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Proinsulin maturation disorder is a contributor to the defect of subsequent conversion to insulin in β-cells ☆

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ABSTRACT

Disproportionate hyperproinsulinemia is an indicator of β-cell dysfunction in diabetes and the basis underlying this abnormality remains obscure. Recently, we have found proinsulin is an aggregation-prone molecule inherent with a low relative folding rate and maintains a homeostatic balance of natively and plentiful non-natively folded states (i.e., proinsulin homeostasis, PIHO) in normal β-cells as a result of the integration of maturation and disposal processes. PIHO is susceptible to environmental and genetic influences. Perturbation of PIHO produces a number of toxic consequences with known association to β-cell failure in diabetes. To explore whether the perturbation of PIHO has a link to disproportionate hyperproinsulinemia, we investigated proinsulin conversion and the involved prohormone convertase 1/3 (PC1/3) and 2 (PC2) in mouse *Ins2*$^{+/Akita}$ islets/β-cells that preserve a primary PIHO disorder due to a mutation (C96Y) in the insulin 2 (*Ins2*) gene. Our metabolic-labeling studies found an increased ratio of proinsulin to insulin in the cellular or released proteins of *Ins2*$^{+/Akita}$ islets. Histological, metabolic-labeling, and RT-PCR analyses revealed decreases of the PC1/3 and PC2 immunoreactivities in the β-cells of *Ins2*$^{+/Akita}$ islets in spite of no declines of these two convertases at the transcriptional and translational levels. Immunoblot analyses in cloned *Ins2*$^{+/Akita}$ β-cells further confirmed the increased ratio of proinsulin to insulin despite the levels of PC1/3 and PC2 proteins were not reduced somehow. The findings demonstrate that the perturbation of PIHO results in defects in the subsequent conversion process of proinsulin and is a contributor to the occurrence of disproportionate hyperproinsulinemia in diabetes.

1. Introduction

Proinsulin is the dominant form of insulin precursor in the early secretory pathway following the rapid removal of signal peptide from preproinsulin, achieves its native conformation mainly in the endoplasmic reticulum (ER) of β-cells [1]. After a general 40–60-min trip through the ER and Golgi, proinsulin moves into the *trans* Golgi compartment and/or immature granules for initiation of conversion. Therein C-peptide is removed by prohormone convertase 1/3 (PC1/3) and 2 (PC2), and its two sets of flanking dibasic cleavage sites (lysine/arginine and arginine, i.e., KR and RR) are digested by carboxypeptidase. As a result, proinsulin is converted to insulin and stored in mature granules ready for secretion [1].

Disproportionate hyperproinsulinemia, i.e., elevated levels of blood proinsulin or proinsulin-like material versus insulin, is an indicator of β-cell dysfunction recognized in general forms of diabetes [2–9] in addition to some monogenetic subjects. Atypical elevations of fasting proinsulin levels and the proinsulin to C-peptide or insulin ratio are also found in pre-diabetic patients with type 1 diabetes (T1D) [2,3]. These abnormalities have been suggested to be complement immune markers in identifying individuals with high risk of bonding T1D despite the underlying mechanisms are unknown [4]. In patients with type 2 diabetes (T2D), the well-demonstrated progressive hyperproinsulinemia [5–9] is suggested to reflect inefficiencies of proinsulin conversion [6] and may result from increases in β-cell secretory demand [6,10,11]. Obesity or free fatty acid elevation in most subjects with T2D [12–14] is suggested to be also implicated in the occurrence of hyperproinsulinemia. Long-term exposure of MIN6 β-cells to high-level free fatty acids leads to the delayed processing of proinsulin and involved convertases [15]. This is a result supporting the proposition; however, there are some clinic data that do not support the link of obesity and hyperproinsulinemia. For example, the levels of proinsulin do no show a difference between lean and obese subjects with normal glucose tolerances or with T2D [7,16]. The actual β-cell defects contributing to hyperproinsulinemia of diabetes remain to be investigated.

