀)/*v*_t. The columns were calibrated with synthetic GLP-1, glucagon, glicentin and oxyntomodulin, which yielded slender peaks at the expected positions. The eluted fractions were analysed with sequence-specific RIAs as described below.

Radioimmunoassay

Four different RIA assays were employed. An assay for the C-terminus of glucagon, using the antibody (Ab) 4305 (Holst 1982). This antibody has an absolute requirement for the intact C-terminus of the glucagon molecule, and therefore does not react with C-terminally elongated forms, including oxyntomodulin or glicentin. The standards used were human glucagon and tracer ¹²⁵I-labelled human glucagon. The detection limit was below 1 pmol/l and the intra-assay coefficient of variation was below 5%.

A 'side-viewing' glucagon assay was based on Ab 4304, which reacts with a mid-region (residues 6–15) of the glucagon sequence (Holst 1982). This antibody binds all molecular forms of glucagon and proglucagon (including glicentin and oxyntomodulin) with equal strength, regardless of modifications of the termini, and therefore provides a measure of the total amounts of glucagon and proglucagon. Assay conditions were as for the C-terminal assay. The detection limit was 5 pmol/l and the intra-assay coefficient of variation was below 6%.

The assay for the amidated C-terminus of GLP-1 was based on Ab 390 (Ørskov *et al.* 1994), which has an absolute requirement for this region and reacts with neither the shortened nor the elongated molecular form, or with non-amidated, glycine-extended GLP-1 (GLP-1 7–37) or proglucagon 78–108. The standards used were human GLP-1, and monoiodinated ¹²⁵I-labelled GLP-1 was used as the tracer. The detection limit was 1 pmol/l and the intra-assay coefficient of variation was 5%.

The 'side-viewing' GLP-1 assay was based on Ab 2135, which reacts with a mid-region of the GLP-1 sequence (Ørskov *et al.* 1991). This antibody binds all molecular forms of GLP-1 with equal strength, regardless of modifications of the termini, and therefore provides a measure of the total amount of GLP-1-containing molecules. Assay conditions were as for the C-terminal assay. The detection limit was 5 pmol/l and intra-assay coefficient of variation was 6%.

Table 1 Tissue concentrations of amidated GLP and of total proglucagon product measured by RIA in five different tissues in wild-type (*n*=5) and *fat/fat* mice (*n*=4). The values (means ± S.E.M.) are in fmol/g. The total number of peptides containing the glucagon sequence (=glucagon, proglucagon, glicentin and oxyntomodulin) was measured using side-viewing antiserum 4304. This antibody recognizes all fragments derived from the proglucagon precursor containing the mid-portion of glucagon (Fig. 1). Amidated GLP-1 was measured using antiserum 390, which is specific for amidated GLP-1 (Fig. 2) and does not recognise C-terminally extended forms of GLP-1 (see Materials and Methods)

	Amidated GLP-1*		Glucagon+proglucagon	
	<i>fat/fat</i>	Wild-type	<i>fat/fat</i>	Wild-type
Pancreas	163 ± 57	1160 ± 740	66 500 ± 8600	62 300 ± 8400
Brain	7.5 ± 1.7	33 ± 9	200 ± 23	188 ± 15
Stomach	247 ± 67	3100 ± 930	4560 ± 370	6100 ± 1200
Intestine	568 ± 87	3960 ± 640	18 700 ± 6400	10 700 ± 2900
Colon	664 ± 98	9300 ± 2300	26 300 ± 2700	30 100 ± 5400

*P<0.05.

Results

Reduced concentrations of amidated GLP-1 in fat/fat mice

The total concentrations of glucagon and proglucagon were measured using Ab 4304, which recognises all peptides containing the mid-glucagon sequence. We found the expected expression pattern for proglucagon: high expression in the pancreas and gut, and lower expression in the brain (Table 1). There were no significant differences in the concentrations of glucagon+proglucagon between *fat/fat* and wild-type mice in any of the tissues examined (Table 1). Because of the tissue-specific processing, the fraction of amidated GLP-1 (of total glucagon-containing peptides) in tissues from wild-type mice varies from 2 to 51% (Table 2) according to the pattern shown in Fig. 1. In the pancreas, glucagon expression is so high that even though only a minor fraction (~2%) of proglucagon is processed to GLP-1, the GLP-1 concentrations amount to one-third of that observed in the small intestine.

