Perspectives in Diabetes

What β-cell Defect Could Lead to Hyperproinsulinemia in NIDDM?

Some Clues From Recent Advances Made in Understanding the Proinsulin-Processing Mechanism

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Pancreatic β-cell dysfunction is a characteristic of non-insulin-dependent diabetes mellitus (NIDDM). An aspect of this dysfunction is that an increased proportion of proinsulin is secreted, but an actual β-cell defect that leads to hyperproinsulinemia is unknown. Nevertheless, an impairment in β-cell proinsulin conversion mechanism has been suggested as the most likely cause. Insulin is produced from its precursor molecule, proinsulin, by limited proteolytic cleavage at two dibasic sequences (Arg2, Arg52 and Lys54, Arg62). Two endopeptidase activities catalyze this cleavage: PC2 and PC3. PC2 endopeptidase cleaves predominately at Lys64, Arg66, and PC3 endopeptidase cleaves at Arg22, Arg32. The recent identification and characterization of these endopeptidases has enabled a better understanding of the human proinsulin-processing mechanism. In particular, experimental evidence suggests that the majority of human proinsulin processing is sequential. PC3 cleaves proinsulin first to generate a proinsulin conversion intermediate that is the preferred substrate of PC2. Both PC2 and PC3 activities are influenced by Ca2+ and pH, but the more stringent Ca2+ and pH requirements of PC3 suggest it as the most likely enzyme to regulate proinsulin conversion, as well as initiate it. When an increased demand is placed on the proinsulin-processing mechanism by a glucose-stimulated increase in proinsulin biosynthesis, there is a coordinate increase in PC3 biosynthesis (but not in PC2). This supports PC3 as the key endopeptidase that regulates proinsulin processing. In this perspective, the current concepts of the enzymology and regulation of proinsulin conversion at a molecular level are reviewed. Then, several hypothetical points of β-cell dysfunction that might affect the proinsulin-processing mechanism and lead to hyperproinsulinemia are considered. Because the term NIDDM encompasses a wide variety of different disorders, any one or a combination of these possible points of β-cell dysfunction could result in hyperproinsulinemia for a certain subset of NIDDM. Diabetes 43:511-17, 1994

That an increased proportion of proinsulin and proinsulin conversion intermediates exists in the circulation of patients with NIDDM is well established (1-4). Conventional radioimmunoassay (RIA) determinations of serum insulin levels also measure proinsulin-like molecules. However, use of techniques such as high-performance liquid chromatography (5) and proinsulin-specific RIAs (6) have measured the proinsulin component separately from insulin. Because proinsulin and proinsulin conversion intermediates are biologically inactive compared with insulin, and an increased proportion of the insulin measured with conventional RIAs is proinsulin and conversion intermediates, patients with NIDDM could be insulin deficient (1,2,4,7). For the most part, hyperproinsulinemia accompanies hyperglycemia (1,4,8). Even under relatively mild hyperglycemic circumstances (fasting plasma glucose levels of 6.1-7.8 mM), the proportion of circulating proinsulin rises from ~10% normally to 15-22% (8). As hyperglycemia worsens, the relative contribution of proinsulin in the circulation can rise to >40% (1,8). However, hyperproinsulinemia also has been observed to occur in certain individuals without hyperglycemia (1,2,9,10). Currently, the cause of hyperproinsulinemia is thought to be a pancreatic β-cell defect that is augmented by the increased demand placed on the β-cell by hyperglycemia, rather than a decreased clearance of proinsulin-like molecules from the circulation (although this latter possibility has not been ruled out yet) (1-4). A β-cell defect could be reflected either as a primary dysfunction of the proinsulin conversion machinery itself or a malfunction in related β-cell regulatory mechanisms that affect insulin production secondarily. Over the last 2-3 years, great strides have been made for a better understanding of the proinsulin conversion mechanism in β-cells. After 25 years of searching, the specific enzyme activities that catalyze human proinsulin conversion have now been identified. In addition, other β-cell biochemical investigations have led to a clearer picture of the mechanics and regulation of proinsulin processing. In this perspective, we review these recent advances and hypothesize how certain defects in the human proinsulin conversion mechanism could contribute to hyperproinsulinemia.
FIG. 1. Cleavage sites of the two distinct proinsulin-processing endopeptidases on human proinsulin.