Insulin is the most abundant protein product of β-cells and constitutes up to 14% of the dry weight of rodent islets/β-cells [17,18]. Studies of protein biosynthesis in rodent/carp islets have shown

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incorporation of 6–30% of radioactive amino acids into preproinsulin in basal or glucose-stimulated conditions [19,20], although islets/β-cells produce more than 20,000 proteins. Thus, proinsulin bears the greatest burden in β-cell protein folding. Moreover, it is recently found that proinsulin is an aggregation-prone molecule inherent with a low relative folding rate and removed heavily at the early stage of insulin biosynthesis. Proinsulin hence maintains a homeostatic balance of natively and plentiful non-natively folded states (i.e., proinsulin homeostasis, PIHO) in normal β-cells as a result of the integration of maturation and disposal processes for adaptation [21].

Also, it is recently shown that PIHO is susceptible to various environmental and genetic influences. PIHO disorders are induced by mutations in the insulin gene, cellular energy and calcium changes, ER and reductive or oxidative stress, and insults by thiol reagent and cytokine [21]. Moreover, we have recently found that PIHO disorders occur in pre-diabetic non-obese diabetic (NOD) and db/db mice (unpublished data) that are typical models representing for the main forms of diabetes in humans. On the other hand, PIHO disorders result in a number of toxic consequences with known association to β-cell failure in diabetes [21–23] (unpublished observations). These findings uncovered PIHO, an essential post-translational regulation mechanism in insulin biosynthesis that critically links to diabetes. Based on accumulated data in our recent studies, we have suggested that the perturbation of PIHO is an early defect of β-cells that contributes to subsequent abnormalities possibly including hyperproinsulinemia in diabetes [21].

To test this hypothesis, we investigated the conversion of proinsulin to insulin and the involved prohormone convertase 1/3 (PC1/3) and 2 (PC2) in the *Ins2*$^{+/Akita}$ islets and cloned *Ins2*$^{+/Akita}$ β-cells [22] that are inherent with a primary disorder in PIHO due to a point mutation (C96Y, i.e., *Ins2*$^{+/Akita}$) in the homolog of the human insulin gene [21,23]. Our metabolic-labeling, histological, and immunoblotting studies demonstrated that the PIHO disorder is a contributor in the defect of subsequent conversion to insulin in β-cells.

2. Materials and methods

2.1. Reagents, cell lines, and mice

In this study, we applied antibodies against glucagon or rat C-peptide II (Millipore), insulin (Dako North America, Inc.), tubulin (Sigma–Aldrich), PC1/3 and PC2 (produced in Dr. Donald F. Steiner lab). Second antibodies were purchased from Jackson ImmunoResearch Laboratories, Inc. We obtained N-ethylmaleimide, dithiothreitol, collagenase, bovine serum albumin (BSA), and Ficoll 400 from Sigma–Aldrich; TRIZOL and reverse transcription (RT)-PCR reagents, fetal bovine serum (FBS), RPMI 1640 and DMEM media from Invitrogen; and protease inhibitor cocktail from Roche Applied Science. We obtained Immobilon-P^{SQ} membrane from Millipore; [^{35}S]-methionine (Met) from Perkin Elmer. The *Ins2*$^{+/+}$ and *Ins2*$^{+/Akita}$ β-cell lines were kindly provided by Drs. H. Kubota and K. Nagata (Kyoto University Kyoto, Japan).

The C57BL/6J and *Ins2*$^{+/Akita}$ mice were purchased from The Jackson Laboratory. All animal and tissue sample experiments have been approved by the Institutional Animal Care and Use Committee of The Ohio State University and were performed in accordance with the guidelines of the National Institutes of Health and The Ohio State University.

2.2. Islet preparation and cell culture

Islet isolation and islet/β-cell culture procedures were described previously [21–23].

2.3. Pulse-chase, immunoprecipitation, and tricine–SDS–PAGE

Islets were subjected to pulse and chase incubations as described [21,23] at 37 °C with 5% CO_2/95% O_2 condition. Briefly, pre-incubated islets were labeled with [^{35}S]-Met for 45 (Fig. 1) or 30 (Fig. 3B) min. For chase experiments, labeled islets were divided into two groups with equal numbers. One group of islets was frozen immediately and another group was incubated in 10% FBS/RPMI 1640 complete media (containing 11 mM glucose) for a 2 h chase. Cellular proteins were extracted in the immunoprecipitation (IP) buffer (50 mM Tris–HCl, 100 mM NaCl, 2.5 mg/mL bovine serum albumin, 1% Triton X-100, 20 mM N-ethylmaleimide, pH 7.4) containing a protease inhibitor cocktail with sonication. Cellular or medium proteins were then subjected to IP with specific antisera. Immunoprecipitates eluted in tricine sample buffers (Bio-Rad) were resolved by tricine–SDS–PAGE for autoradiography as described [21].

