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Research Article

Degradation of proinsulin C-peptide in kidney and placenta extracts by a specific endoprotease activity

E. Melles[a], H. Jörnvall[a], S. Tryggvason[a], K. Gemzell Danielsson[b], K. Ekberg[c], K. Tryggvason[a], J. Wahren[c] and T. Bergman[a,*]

[a] Department of Medical Biochemistry and Biophysics, Karolinska Institutet, 171 77 Stockholm (Sweden), Fax: +46 8 337 462, e-mail: Tomas.Bergman@mbb.ki.se
[b] Department of Obstetrics and Gynecology, Karolinska University Hospital, 171 76 Stockholm (Sweden)
[c] Department of Surgical Sciences, Karolinska Institutet, 171 77 Stockholm (Sweden)

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Abstract. Degradation of proinsulin C-peptide in mouse kidney and human placenta extracts was studied using reverse-phase high-performance liquid chromatography and nano-electrospray mass spectrometry. In total, 15 proteolytic cleavage sites were identified in human and mouse C-peptides. Early sites included the peptide bonds N-terminal of Val/Leu10, Leu12, Leu21, Leu24 and Leu26 in different combinations for the two tissues and two peptides. Notably, these cleavages were N-terminal of a hydrophobic residue, and all but one N-terminal of Leu. A late degradation product of the human peptide detected in the kidney extract was the C-terminal hexapeptide, containing just one residue more than the biologically active C-terminal pentapeptide of C-peptide. We conclude that the degradation of C-peptide in kidney and placenta follows similar patterns, dominated by endopeptidase cleavages N-terminal of Leu.

Key words. Proinsulin C-peptide; kidney; placenta; HPLC; mass spectrometry.

C-peptide constitutes the proinsulin segment positioned between the A and B chains of insulin. After cleavage of the parent molecule, C-peptide is secreted from the pancreatic β cells into the blood circulation in amounts equimolar with those of insulin [1]. C-peptide has an important function in linking the A and B chains in a manner that allows correct folding and formation of the interchain disulfide bonds in insulin, cf. [2]. C-peptide has for many years been considered biologically inactive but is now known to bind to target cells, to activate a Ca^{2+}-signaling response [3, 4] and to have effects on Na^+,K^+-ATPase [5–7], endothelial NO synthase (eNOS) [8, 9], and MAP kinases [7, 10; M. Henriksson et al., unpublished data]. It also appears to have some insulin-like properties [11; M. Henriksson et al., unpublished data] and is beneficial for clinically observable parameters [2]. The latter include improvements in renal and nerve function and increased blood flow in type I diabetic patients. The interest in C-peptide is now focused on its therapeutic potential in preventing type I diabetic complications [2].

Most degradation and removal of C-peptide takes place in the kidneys [12, 13] but little is known about the proteolytic enzyme(s) involved there and the mode of degradation. Tests with a neutral metallo-endopeptidase purified from rat kidney revealed that this enzyme has the capacity to cleave proinsulin at peptide bonds in or near the C-peptide moiety and that incubations with isolated bovine C-peptide followed a similar time course [14]. In another study, N-terminally ^{125}I-tyrosylated human C-peptide, introduced into the maternal circulation of a pregnant

* Corresponding author.

Rhesus monkey, was shown to be degraded in the uterus during transfer to the fetus [15], with major products of approximately 12 and 25 residues and with an increased C-peptide degradation rate during pregnancy [15]. Degradations have also been traced in the pancreas [16] and in a neuroendocrine tumor of the pancreas (insulinoma) [17], identifying C-peptide fragments resulting from cleavages in the C-terminal region, and in the latter case ascribing the cleavages to cathepsin B.

We have previously investigated C-peptide degradation in serum and found evidence for an endoprotease degradation mode, while similar studies with the C-terminal pentapeptide fragment showed sensitivity of that segment to aminopeptidase degradation [18]. We have continued these studies by analysis of C-peptide degradation at physiologically important locations and studied the degradation in mouse kidney and human placenta, identifying cleavage products with high-performance liquid chromatography (HPLC) and mass spectrometry. The proteolytic pattern detected resembles that of insulin [19]. Identification of the C-peptide degradation products may allow design of structural modifications to increase the peptide half-life which may be important for therapeutic applications in type I diabetes.

