Kinetics of Proinsulin Conversion in Human Islets

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Islets isolated from human cadaver pancreas were pulse-labeled (10 min with [^{3}H]leucine) and then incubated for a 180-min chase. Islets and chase medium were collected every 15 min and analyzed by reversed-phase HPLC to quantify the percentage of radioactively labeled proinsulin, conversion intermediates, and fully processed insulin. Release of proinsulin-related labeled products into the chase medium was <10% of total. Whereas 50% of labeled proinsulin had been lost by conversion within 45 min, fully processed insulin only appeared with a half-time of 100 min. This discrepancy is attributable to accumulation of radioactive conversion intermediates. Des 64.65 split proinsulin was a minor component, reaching a maximum of 5.2 ± 1.7% (n = 4) at 60 min of chase. By contrast, des 31.32 split proinsulin—and a truncated form lacking the first three residues of C-peptide—rose progressively to 29.3 ± 1.4% by 75 min, and declined thereafter. The accumulation of des 31.32 split proinsulin rather than the des 64.65 split form during the conversion of human proinsulin reflects slower conversion at the C-peptide/A-chain than at the B-chain/C-peptide junction, and is consistent with the appearance of this particular conversion intermediate in the circulation. *Diabetes* 42:933–36, 1993

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KRBB, Krebs-Ringer bicarbonate buffer; BSA, bovine serum albumin; HPLC, high-performance liquid chromatography; RIA, radioimmunoassay; type II diabetes, non-insulin-dependent diabetes mellitus.

It has been known for many years that proinsulin is converted by endoproteolytic cleavage at the two pairs of basic residues that link the insulin A- and B-chains to C-peptide. This cleavage is followed by the trimming of the residual COOH-terminal basic residues by carboxypeptidase E/H (1). Two endoproteases, PC1, also known as PC3 (2,3), and PC2 (4,5), are now thought to be implicated, each apparently being responsible for cleavage at predominantly one of the two conversion sites (6,7). The relative activity of these two enzymes thus leads to the accumulation of one or the other of the two proinsulin conversion intermediates, des 31.32 or des 64.65 split proinsulin (cleaved only between the B-chain and C-peptide or C-peptide and A-chain, respectively) (1).

In humans, des 31.32 split proinsulin (cleaved and trimmed only between the B-chain and C-peptide) is found as a minor component in the circulation, along with proinsulin and insulin, which are the dominant conversion products (8,9). Note that this particular conversion intermediate is found at much higher levels in the circulation of type II diabetic patients (10). The appearance of this intermediate in the circulation could result from its accumulation in B-cell granules during proinsulin conversion with release into the circulation if a granule in which conversion is not complete is marshalled for exocytosis.

We have now studied the kinetics of proinsulin conversion in isolated human islets. The results show that des 31.32 split proinsulin is indeed the major conversion intermediate.

RESEARCH DESIGN AND METHODS

Isolation of human islets. Islets were isolated from human adult pancreas procured from three brain dead multiple organ donors (2 females, 38 and 48 yr old, and 1 male, 31 yr old). The cold ischemia time in University of

Wisconsin solution was <12 h. After pancreatic duct cannulation and injection with collagenase (Boehringer-Mannheim, Mannheim, Germany, lot 1213 873, 2 mg/ml, 250 ml), the pancreases were digested by the automated method of Ricordi et al. (11). Islets were then purified from the dispersed pancreatic tissue on a 4-layer, top-loaded Ficoll density gradient (Ficoll 400,000, Sigma, St. Louis, MO in Eurocollins solution) (12), using a Cobe cell centrifugator (Denver, CO). The purity of each preparation was evaluated after differential staining of endocrine tissue with diphenylthiocarbizone (dithizone). All preparations consisted of 80–90% endocrine tissue. For three experiments, the islets were maintained overnight in tissue culture (CMRL-1066 medium, 10% fetal calf serum) at 37°C in a humidified atmosphere of 5% CO_2/95% air. In a fourth experiment, half of the islets were maintained for a further 2 days in culture.