2.4. PC1/3 and PC2 transcripts in islets

Quantitative RT-PCR procedures were described previously [23,24]. Islet RNAs of 12-week-old mice were prepared by using TRIZOL reagent. cDNAs were subjected to PCR by using the primer pairs for PC1/3 (5′-AAGTGTGGGGTTGGAGTTGC-3′, 5′-CGCTTG TTATTCGCTGGTCTG-3′), PC2 (5′-GTTACCCCCGACACAAGCAATCC-3′, and 5′-CATCAGGCACCTCAGTTTACATCC-3′), and β-actin (5′-CGT AAAGACCTCTATGCCAA-3′, 5′-AGCCATGCCAATGTTGTCTC-3′). The expected size of PCR product was 433 bp for PC1/3, 403 bp for PC2, and 349 bp for β-actin.

2.5. Immunohistochemistry and immunoblot

The procedures were described previously [21,23,24]. Double-staining on pancreas sections was carried out with antibodies to PC1/3 or PC2 and glucagon or C-peptide. For histological studies, PC1/3 antisera were further purified by incubation with a membrane bound with proteins extracted from pancreatic α-cells (αTC1.6). Fluorescent images were examined with an Axiovert 200

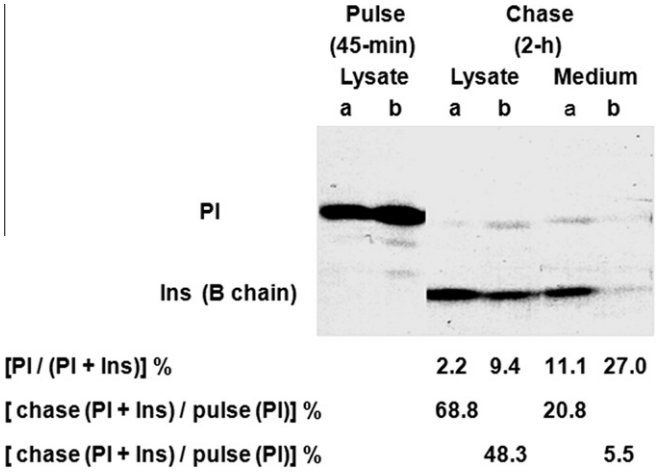

Fig. 1. Inefficient conversion and atypical loss of nascent proinsulin in the *Ins2*$^{+/Akita}$ islets. One hundred islets isolated from 4-week-old wild-type (*Ins2*$^{+/+}$) or mutant (*Ins2*$^{+/Akita}$) mice were labeled with [^{35}S]-Met for 45 min. Labeled islets were divided into two groups with equal numbers. One group of islets was frozen immediately and another group was incubated in 10% FBS/RPMI 1640 complete media (containing 11 mM glucose) for 2 h. Cellular and medium (pro)insulin immunopurified with insulin and C-peptide antisera were resolved by 10% reduced tricine–SDS–PAGE for radioautography. (Pro)insulin amounts were determined by densitometry and radioactivity of (pro)insulin bands. Lane a, *Ins2*$^{+/+}$ islets; lane b, *Ins2*$^{+/Akita}$ islets; PI, proinsulin; Ins, insulin.

microscope. Protein extracts in tricine sample buffer were resolved by 16.5% tricine–SDS–PAGE for immunoblot analyses [21,23].

2.6. Data analysis

Radioactivity, densitometry and immunoreactivity of protein bands (area and/or gel slices) were quantified by liquid scintillation counter (Beckman Coulter, Inc.) and NIH ImageJ software. Data are shown as the mean or the mean ± standard deviation (SD). Statistical significance ($^{*}P < 0.05$; $^{**}P < 0.005$) was assessed by Student's *t*-test (two-tailed). All experiments were carried out at least three times.