Materials and methods

Mouse kidneys were dissected and recovered from anesthetized animals (approved by the local ethics committee) and stored at −80 °C. Kidneys were disintegrated in 100 mM HEPES buffer, pH 7.1, using a Potter Elvehjem homogenizer. For one kidney (0.180 g), 300 µl buffer was used, followed by the addition of 400 µl buffer. Aliquots of 50 µl homogenate were stored at −80 °C. Human placenta (immediately frozen upon parturition) was obtained from Karolinska University Hospital (approved by the local ethics committee). After thawing, 14 g was disintegrated in 100 mM HEPES buffer, pH 7.1, using an Ultraturrax T25 mixer, resulting in 5 ml homogenate that was stored in 500-µl aliquots at −80 °C. For degradation studies, human and mouse C-peptides (PolyPeptides), 26 nmol dissolved in 78 µl water, were incubated with 40 µl tissue homogenate and 282 µl HEPES buffer at 37 °C which means a ten-fold dilution of the crude homogenates. As a blank incubation, 40 µl kidney or placenta homogenate and 360 µl HEPES buffer was used. Aliquots of 66.7 µl corresponding to 4.3 nmol C-peptide were withdrawn at specific time points (0, 15, 20, 40, 60 min and 3 h for the kidney incubations; 0, 20, 40, 60 min, 3 and 6 h for the placenta incubations). The aliquots were acidified (pH 2) by addition of 200 µl 0.13% trifluoroacetic acid (TFA) and then 10 µl 10% TFA, followed by centrifugation at 10,000 rpm for 10 min. After filtration through a 0.2-µm membrane filter (NanoSep; Pall Gelman Laboratory), the sample was analyzed by reverse-phase HPLC (Äkta system; Amersham Pharmacia) using a Vydac C_4 column (4.6 × 250 mm) with a linear gradient of 0–60% acetonitrile in 0.1% TFA for 41.5 min at 1 ml/min. Fractions were collected in Eppendorf tubes, dried under a stream of nitrogen and dissolved in 60% acetonitrile containing 1% acetic acid for nano-electrospray mass spectrometry.

Mass spectra were recorded using a quadrupole time-of-flight tandem mass spectrometer (Q-TOF; Micromass) equipped with an orthogonal sampling electrospray ionization (ESI)-interface (Z-spray; Micromass), and metal-coated nano-ESI needles (Proxeon) which gave a spraying orifice of about 5 µm and a flow of approximately 20–50 nl/min at a capillary voltage of 0.8–1.2 kV. For acquisition of collision-induced dissociation (CID) spectra, the collision energy was optimized in the range 30–80 eV with argon as the collision gas. Some fractions were also analyzed by Edman degradation using a Procise HT instrument (Applied Biosystems).

Results and discussion

Procedure

The degradation of human C-peptide and the cleavage products generated were studied after incubations in mouse kidney and human placenta extracts. To decrease the background of proteins and peptides, we diluted the extracts 1:10 before mixing with C-peptide. This dilution still led to efficient degradation of C-peptide within reasonable time frames. Aliquots were taken at specific time points and were immediately acidified with TFA (to pH 2) to stop proteolytic activities. Samples were centrifuged to separate protein precipitates, and the supernatants were filtered, followed by reverse-phase HPLC. The human C-peptide eluted after 38 min, while the degradation products eluted earlier and in order of increasing hydrophobicity (fig. 1). The fractions containing C-peptide cleavage products (as judged from comparison with a blank run in which an incubation without C-peptide was injected) were collected and analyzed by nano-electrospray mass spectrometry to identify the C-peptide segments. The degradation of mouse C-peptide was similarly studied in the mouse kidney extract.

Degradation products of C-peptide

The degradation of C-peptide in the two systems, mouse kidney and human placenta extracts, was established. An early and major degradation product in the kidney incubation with the human peptide revealed a mass of 1868.92 Da, corresponding to the 20-residue segment Glu1–Ser20 resulting from a cleavage at Ser20–Leu21 (figs. 1A, 2). This fragment was also confirmed by complete Edman degradation. In the placenta extract, an early