Pulse-chase protocol. Islets were washed 3 times in KRBB containing 10 mM HEPES, 0.25% BSA, pH 7.4 (KRBB), and 16.7 mM glucose. After 15 min preincubation in this same buffer at 37°C, the islets were labeled for 10 min in 2 ml KRBB-16.7 mM glucose, containing 200 μCi [^{3}H]leucine (specific radioactivity 100 Ci/mmol, American Radiolabeled Chemicals, St. Louis, MO). The labeled islets were washed 3 times in ice-cold KRBB-1.67 mM glucose and then dispensed in 1-ml aliquots for the chase incubation at 37°C. Every 15 min, one tube was centrifuged at 1000 *g* for 2 min. The supernatant (chase medium) was acidified (20 μl of 1M HCl/ml KRBB) before injection into HPLC. The islet pellet was extracted by sonication in 100–200 μl 1M acetic acid, 0.1% BSA. Before injection into HPLC, the extracts were clarified by centrifugation at 11,000 *g* for 5 min.

Reversed-phase HPLC. Proinsulin-related peptides were separated by HPLC, essentially as described previously (13). A LiChroCart 250–4 (5 μm C18 column, 250 x 4 mm, Merck, Darmstadt, Germany) was fitted to a Beckman System Gold HPLC apparatus (Fullerton, CA). Buffer A was 50 mM H_3PO_4, 100 mM $NaClO_4$, 10 mM heptanesulfonic acid, pH 3; buffer B was 9:1, acetonitrile: H_2O. The elution program was as follows: 37% B isocratic from 0 to 25 min; at 25 min linear gradient from 37 to 41% B over 70 min. The column was then washed by raising buffer B to 60% over 10 min, maintaining this percentage for 5 min, and then returning to 37% B over 10 min. The elution times of insulin, the two conversion intermediates, and proinsulin were established by coinjection of authentic standards for each sample (generously provided by Dr. Ronald Chance, Eli Lilly, Indianapolis, IN).

RESULTS

Representative HPLC elution profiles for extracts of islets taken at early (15 min), intermediate (60 min), and late (180 min) times of chase are shown in Fig. 1. There is a progressive decrease of radioactivity associated with proinsulin in favor of conversion intermediates and then insulin.

The appearance of a radioactive product eluting between des 31.32 split proinsulin and des 64.65 split proinsulin was quite unexpected. Because the extracts were injected into HPLC without prior purification, this product could have been unrelated to proinsulin. This was not the case. The product was found to be both immunoprecipitable with anti-insulin serum and recognized in an insulin RIA. It did not coelute with any of the other known conversion intermediates (i.e., split proinsulin intermediates still carrying COOH-terminal basic amino acids). Systematic analysis of this product, and of the modified C-peptide generated from it by digestion with trypsin and carboxypeptidase (6), showed it to be related to des 31.32 split proinsulin. The modified C-peptide was therefore purified by HPLC and the first 11 residues sequenced. The sequence data showed unambiguously that the first three residues of C-peptide (Glu, Ala,Glu) were missing (NH_2-terminal sequence obtained was Asp,Leu,Gln,Val,Gly,Gln,Val,Asp,Leu,Gly,Gly. . .).