3. Results

3.1. Inefficient conversion and atypical loss of nascent proinsulin in the $Ins2^{+/Akita}$ islets

PIHO disorder in the early stage of insulin biosynthesis may result in defects in the subsequent conversion of proinsulin to insulin. To test this hypothesis, we labeled mutant ($Ins2^{+/Akita}$) and normal control islets with ^{35}S-Met for 45 min (applying ^{35}S-Met simplifies the experiment because its residue occurs in the dominant proinsulin 2 form and not in proinsulin 1 in mice). We then analyzed the proportional composition of cellular or secreted proinsulin and insulin products after a 2 h chase. A 2 h chase course generally covers a complete post-translational process from the appearance of nascent proinsulin to the release of insulin [1]. In a radioautograph that showed (pro)insulin immunopurified from intracellular or secreted proteins of the labeled islets (Fig. 1), an increase ($P < 0.05$, $n = 3$) of proinsulin percentage (9.4% intracellular and 27.0% medium) in cellular or secreted proinsulin and insulin products of the mutant islets was exposed by comparison to the normal islet controls (2.2% and 11.1%). The result suggests that the PIHO disorder in the mutant islets result in the inefficient conversion of proinsulin to insulin.

After the 2 h chase, 48.3% and 5.5% of nascent proinsulin (that was synthesized in the 45 min pulse) were retained in the intracellular or secreted proinsulin and insulin products of mutant islets. By contrast, up to 68.8% and 20.8% were retained in the normal controls (Fig. 1). In consistent with our previous observations [21,23], this result showed that a large fraction of nascent (pro)insulin was atypically removed in the mutant $Ins2^{+/Akita}$ islets during the 2 h post-translational processing.

3.2. Decrease of PC1/3 and PC2 immunoreactivities in the β-cells within $Ins2^{+/Akita}$ islets

To investigate whether the PIHO disorder has an effect on the convertase functioning in proinsulin conversion, we examined the levels of PC1/3 and PC2 convertase in the mutant islets by histological studies (Fig. 2). We applied C-peptide antisera and determined the area of β-cells that was clearly distinguished from α-cells (marked by immunoreactive glucagon) within sectional islets (Fig. 2A and B). The results showed that the PC1/3 or PC2 immunoreactivities per unit of β-cell area of mutant islets was 28.5 ± 17.8 and 26.0 ± 15.1 percentage of the normal controls, respectively (Fig. 2A–C). The results indicate both PC1/3 ($P < 0.05$) and PC2 ($P < 0.005$) immunoreactivities were decreased in the mutant islets (Fig. 2A and B, lower panels). Moreover, the decreases occurred specifically in β-cells rather than in α-cells. This is because no decrease of PC2 immunoreactivities was evident in α-cells of the mutant islets (Fig. 2B, lower panel) although PC2 is normally expressed in both α- and β-cells within islets regardless of no natural PC1/3 expression in α-cells [1].

3.3. No declines of PC1/3 and PC2 at the transcriptional and translational levels in the $Ins2^{+/Akita}$ islets

Further analysis with quantitative RT-PCR (Fig. 3A) and metabolic-labeling (Fig. 3B) studies demonstrated no decline of PC1/3 and PC2 convertase occurred at the transcriptional and translational levels in the mutant islets. In agreement with the result of quantitative RT-PCR, the transcripts of PC1/3 and PC2 convertase did not show a reduction in our microarray analysis on $Ins2^{+/Akita}$ islets (unpublished data). These findings overall suggest that abnormalities responsible for the decrease of PC1/3 and PC2 immunoreactivities (Fig. 2) occurred in post-translational processes in the mutant islets.