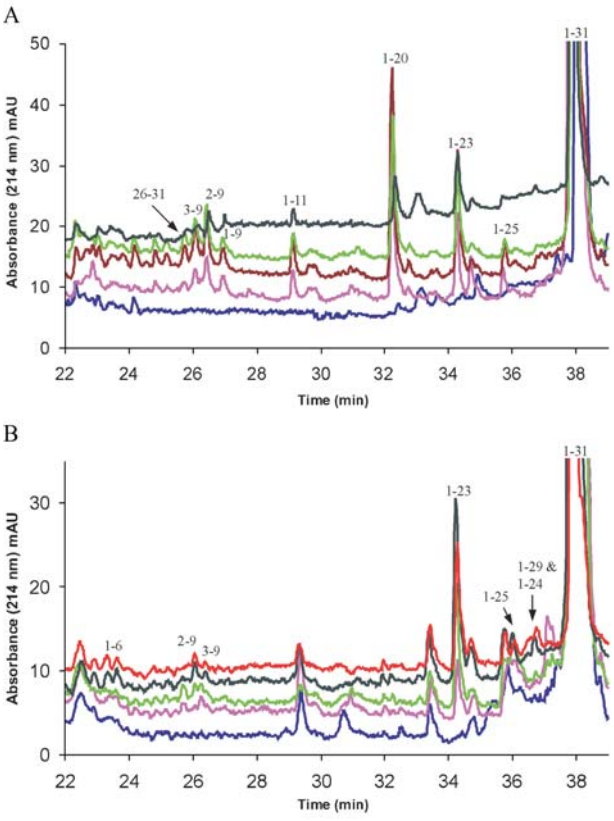

Figure 1. Reverse-phase HPLC of human C-peptide (residues 1–31) and its degradation products generated during incubation in kidney extracts (*A*) for 0 min (blue), 20 min (purple), 40 min (brown), 1 h (green) and 3 h (black) and in placenta extracts (*B*) for 0 min (blue), 20 min (purple), 1 h (green), 3 h (black) and 6 h (red). Identification was by nano-electrospray mass spectrometry, and by tandem mass spectrometry or Edman degradation when necessary to ascertain identification.

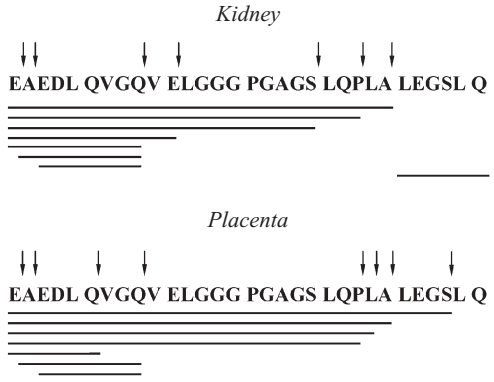

Figure 2. Fragments identified after digestion of human C-peptide in kidney and placenta extracts. Arrows indicate peptide bonds found to be susceptible to proteolytic cleavage. Lines indicate the resulting cleavage products detected.

and major degradation product of the human peptide was a 23-residue fragment with a mass of 2207.16 Da, corresponding to the segment Glu1–Pro23 (figs. 1B, 2). This fragment was also found early in the kidney extract, and the corresponding fragment (Glu1–Thr23) was found with the mouse peptide in the kidney extract. In these three fragments, the cleavage site is N-terminal of a Leu residue. Other early fragments detected in both extracts consisted of the first 25 residues of human C-peptide (fig. 1), and in the kidney homogenates, peptides ending at position 9 for both the human (fig. 1A) and mouse C-peptide degradations, and at position 11 for the human peptide (fig. 1A), all but one also with the cleavage site N-terminal of a Leu residue (fig. 2). Additional early but minor fragments in the placental degradation had the masses