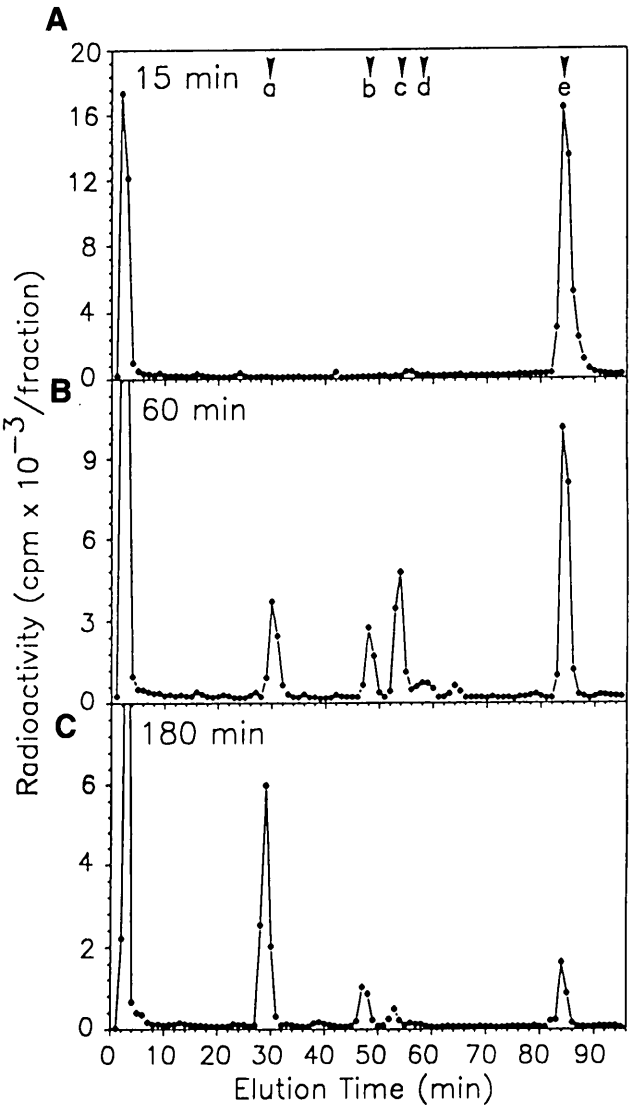

FIG. 1. Elution of radioactive products from HPLC. Pulse-labeled human islets were extracted in acid after 15 (*A*), 60 (*B*), or 180 (*C*) min of chase, and the extracts analyzed by reversed-phase HPLC. The elution times of standards are given by the arrowheads: *a*, insulin; *b*, des 31.32 split proinsulin; *c*, des 31–35 split proinsulin (des 31.32 split proinsulin that has lost the first 3 residues of C-peptide, and which is believed to be an artefact of extraction); *d*, des 64.65 split proinsulin; and *e*, proinsulin.

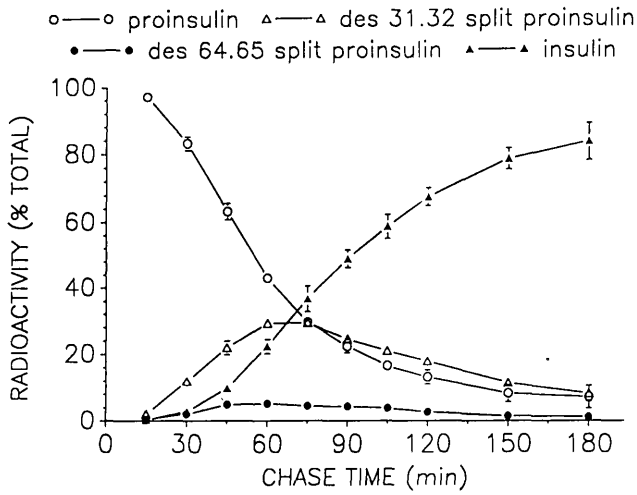

FIG. 2. Time course of conversion of proinsulin to conversion intermediates and insulin. Pulse-labeled islets were extracted every 15 min during a 180-min chase incubation, and the extracts analyzed by HPLC. The radioactivity eluting as proinsulin, conversion intermediates, and insulin was summed for each time point, and the contribution of each product was expressed as a percentage of this value. For the purposes of this figure, des 31–35 split proinsulin (see Fig. 1) is considered identical to des 31.32 split proinsulin. Data are means ± SE; *n* = 3–4.

The product eluting between the two known conversion intermediates is thus des 31–35 split proinsulin. The truncated C-peptide (des 1–3 C-peptide) generated by complete conversion of this intermediate also was found in islet extracts. Note, neither the truncated intermediate nor the truncated C-peptide were released into the medium (data not shown). We tentatively conclude that the truncation occurs as an artifact of acid extraction. For the purposes of evaluating proinsulin conversion kinetics, the truncated intermediate is thus considered equivalent to unmodified des 31.32 split proinsulin. Further studies will be necessary, however, before it can be concluded unequivocally that this enigmatic truncation (which, as it happens, removes an acidic domain highly conserved throughout the animal kingdom [14]) does not occur naturally within human B-cells. If it does, it will be interesting to discover why neither this intermediate nor the C-peptide generated from it, can be secreted.