3.4. Conformation of the inefficient conversion and atypical loss of proinsulin in cloned $Ins2^{+/Akita}$ β-cells

The above demonstrated changes all occurred in the islets of 4- to 12-week-old diabetic Akita mice that have preserved hyperglycemia since diabetes onset at 4 weeks of age [23]. Hyperglycemia, a principal indicator of developed diabetes, is known to have a demonstrable glucotoxicity contributing to β-cell failure in diabetes. To clarify the above described changes that result primarily from PIHO disorders or glucotoxicity, we further analyzed the levels of proinsulin, insulin, PC1/3, PC2, and internal control tubulin proteins in cloned $Ins2^{+/Akita}$ and control $Ins2^{+/+}$ β-cells. These two β-cell lines have been cultured under the high (25.5 mM) glucose condition with same media since they were established from Akita and wild-type littermate mice [22]. The results of our immunoblot analysis clearly showed that the cloned $Ins2^{+/Akita}$ β-cells have an inefficient conversion of proinsulin to insulin compared to controls (Fig. 4A and B). In addition, the low proinsulin and insulin content evident in the mutant islets previously [21,23] showed in the cloned $Ins2^{+/Akita}$ β-cells as well herein (Fig. 4A). Interestingly, no decrease of immunoreactive PC1/3 and PC2 proteins was observed in the cloned $Ins2^{+/Akita}$ β-cells (Fig. 4A) despite the decrease of PC1/3 and PC2 immunoreactivities evident clearly in the primary β-cells of $Ins2^{+/Akita}$ islets (Fig. 2). These data indicate that the PIHO disorder rather than glucotoxicity primarily results in the inefficient conversion in addition to atypical loss of proinsulin in the $Ins2^{+/Akita}$ islets/β-cells. Additionally, these data also suggest that some *in vivo* alterations responsible for the decrease of PC1/3 and PC2 immunoreactivities are alleviated somehow in the cloned $Ins2^{+/Akita}$ β-cells culturing at the *in vitro* conditions.

4. Discussion

Disproportionate hyperproinsulinemia is accepted to result from uncharacterized defects and/or secretory demand increases of β-cells in general diabetes [2,4,11]. In subjects with T1D, this abnormality is more prominent in pre-diabetic individuals sampled within 40 months prior to disease onset than in pre-diabetic subjects sampled 40–110 months before [4]. In patients with T2D, suppression of β-cell secretion with administration of somatostatin partially improved their hyperproinsulinemia [25]. These recent findings support the notion that β-cells' uncharacterized defects that link to hyperproinsulinemia may be aggravated by secretory demand increases [11,25]. In the course of insulin biosynthesis and secretion, distal processes seem not to be associated with the occurrence of hyperproinsulinemia. This is because 8-week sulfonylurea therapy did not improve the hyperproinsulinemia of T2D [26] despite sulfonylurea administration known is generally beneficial to the activities of K_{ATP} channels coupled insulin secretion on the plasma membrane of β-cells.