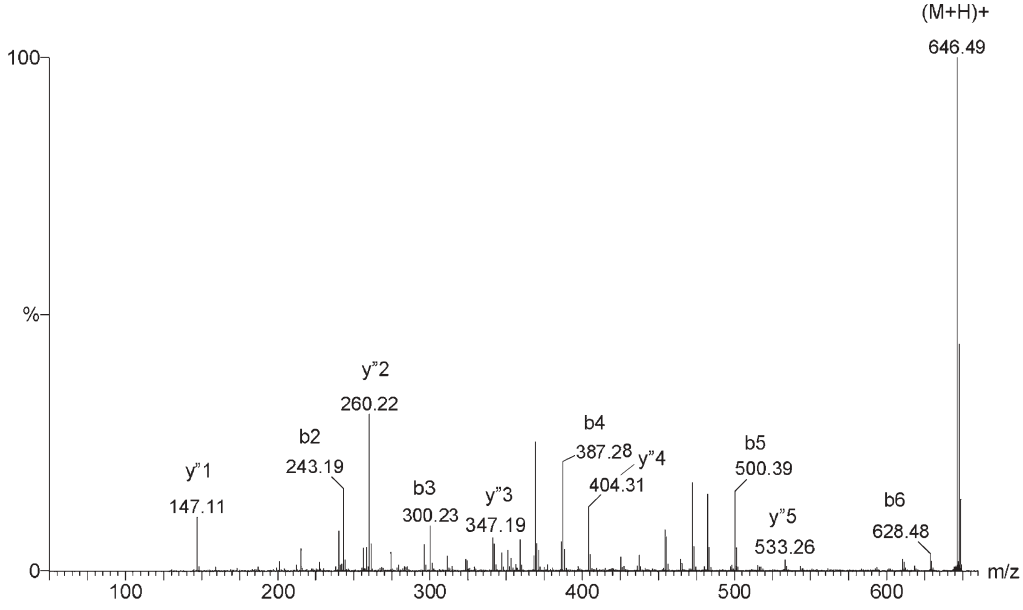

Figure 3. CID spectrum of fragment 645.48 Da generated during incubation of human C-peptide in the kidney extract (residues 26–31, fig. 1A) corresponding to the hexapeptide LEGSLQ that appeared as a singly charged peptide in the mass spectrum.

2320.20 Da (the 24-residue Glu1–Leu24) and 2777.46 Da (the 29-residue peptide Glu1–Ser29) (figs. 1B, 2), also with cleavages N-terminal of hydrophobic residues, Ala and Leu, respectively. The pattern with cleavages N-terminal of Leu residues partly overlaps with that from cleavages with insulin protease [19] and cathepsin B [17].

Short fragments consisting of 8- and 7-residue peptides derived from both the C-peptides in the kidney extract, and the 8-, 7- and 6-residue peptides in the placenta extract were also detected (figs. 1, 2). The pattern in these products is different since they result from secondary cleavages. Two of these peptides in each set, the 8- and 7-residue peptides, are also N-terminally truncated and identical between extracts (fig. 2). The 8-residue peptide is truncated by one residue and starts at Ala2, while the 7-residue peptide is truncated by two residues and starts at Glu3 (fig. 2). Thus, the late-occurring fragments result from aminopeptidase-like cleavages at one end plus secondary endopeptidase cleavages at the other, Gln9–Val10 (kidney) and Gln6–Val7 (placenta).

A fragment detected after incubation with the kidney extract consisted of 6 residues, with a mass of 645.48 Da and corresponding to the C-terminal segment LEGSLQ (figs. 1A, 2) identified by CID tandem mass spectrometry (fig. 3). This hexapeptide contains just one residue more than the biologically active C-terminal pentapeptide EGSLQ [5] known to have in vivo beneficial functional activity [20]. The degradation rate of the intact human C-peptide, as monitored by the disappearance of the HPLC peak, was 13 nmol/min per gram in the kidney extract and 0.6 nmol/min per gram in the placenta extract.

In conclusion, we found that the degradation patterns detected when C-peptide is incubated in kidney and placenta extracts are largely overlapping, and that a majority of the fragments are generated via cleavages at sites N-terminal of a Leu residue, as in insulin and other degradations [13, 17, 19], indicating that amino acid-specific proteolytic processing of C-peptide occurs in the kidney, the major site of C-peptide degradation.