The radioactivity eluting with proinsulin, conversion intermediates, and insulin (after correcting for the loss of the 6 Leu residues in C-peptide; human insulin has 6 Leu residues itself) was summed and taken as 100%. The percentage of each product was then determined (Fig. 2). Because the results from each experiment were quantitatively similar, and the time in culture failed to affect the kinetics of conversion, the data have been pooled and expressed as mean ±SE. Radioactive proinsulin declined from 100% to 63.3 ± 2.5% by 45 min, and to 7.2 ± 3.4% by 180 min of chase. Radioactivity in insulin increased from 0% to 48.8 ± 2.7% by 90 min and to 83.6 ± 5.5% by 180 min. Des 64.65 split proinsulin was only a minor component, reaching a maximum of 5.2 ± 1.7% at 60 min. Des 31.32 split proinsulin (together with its truncated form) accounted for 29.3 ± 1.4% of radioactivity at 75 min and declined to 8.2 ± 2.4% by 180 min.

DISCUSSION

The kinetics of proinsulin conversion are presumed to reflect not only the absolute levels and intrinsic activities of the two endoproteases, PC1 and PC2, but also the molecular characteristics of the substrate, proinsulin (14). Thus, it has been shown that rat proinsulin II is converted less rapidly than proinsulin I, due to unusually slow cleavage at the B-chain/C-peptide junction (13). We attribute this (13) to the presence of methionine, rather than lysine as in most other proinsulin species, including human (1), at residue B29 of rat proinsulin II. Residue B29 lies at the −4 position (i.e., 4 residues NH_2-terminal) relative to the site of cleavage between the B-chain and the C-peptide. The endoproteases responsible for proprotein conversion in the constitutive secretory pathway also have been shown to favor a basic amino acid (Arg by preference) in the −4 position (15,16).

Assuming that rat and human islets have essentially equivalent activities of PC1 and PC2, it was predicted that human proinsulin, with its Lys^{B29}, would be cleaved at the B-chain/C-peptide junction as efficiently as rat proinsulin I. Indeed, the rate of disappearance of these two proinsulin species is quite similar (13; Fig. 2). For rat proinsulin I, however, des 31.32 split proinsulin never exceeded 15% of total proinsulin-related radioactivity (13); whereas, for human proinsulin, we now find close to 30% of radioactivity in this form during the course of the chase incubation. This suggests that cleavage at the C-peptide/A-chain junction of human proinsulin (or of des 31.32 split proinsulin) is much slower than for rat I. Examination of the sequence immediately NH_2-terminal to Lys^{64},Arg^{65}, the cleavage site between C-peptide and the insulin A-chain, may provide the explanation. Rat proinsulin I has a basic amino acid, Arg, at residue 62, which corresponds to the −4 position relative to this cleavage site. In human proinsulin, this residue is Leu. Using the same reasoning applied to the B-chain/C-peptide junction (13), the data at hand would suggest that PC2, the enzyme believed responsible for cutting the C-peptide/A-chain junction (4–6), in addition to favoring des 31.32 split proinsulin as its substrate rather than unprocessed proinsulin (17), prefers a basic amino acid in the −4 position. This, in turn, suggests that a −4 basic amino acid is preferred by both endoproteases implicated in the regulated secretory pathway (PC1 and PC2), as well as by the enzyme(s) implicated in the constitutive pathway (15,16).

The relative amounts of radioactive products generated during a pulse-chase protocol do not, of course, reflect their steady-state levels. Thus, when HPLC fractions were assayed by insulin RIA (which will detect all insulin-related products present in islets, whether labeled or not), insulin itself was the major form in human islets (data not shown). Des 31.32 split proinsulin and proinsulin also were present in equal, albeit relatively small, amounts. These islets, however, were taken from the pancreas of individuals not known to be suffering from any condition affecting pancreatic endocrine function. Because our data show that cleavage at the C-peptide/A-chain junction is the rate-limiting step in proinsulin conversion, des 31.32 split proinsulin will be a major

presence only in maturing granules, the proinsulin conversion compartment, which are in the minority under normal circumstances (18). If, as seems likely in type II mellitus, there is an increased demand on the B-cells to produce unusually large amounts of insulin relative to their granular stores, granule turnover will be increased, and maturing granules will be required to release their contents before conversion is complete. This scenario could explain the high levels of des 31.32 split proinsulin in the circulation of type II diabetic individuals without implication of any defect in conversion per se. Although such a defect, if it preferentially affected cleavage at the C-peptide/A-chain junction, could further exacerbate the situation (19).

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