IDENTIFICATION OF THE PROINSULIN-CONVERTING ENZYMES

Insulin is produced posttranslationally from its precursor molecule, proinsulin, by limited proteolysis, primarily in newly formed β-granules of the β-cell (11,12). An endoproteolytic cleavage can be made on the carboxylic side of two dibasic sequences in the proinsulin molecule: Arg₃¹, Arg₃² and at Lys₆⁴, Arg₆⁵ (13). This is followed by the specific removal of the newly exposed COOH-terminal basic amino acids by the rapid action of an exopeptidase, carboxyypeptidase-H (CP-H) (14,15), to yield the products insulin and C-peptide. A proinsulin-processing endopeptidase activity was first observed as a Ca²⁺-dependent β-granule-specific site, whereas the other (type-I) cleaved preferentially at Lys₃⁴, Arg₆⁵ (13). This is followed by the specific removal of the newly exposed COOH-terminal basic amino acids by the rapid action of an exopeptidase, carboxyypeptidase-H (CP-H) (14,15), to yield the products insulin and C-peptide. A proinsulin-processing endopeptidase activity was first observed as a Ca²⁺-dependent β-granule-specific site, whereas the other (type-I) cleaved preferentially at Lys₃⁴, Arg₆⁵ (13). Around the time of the discovery of the type-I and type-II proinsulin-processing endopeptidases, the only unequivocal proprotein-processing endopeptidase was the Kex2 protease found in Saccharomyces cerevisiae (20-22). The Kex2-protease was cloned (23), and its cDNA sequence provided means whereby mammalian Kex2-homologues could be isolated. Over the last 3 years, a new family of mammalian Kex2-related proprotein-processing endopeptidases has emerged (21,24,25). Among this family of novel enzymes are two neuroendocrine specific proteases named PC2 (26,27) and PC3 (28) (PC3 also has been named mPC1 (29)). After the discovery of PC2 and PC3, it was subsequently established that PC2 and the type-II proinsulin-processing activity are equivalent (30) and that the PC3 is immunologically (31) and biochemically (32) indistinguishable from the type-I proinsulin-processing activity. Cotransfection experiments of PC2 and PC3 with proinsulin have substantiated PC3 and PC2 as the proinsulin-processing type-I and type-II endopeptidase activities, respectively (33).

SEQUENTIAL PROCESSING OF HUMAN PROINSULIN

Theoretically, proinsulin processing can take one of two routes (Fig. 2). Either PC3 will cleave intact proinsulin first at the Arg₃¹, Arg₆⁵ site to generate the split 32, 33 proinsulin conversion intermediate or PC2 will cleave at the Lys₆⁴, Arg₆⁵ site to yield the split 65, 66 proinsulin conversion intermediate. These split proinsulin intermediates are rarely detected in vivo because of the relatively high quantity of CP-H activity present in a β-granule (34). The more commonly seen proinsulin intermediates are the CP-H trimmed des 31, 32 proinsulin and des 64, 65 proinsulin. Another round of appropriate PC2/PC3 cleavage, plus CP-H trimming of the des proinsulin intermediates, then generates the products, insulin and C-peptide.