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1 Steiner D. F., Cunningham D., Spiegelman L. and Aten B. (1967) Insulin biosynthesis: evidence for a precursor. Science **157:** 697–700
2 Wahren J., Ekberg K., Shafqat J., Johansson J., Johansson B.-L. and Jörnvall H. (2004) Biological effects of C-peptide and proinsulin. In: International Textbook of Diabetes Mellitus, 3rd edn., pp. 165–181, De Fronzo R. A., Ferrannini E., Keen M., Zimmet P. (eds), Wiley, Chichester
3 Ohtomo Y., Aperia A., Sahlgren B., Johansson B.-L. and Wahren J. (1996) C-peptide stimulates rat renal tubular Na^+, K^+-ATPase activity in synergism with neuropeptide Y. Diabetologia **39:** 199–205
4 Shafqat J., Juntti-Berggren L., Zhong Z., Ekberg K., Kohler M., Berggren P. O. et al. (2002) Proinsulin C-peptide and its analogues induce intracellular Ca^{2+} increases in human renal tubular cells. Cell. Mol. Life Sci. **59:** 1185–1189
5 Ohtomo Y., Bergman T., Johansson B.-L., Jörnvall H. and Wahren J. (1998) Differential effects of proinsulin C-peptide fragments on Na^+, K^+-ATPase activity of renal tubule segments. Diabetologia **41:** 287–291
6 Tsimaratos M., Roger F., Chabardes D., Mordasini D., Hasler U., Doucet A. et al. (2003) C-peptide stimulates Na^+,K^+-ATPase activity via PKC alpha in rat medullary thick ascending limb. Diabetologia **46:** 124–131
7 Zhong Z., Kotova O., Davidescu A., Ehrén I., Ekberg K., Jörnvall H. et al. (2004) C-peptide stimulates Na^+,K^+-ATPase via activation of ERK1/2 MAP kinases in human renal tubular cells. Cell. Mol. Life Sci. **61:** 2782–2790
8 Scalia R., Coyle K., Levine B., Booth G. and Lefer A. (2000) C-peptide inhibits leukocyte endothelium interaction in the microcirculation during endothelial dysfunction. FASEB J. **14:** 2357–2364
9 Kitamura T., Kimura K., Makondo K., Furuya T., Suzuki M., Yoshida T. et al. (2003) Proinsulin C-peptide increases nitric oxide production through mitogen-activated protein kinase-dependent transcriptional enhancement of endothelial nitric oxide synthase in rat aortic endothelial cells. Diabetologia **46:** 1698–1705
10 Kitamura T., Kimura K., Okamoto S., Canas X., Sakane N., Yoshida T. et al. (2001) Proinsulin C-peptide rapidly stimulates mitogen-activated protein kinases in Swiss 3T3 fibroblasts: requirement of protein kinase C, phosphoinositide 3-kinase and pertussis toxin-sensitive G-protein. Biochem. J. **355:** 123–129
11 Grunberger G., Qiang X., Li Z., Mathews S.T., Sbrissa D., Shisheva A. et al. (2001) Molecular basis for the insulinomimetic effects of C-peptide. Diabetologia **44:** 1247–1257
12 Faber O. K., Kehlet H., Madsbad S. and Binder C. (1978) Kinetics of human C-peptide in man. Diabetes **27:** 207–209
13 Zavaroni I., Deferrari G., Lugari R., Bonora E., Garibotto G., Dall'Aglio E. et al. (1987) Renal metabolism of C-peptide in man. J. Clin. Endocrinol. Metab. **65:** 494–498
14 Varandani P. T. and Shroyer L. A. (1981) Degradation of proinsulin and isolated C-peptide by rat kidney neutral metalloendopeptidase. Biochim. Biophys. Acta **661:** 182–190
15 Gruppuso P. A., Susa J. B., Sehgai P., Frank B. and Schwartz R. (1987) Metabolism and placental transfer of ^{125}I-proinsulin and ^{125}I-tyrosylated C-peptide in the pregnant Rhesus monkey. J. Clin. Invest. **80:** 1132–1137
16 Verchere C. B., Paoletta M., Neerman-Arbez M., Rose K., Irminger J.-C., Gingerich R. L. et al. (1996) *Des*-(27-31)C-peptide: a novel secretory product of the rat pancreatic beta cell produced by truncation of proinsulin connecting peptide in secretory granules. J. Biol. Chem. **271:** 27475–27481
17 Conlon J. M., Höög A. and Grimelius L. (1995) Intracellular degradation of the C-peptide of proinsulin in a human insulinoma: identification of sites of cleavage and evidence for a role for cathepsin B. Pancreas **10:** 167–172
18 Melles E., Bergman T., Alvelius G., Jonsson A., Ekberg K., Wahren J. et al. (2003) Proinsulin C-peptide and its C-terminal pentapeptide: degradation in human serum and Schiff base formation with subsequent CO_2 incorporation. Cell. Mol. Life Sci. **60:** 1019–1025
19 Stentz F. B., Kitabchi A. E., Schilling J. W., Schronk L. R. and Seyer J. M. (1989) Identification of insulin intermediates and sites of cleavage of native insulin by insulin protease from human fibroblasts. J. Biol. Chem. **264:** 20275–20282
20 Sato Y., Oshida Y., Han Y.-Q., Morishita Y., Li L., Ekberg K. et al. (2004) C-terminal fragments of C-peptide stimulate whole body glucose utilization in diabetic rats. Cell. Mol. Life Sci. **61:** 727–732