In all *fat/fat*-mice tissues examined, the concentrations of amidated GLP-1 were reduced 7–14-fold) compared with those in tissue from wild-type mice (Table 1). To clarify whether this was due to altered liberation of GLP-1

or reduced amidation, we performed gel chromatography of the intestinal and pancreatic extracts (chosen to represent each of the two processing patterns) (see Fig. 1). Analysis of the brain processing pattern failed because of the small amounts of peptide present. In the pancreatic extracts of *fat/fat* mice, there was a minor increase in GLP-1 relative to the amount in the wild type (Fig. 3); the opposite pattern was seen in the intestinal extracts (Figs 3 and 4). However, the small changes in GLP-1 processing alone cannot explain the reduction in carboxyamidated GLP-1. We assume, therefore, that this is primarily due to a reduction amidation of GLP-1, and only to a lesser extent due to altered liberation of GLP-1 from proglucagon.

The ratio between GLP 1–36 amide/GLP1–37/38 and GLP 7–36 amide/GLP7–37/38 was unaffected by the loss of CPE, suggesting that the monobasic cleavage of GLP 1–36 amide/GLP1–37/38 to GLP 7–36 amide/GLP 7–37/38 is unaffected.

Moderately attenuated processing of proglucagon by prohormone convertases in fat/fat mice

To clarify the consequences of lack of CPE on prohormone convertase processing of proglucagon in both the

Table 2 The fraction of amidated GLP-1 (GLP-1 1-36 amide+GLP-1 7-36 amide) out of the total glucagon+proglucagon in CPE-deficient *fat/fat* mice

	GLP-1 amide, <i>fat/fat</i>	GLP-1 amide, wild-type
	Glucagon+proglucagon, <i>fat/fat</i>	Glucagon+proglucagon, wild-type
Pancreas	0.25%	2%
Brain	3.80%	18%
Stomach	5.40%	51%
Intestine	3.00%	37%
Colon	2.50%	31%

Figure 3 Gel chromatography of pools of pancreatic extracts from *fat/fat* mice (right) and wild-type controls (left). The upper graphs show the elution of glucagon and glicentin and other fragments of preproglucagon containing a mid-portion of the glucagon sequence measured using antiserum 4304. The lower graphs show the elution of peptides containing the mid-portion of GLP-1 studied using the side-viewing antiserum 2135. The peaks are identified relative to elution positions of the synthetic glucagon and glicentin peptides used for calibration.

pancreas and the gut, the extracts were monitored with antibodies against the glucagon sequence. In the gel filtration from the pancreatic extracts from *fat/fat* mice, a peak corresponding to the elution position of glicentin 1–61 (Baldissera & Holst 1986) was identified by using both antiserum 4304 and antiserum 4305 (data not shown); this was not present in extracts from the wild-type mice (Fig. 3). Furthermore, the intestinal processing of proglucagon was also affected, as demonstrated by a reduction in the amount of oxyntomodulin (Fig. 4). Thus, in both tissues a lack of CPE attenuated cleavage in proglucagon at the dibasic site Lys 31/Arg 32 in the *fat/fat* mice.

To evaluate the C-terminal removal of Lys 62 and Arg 63 from glucagon and glicentin 1–61, we monitored the elution with Ab 4305 (data not shown). As there were similar elution profiles in *fat/fat* and wild-type mice, we conclude that CPE is not important for the C-terminal processing of the glucagon sequence.

The attenuated carboxypeptidase processing potentially could also affect the removal of the Lys 31 and Arg 32 from GRPP, Lys 70 and Arg 71 from IP-1, and glicentin and oxyntomodulin and Arg 124 and Arg 125 from IP-2. However, as we do not have antibodies that would allow us to detect altered processing at these amino acids we are unable to investigate this possibility.

Figure 4 Gel chromatography of pools of small intestinal extracts from *fat/fat* mice (right) and wild type controls (left). The upper panels shows the elution of glucagon, oxyntomodulin and glicentin measured using antiserum 4304, that is a side viewing antibody, recognising the mid portion of glucagon. The lower panels shows the elution of peptides containing the mid portion of GLP-1 studied using the side viewing antiserum 2135. The peaks are identified relative to elution positions of the peptides used for calibration. MPGF, major proglucagon fragment.