However, the proinsulin-processing scenario outlined in Fig. 2 is probably not applicable to human proinsulin conversion. Several lines of evidence suggest that human proinsulin conversion has a preferred sequential route (Fig. 3). First, des 31, 32 proinsulin is present in higher quantities in the circulation and in pancreas extracts, whereas des 64, 65 proinsulin is low (4-6). In NIDDM, hyperproinsulinemia consists of elevated intact proinsulin and des 31, 32 proinsulin levels; des 64, 65 proinsulin levels are difficult to measure and most likely do not contribute to increased levels of proinsulin-like molecules (4,6). Second, kinetic studies of proinsulin conversion in isolated human pancreatic islets have shown that des 31, 32 proinsulin appears as a transient conversion intermediate at a greater than sevenfold higher level than des 64, 65 proinsulin (35). Third, an Arg₃¹, Arg₆⁵ to Arg₃⁴, Gly₃⁵ proinsulin mutation (which is not processed by PC3 activity, because the Arg-Arg dibasic processing site is no longer present) was processed inefficiently by PC2 activity at Lys₆⁴, Arg₆⁵, even though this site was present in the Gly₃⁵ proinsulin variant (19,36). This finding led to the idea that some degree of secondary structure, as well as dibasic specificity, is necessary for efficient PC2 cleavage of proinsulin. Indeed, nuclear magnetic resonance studies have shown that although most of the C-peptide moiety within the proinsulin molecule is unstructured, a degree of ordered structure occurs around the Lys₆⁴, Arg₆⁵ site and has been termed the CA-knuckle (37). It has been proposed that the CA-knuckle might represent a structural element necessary for PC2 processing of proinsulin (37). Finally, it has been demonstrated that PC2 activity has a much greater preference for des 31, 32 proinsulin as a substrate than intact proinsulin (31,38). Furthermore, intact proinsulin appeared to be inefficiently processed at Lys₆⁴, Arg₆⁵ by PC2 compared with PC3 cleavage at Arg₃¹, Arg₆⁵ (38). This led to a proposal that initial cleavage of intact proinsulin by PC3 at Arg₃¹, Arg₆⁵ is necessary to release a structural constraint around the Lys₆⁴, Arg₆⁵ site (perhaps represented by the CA-knuckle) that then renders easier accessibility for PC2 to cleave des 31, 32 proinsulin at Lys₆⁴, Arg₆⁵ (38).

Collectively, these lines of evidence indicate that the route via des 31, 32 proinsulin is the predominant pathway for proinsulin to insulin conversion in human β-cells (Fig. 3).
The strong preference of PC2 for processing des 31, 32 proinsulin ensures that the conversion mechanism strongly favors insulin and C-peptide production in β-granules, which compliments the observations made in vivo where >95% of proinsulin is fully converted to insulin (35,39). At most, <10% of human proinsulin is converted to insulin via des 64, 65 proinsulin (35). The vast majority of proinsulin processing occurs in newly formed β-granules (11,40), where the intraorganellar environment of Ca²⁺ and pH ideally suits maximal activity of the PC2 and PC3 endopeptidases (see below) (17,38). The more stringent Ca²⁺ and pH requirements of the PC3 activity ensure that it is only active in β-granules (17,38). In contrast, the relatively broad biochemical requirements for PC2 activity suggest that it might also be partially active in the trans-Golgi network (the preceding organelle to the β-granule of the β-cell's secretory pathway) (2,17,38). Cleavage of proinsulin by partially active PC2 in the trans-Golgi network (17,38) could account for a small amount of des 64, 65 proinsulin produced in human β-cells. However, note that PC2 is relatively inefficient at cleaving intact proinsulin, as well as not being fully active in the trans-Golgi compartment (17,31,38). Thus, the path via des 64, 65 proinsulin would be a relatively minor route of proinsulin conversion. Once in the β-granule compartment, >90% of proinsulin is converted by the preferred sequential route via des 31, 32 proinsulin (Fig. 3). In humans this hypothesis predicts that des 64, 65 proinsulin would be produced in negligible amounts, and that des 31, 32 proinsulin would be the major proinsulin conversion intermediate. This is actually what has been observed in both the human circulation and islets (4,6,35).