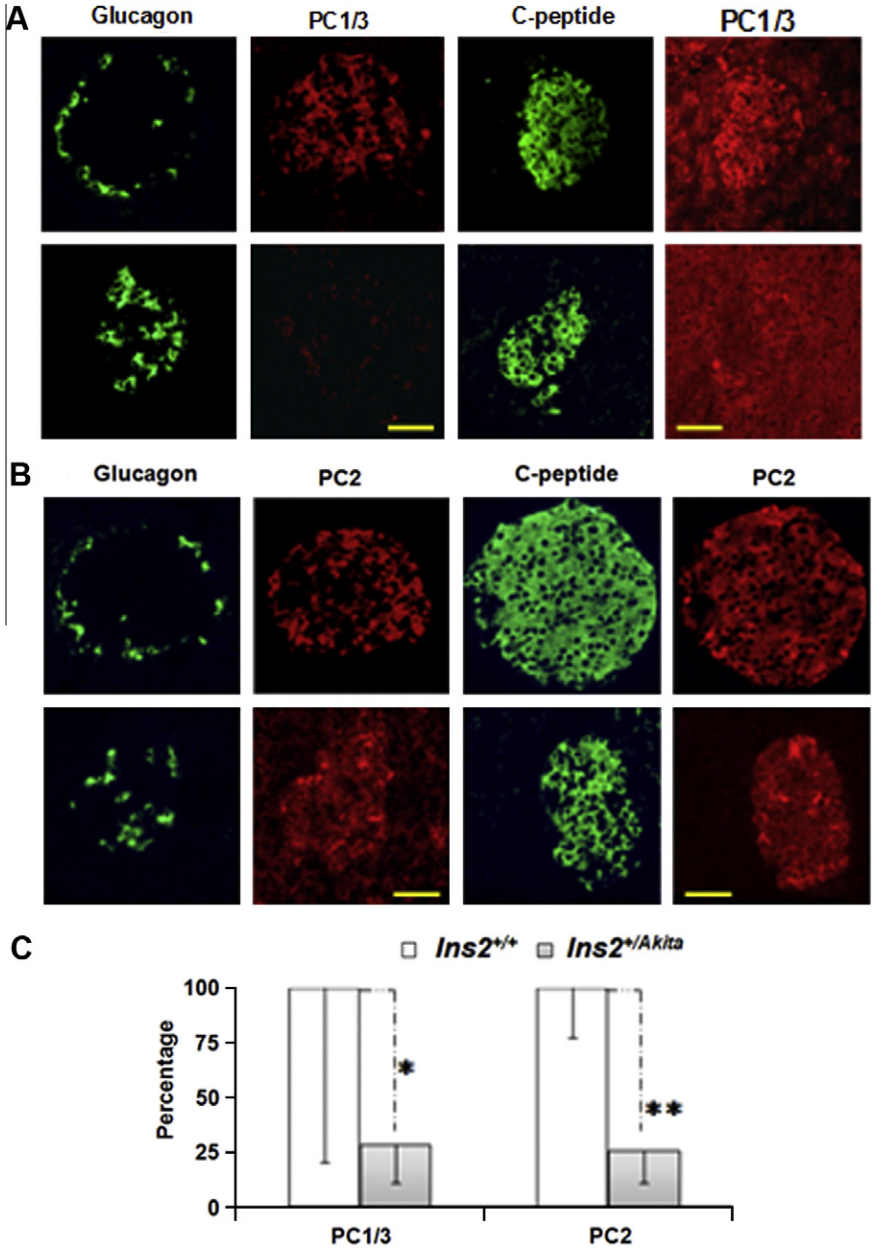

Fig. 2. Decrease of PC1/3 and PC2 immunoreactivities in the β-cells of *Ins2*$^{+/Akita}$ islets. Double immunofluorescent detection of PC1/3 (A) or PC2 (B) with glucagon or C-peptide in pancreatic sections of the wild-type *Ins2*$^{+/+}$ (upper panels) and mutant *Ins2*$^{+/Akita}$ (lower panels) mice at 12 weeks of age. Scale bar, 50 μm. (C) PC1/3 or PC2 immunoreactivities per unit of β-cell area in the mutant islets versus in the normal control islets. The β-cell area of each sectional islet was determined by C-peptide immunoreactivities, which was clearly distinguished from the area of α-cells marked by glucagon immunoreactivities. Data are presented as the mean ± SD. *n*, 30; ∗, $P < 0.05$; ∗∗, $P < 0.005$.

In this study, the findings demonstrated that the PIHO disorder in the early stage of insulin biosynthesis results in the abnormal high proinsulin/insulin ratio in both cellular and released proteins of the dysfunctional β-cells. As shown in Figs. 1 and 4, the abnormal high proinsulin/insulin ratio was exposed in the *Ins2*$^{+/Akita}$ islets and in the cloned dysfunctional *Ins2*$^{+/Akita}$ β-cells. Because the cloned dysfunctional and normal control β-cells have been all maintained at the high (25.5 mM) glucose condition since their establishment [22], the high proinsulin/insulin ratio preserved only in the *Ins2*$^{+/Akita}$ β-cells (rather than developed in the *Ins2*$^{+/+}$ β-cells) (Fig. 4) clearly showed that this abnormality does not result primarily from glucotoxicity. In fact, this abnormality is a consequence of the PIHO disorder in the *Ins2*$^{+/Akita}$ β-cells.

Our further analysis (Figs. 2 and 3) demonstrated a post-translational decline of the PC1/3 and PC2 convertase in the β-cells of *Ins2*$^{+/Akita}$ islets, which is a consequence of the PIHO disorder too. This consequence may contribute to the defective conversion of proinsulin to insulin *in vivo* (Fig. 1). Interestingly, the analyses in cloned *Ins2*$^{+/Akita}$ β-cells showed the preservation of the inefficient conversion of proinsulin despite the levels of PC1/3 and PC2 proteins were not reduced (Fig. 4), suggesting there are additional toxic consequences of the PIHO disorder that are implicated in the defective conversion of proinsulin to insulin *in vivo*. The additional toxic consequences may include the known ER and mitochondrial dysfunction [23] and uncharacterized disorders, which somehow affect the maturation and conversion of proinsulin and/or the function of PC1/3 and PC2 convertase.