Discussion

This study demonstrates that CPE is important for the maturation of glucagon and GLP-1. CPE is presumably the enzyme primarily responsible for the C-terminal removal of the arginyl residue from the GLP-1 peptides generated after endopeptidolytic cleavage of proglucagon. Thus, GLP-1 processing follows the pattern seen for other peptide hormones studied in *fat/fat* mice, i.e. gastrin, CCK, insulin, melanin concentrating hormone, POMC (Naggert *et al.* 1995, Rovere *et al.* 1996, Cain *et al.* 1997, Lacourse *et al.* 1997, Udipi *et al.* 1997). Even though the point mutation Ser220Pro in *fat/fat* mice abolishes carboxypeptidase E activity, tissues from *fat/fat* mice still

contain some amidated GLP-1, indicating the presence of other sources of carboxypeptidase activity. This has also been observed in other peptide hormone systems. Two candidates for this activity are the recently discovered carboxypeptidase D (CPD) and carboxypeptidase X (CPX), which are present in endocrine cell lines and neurons (Dong *et al.* 1999, Varlamov *et al.* 1999a,b). However, it remains to be determined as to whether one or both account for the residual carboxypeptidase activity in the cells expressing proglucagon.

The accumulation of that which elutes as glicentin 1–61 in pancreatic extracts, and the reduced formation of that which elutes as oxyntomodulin in intestinal extracts, indicate that a lack of CPE also interferes with the

prohormone convertase-mediated processing of the proglucagon precursor. This effect is primarily at Lys 31/Arg 32. PC2 has been shown to be responsible for the dibasic at Lys 31/Arg 32 cleavage and to participate in the Lys 62/Arg 63 cleavage (Furuta *et al.* 1997, Westphal *et al.* 1999). Thus, PC2 is necessary for liberating glucagon from proglucagon. The changes in dibasic cleavage of proglucagon described here resemble – but are less pronounced than – those seen in mice lacking PC2 activity (Furuta *et al.* 1997, Westphal *et al.* 1999). This suggests that the attenuated processing is due to reduced PC2 activity in the *fat/fat* mice. It has been shown that processing of the inhibitory 7B2 C-terminal peptide by CPE greatly reduces its inhibitory potency against PC2 (Zhu *et al.* 1996). Hence, loss of active CPE could result in increased concentrations of the inhibitory 7B2 C-terminal peptide, which would inhibit PC2 action. This is one of the first reports to indicate that CPE activity appears to regulate PC2 activity *in vivo*.

A lack of CPE also mildly affected prohormone convertase processing of GLP-1, but the effects seen varied in the two tissues examined. It has been suggested that PC2 is not important for the liberation of GLP-1 (Rouille *et al.* 1997), but, as GLP-1 processing in the PC2 or 7B2 knockouts has not been studied, we cannot tell how our observations correlate with altered PC2 activity.

The biological consequences of reduced GLP-1 amidation are not known. The bioactivity of GLP-1 is confined to the N-terminus of the GLP-1 7–36 amide, which is why C-terminal amidation is not believed to be of major importance for GLP-1 action. Both *in vivo* and *in vitro* studies have indicated that GLP-1 7–37 and GLP-1 7–36 amide have a similar potency for the GLP-1 receptor (Ørskov *et al.* 1993, Wettergren *et al.* 1998). However, there are, at present, no studies that have examined the potency of GLP-1 7–38. Since there seems to be equipotency of GLP-1 7–36 amide and GLP-1 7–37, it is likely that GLP-1 7–38 has the same bioactivity as GLP-1 7–37. Although several immunoreactive forms of GLP-1 are released *in vivo*, including GLP-1 7–36 amide and GLP-1 7–37, most of the GLP-1 in circulation in humans is GLP-1 7–36 amide (Ørskov *et al.* 1994). Carboxyamidation of GLP-1, as well as that of other hormones (e.g. gastrin), seems to protect against C-terminal degradation, and it is therefore likely that GLP-1 7–38 has a shorter half-life than GLP-1 7–36 amide, just like GLP-1 7–37 (Wettergren *et al.* 1998). The consequences of this could be lower GLP-1 plasma concentrations, which might contribute to the diabetic aspects of the phenotype of the *fat/fat* mice. The *fat/fat* mice have mild fasting hyperglycaemia and severe prolonged hyperglycaemia after the oral glucose tolerance test (L Friis-Hansen, unpublished observations), and the same thing is observed in mice lacking the GLP-1 receptor (Scrocchi *et al.* 1996).

It is unclear as to whether incomplete cerebral GLP-1 processing also contributes to the obesity phenotype of the

fat/fat mouse. The GLP-1-receptor knockout mice are not obese, nor do they display disturbances in food intake. However, unlike the GLP-1-receptor-deficient mice, the *fat/fat* mice have multihormone disturbances, and the defects in the proglucagon processing therefore act in concert with other neuroendocrine defects. In future, the contribution of different hormone systems to the diabetic and obesity phenotype of the *fat/fat* mouse could be addressed by studies using cell-type-specific rescue of CPE in this animal model.

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