REGULATION OF PROINSULIN CONVERSION

By examining the biochemistry and cell biology of the PC2 and PC3 endopeptidases, an understanding of the regulation of proinsulin processing is now becoming apparent. Ca²⁺ and pH regulation of endopeptidase activity. Both PC2 and PC3 are Ca²⁺- and pH-dependent endopeptidases; however, they differ from each other in their pH and Ca²⁺ requirements (17,31,38). PC2 is half-maximally activated between 50 and 100 μM Ca²⁺, whereas PC3 is only active above 0.5 mM Ca²⁺ (17,38). Both endopeptidases have an acidic pH 5.5 optimum, but PC2 is more active than PC3 at neutral pH. The major intracellular site of proinsulin processing in the pancreatic β-cell is the β-granule (11), which contains an acidic environment of pH 5.5 (41) and an estimated free Ca²⁺ concentration of 1–10 mM (42). This ideally suits the optimal requirements for full activity of the proinsulin-processing endopeptidases (17,38). The strict biochemical requirements for PC3 activity of high mM Ca²⁺ and narrow acidic pH range (17,38) ensures that it is only active in the inner environment of a β-granule. This then provides means whereby insulin can only be produced in the organelle in which it is stored (17,38). Sequential proinsulin processing in human β-cells (Fig. 3) implies that PC3 makes the initial cut and is the enzyme that is likely to control the process. Thus, it follows that PC3 is more stringently regulated by Ca²⁺ and pH. To render PC3 and PC2 fully active in a β-granule, the key events for initiating proinsulin to insulin conversion will be activation of the proton-pumping ATPase (34,40,41) and Ca²⁺-transporting proteins (43). However, the means of triggering these particular β-granule Ca²⁺ and H⁺ translocation events in a newly formed β-granule have yet to be elucidated.
Proteolysis of PC2 and PC3. The Kex2-related family of mammalian endopeptidases have homology to bacterial subtilisin, which requires a proregion so that the molecule can become a correctly folded, active enzyme. Once folded, the proregion is autocatalytically cleaved (44). Kex2 and another member of the Kex2-related endopeptidase family, furin, undergo autocatalytic cleavage activation (45,46). PC2 and PC3 are initially synthesized as proproteins that are post-translationally cleaved by limited proteolysis (47,48). In pancreatic islets, PC2 is initially synthesized as a 76 kD glycoprotein that is cleaved via 71/68 kD intermediates to a mature 64–66 kD β-granule form (47,48). PC3 is cleaved from a 92/94 kD glycoprotein precursor to a 66 kD mature β-granule form (48). However, it has yet to be demonstrated whether proteolysis of PC2 and PC3 is an autocatalytic and/or activation process (24). Apparently, the precursor form of PC3 has proteolytic activity (32,49). However, it is unclear whether processing of PC3 to its mature 66 kD form affects its processing activity. It has been hypothesized that furin (a putative housekeeping proprotein-processing endopeptidase present in the majority of mammalian cells [21,24]), rather than autocatalytic cleavage, processes PC2 and PC3. For the moment, proteolysis of PC2 and PC3 as a regulatory activation process remains to be demonstrated, but until proven otherwise, it should be considered as a possible regulation of proinsulin processing.

Glucose regulation of PC2 and PC3. Proinsulin biosynthesis can be stimulated by glucose at both translational and transcriptional levels (50-52). An increase in proinsulin biosynthesis places an obvious increased demand on the proinsulin conversion mechanism in β-cells. For short-term glucose stimulation (<2 h), the biosynthesis of PC3 is stimulated in parallel with that of proinsulin at a translational level (48). In contrast, the biosynthesis of PC2 (48) and CP-H (54) are not glucose regulated. However, in the short term, proinsulin processing proceeds just as efficiently at stimulatory or basal glucose concentrations (39), thus implying that enough PC2 and CP-H is already present in β-cells to cater for increased conversion demands. Given that PC3 initiates the majority of sequential processing of proinsulin conversion in human β-cells (Fig. 3), the coordinate glucose regulation of PC3 with its proinsulin substrate at the biosynthetic level (48) further documents PC3 as the key endopeptidase that regulates proinsulin processing in β-cells. Such regulation ensures that proinsulin conversion proceeds efficiently and is adaptable to varying glucose concentrations in the short term (39,53). For longer term situations, it remains to be shown whether PC2, PC3, and CP-H genes are coordinately transcriptionally regulated with the preproinsulin gene by glucose (55–57). This is a distinct possibility, especially because it has been shown that PC2 and PC3 mRNAs are coordinately regulated with that of pro-opiomelanocortin mRNA in pituitary cells (58).

**WHAT DEFECT IN THE β-CELL PROINSULIN CONVERSION MECHANISM COULD CAUSE HYPERPROINSULINEMIA?**

Recent advances in understanding the proinsulin-processing mechanism can now lead to speculation of what might be a β-cell defect that may lead to hyperproinsulinemia in NIDDM. Several possibilities of β-cell dysfunction can be considered.

**Genetic variants of proinsulin conversion endopeptidases.** A mutation in either the PC2 and/or PC3 genes could
adversely affect the activity of these endopeptidases. Such PC2/PC3 mutants in β-cells would result in inefficient processing of proinsulin leading to hyperproinsulinemia, insulin deficiency, and NIDDM. This is likely to be a very rare occurrence. Nevertheless, the possibility is potentially analogous to the recent discovery of glucokinase mutations being a primary cause of maturity onset diabetes of the young (59). PC2/PC3 mutations might also account for the hyperproinsulinemia found in some relatives of insulin-dependent diabetes mellitus patients (10).

A general defective glucose stimulus-coupling mechanism in the β-cell. In some animal models of NIDDM, a selective loss of the glucose-regulated insulin release mechanism occurs in β-cells (3). The glucose stimulus-coupling mechanism for insulin release shares some similarities with other glucose-regulated β-cell functions (60,61). A general dysfunction in the glucose-sensing (e.g., GLUT2 downregulation [3]) and/or signal transduction mechanisms could adversely influence general β-cell function that then secondarily affects proinsulin processing.

Defective transcriptional regulation of the PC3 and/or PC2 genes. Preproinsulin gene transcription is regulated by glucose (55,57,62), and an extended stability of preproinsulin mRNA is observed with increased glucose concentrations (51). A rise in preproinsulin gene expression induced by hyperglycemia should increase proinsulin biosynthesis that, in turn, would place an increased demand on the proinsulin conversion mechanism. However, it has not yet been determined whether the PC3 and PC2 genes are coordinately regulated in β-cells. If the transcriptional machinery for PC2 and/or PC3 gene expression is not coregulated by glucose in parallel to the preproinsulin gene, or an inadequacy of PC2/PC3 gene expression during prolonged states of glucose stimulation (e.g., hyperglycemia in NIDDM) develops, deficient proinsulin conversion and hyperproinsulinemia could result.

Defective translational regulation of glucose-induced PC3 biosynthesis. Glucose specifically regulates PC3 biosynthesis in parallel with that of proinsulin (48). A deficiency in the translational machinery for regulating PC3 biosynthesis versus that of proinsulin could lead to less of the key endopeptidase that initiates the proinsulin conversion mechanism in β-cells. This could result in less efficient processing of proinsulin and hence, hyperproinsulinemia.

A disproportionate ratio between PC3 and PC2 in NIDDM. In contrast to PC3 biosynthesis, PC2 biosynthesis is not translationally regulated by glucose in pancreatic islets (48). Thus, it is possible that an adverse discrepancy might arise in the ratio between PC2 and PC3 present in β-cells. Under conditions of prolonged glucose stimulation of the β-cell, PC3 biosynthesis should be able to keep pace with glucose-stimulated proinsulin biosynthesis (48). However, because PC2 biosynthesis is not glucose regulated, the ratio between PC2 and PC3 would grow ever wider. Eventually, under hyperglycemic conditions, the amount of PC2 present compared with that of PC3 and proinsulin would become rate limiting for proinsulin processing. The second PC2 catalyzed step in sequential processing of human proinsulin would be hindered (Fig. 3). As a result, des 31,32 proinsulin would accumulate, which is what has been observed in hyperproinsulinemia in NIDDM (4,6).

Defective processing of PC2 and PC3. PC2 and PC3 are posttranslationally cleaved by limited proteolysis in β-cells (47,48), but it is not yet clear whether this affects their activities (24). Nevertheless, it is possible that PC2 and PC3 processing might also be affected in NIDDM. A defect in proteolytic processing of PC2 and/or PC3 could limit the activity of these endopeptidases, which results in further inefficient processing of proinsulin and hyperproinsulinemia.

**An adverse change in the inner environment of the β-granule.** The major intracellular site of proinsulin processing in the β-cell is a newly formed β-granule (11,40). Under normal circumstances, the PC2 and PC3 endopeptidases are fully activated in this compartment because their Ca²⁺ and pH requirements are ideally suited by the intragranular environment (17,38). Glucose can affect Ca²⁺ and pH fluxes in the β-cell (61), and furthermore, secretory granule Ca²⁺ and pH are responsive under certain physiological conditions (21,63). Therefore, under hyperglycemic conditions, the possibility arises that the inner environment of the β-granule could be altered, which then adversely affects PC2 and PC3 activity. This would affect proinsulin processing, and hyperproinsulinemia might ensue.

**Mis-targeting of conversion enzymes to the β-granule.** In normal β-cells, >99% of newly synthesized proinsulin is targeted to the β-granule compartment (39,64), i.e., the regulated secretory pathway of β-cells (65). Presumably, PC2 and PC3 are as efficiently targeted to the β-granule, as indicated in that they are both cosecreted with a peptide hormone under regulatory circumstances (47,66). However, a defect in efficient targeting of PC2, PC3, and proinsulin to β-granules, perhaps as a result of overstimulation of β-cell secretory pathway by hyperglycemia, would result in these proteins being constitutively secreted. Proinsulin processing is compromised severely when mistargeted to the constitutive secretory pathway, and hyperproinsulinemia results (64). This is analogous to insulinoma cells, where an increased proportion of proinsulin-like molecules also is secreted (67).

**Increased secretory demand of the β-cell.** Overstimulation of the β-cell’s regulated secretory pathway, arising from hyperglycemia, could result in defective proinsulin conversion. Pulse-chase radiolabeling experiments have determined that ~3 h are required for 90% of newly synthesized proinsulin to be converted to insulin within the β-cell (35,39). Similar experiments have shown preferential release of newly synthesized proinsulin from β-cells and have shown that under stimulated conditions of elevated glucose, ~50% of the newly synthesized (pro)insulin can be released within 3 h with an elevated proportion as proinsulin (39). If hyperglycemia persists, so does glucose stimulation of the β-cell. Although PC2, PC3, and CP-II are cosecreted with (pro)insulin (47,66,68), the environment and dilution of enzyme:substrate ratio on secretion would not be amenable for proinsulin processing to occur outside the β-cell. Therefore, even with the correct inner environment and compliment of conversion enzymes in a β-granule, it is possible that chronic increased demand of the β-cell’s secretory pathway by hyperglycemia results in newly synthesized proinsulin not being retained within the β-granule compartment long enough for complete processing to insulin. As a result, an increased proportion of proinsulin secreted from overstimulated β-cells would gradually accumulate in the circulation and worsen so that chronic hyperproinsulinemia developed.

Note that NIDDM encompasses a family of diabetes disorders, and in all likelihood, hyperproinsulinemia in a given
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