Overall, the findings in this study provide new insights into the basis underlying the abnormality of disproportionate hyperproinsulinemia in diabetes, especially in the patients with diverse

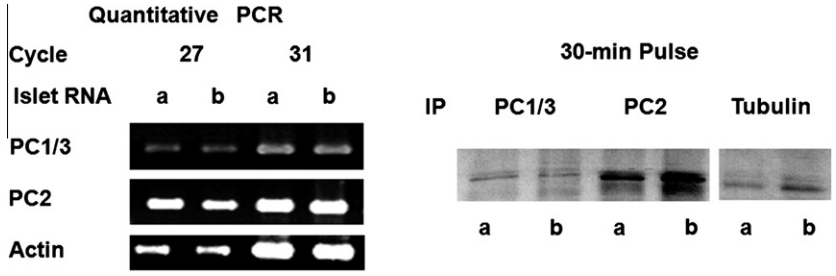

Fig. 3. No declines of PC1/3 and PC2 transcriptional and translational levels in the *Ins2*$^{+/Akita}$ islets. (A) Islet PC1/3 or PC2 transcripts were examined by quantitative RT-PCR. The specific primer sets for PC1/3, PC2, or β-actin and the quantitative RT-PCR approaches were described in Section 2. The expected size of PCR product was 433 bp for PC1/3, 403 bp for PC2, and 349 bp for β-actin. (B) Islet PC1/3 or PC2 protein synthesis was examined by metabolic-labeling approaches. One hundred islets per group were labeled with [^{35}S]-Met in 11 mM glucose for 30 min. PC1/3, PC2, and tubulin proteins were immunopurified with specific antisera and resolved by 16.5% tricine–SDS–PAGE for autoradiography. Islets used in (A) and (B) were isolated from mice at 12 weeks of age. Lane a, *Ins2*$^{+/+}$ islets; lane b, *Ins2*$^{+/Akita}$ islets.

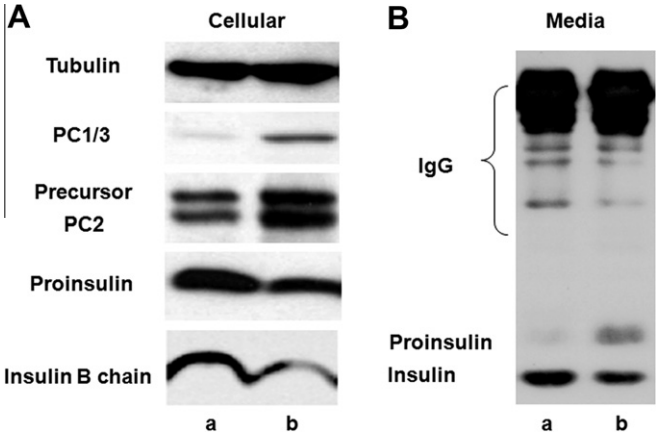

Fig. 4. Conformation of the inefficient conversion and atypical loss of proinsulin in the cloned *Ins2*$^{+/Akita}$ β-cells. (A) The levels of proinsulin, insulin, PC1/3, PC2 and tubulin in the cellular proteins (30 μg) of cloned *Ins2*$^{+/+}$ (lane a) and *Ins2*$^{+/Akita}$ (lane b) β-cells cloned *Ins2*$^{+/Akita}$ β-cells were resolved by 16.5% tricine reduced SDS–PAGE and then subjected to immunoblot analyses with the indicated antisera. (B) Secreted proinsulin and insulin by the cloned *Ins2*$^{+/+}$ (lane a) and *Ins2*$^{+/Akita}$ (lane b) β-cells for 24 h were immunopurified by insulin and C-peptide antisera, resolved by 16.5% tricine non-reduced SDS–PAGE, and subjected to insulin immunoblot analysis.

mutations in the insulin gene [27–29]. Some diversities in the symptom of patients with heterogonous mutations in the preproinsulin molecule exposed differences of varied amino acid residues in the contribution to proinsulin maturation that links to disposal and/or subsequent conversion in β-cells. For example, the H34D mutation results in apparent hyperproinsulinemia as that seen in T2D [27]. Many other mutations beyond the dibasic (KR or RR) cleavage sites lead to the significant insulin deficiency [28,29] and possible proinsulin conversion inefficiency as that exposed in this study. PIHO, the homeostatic balance of natively and non-natively folded proinsulin states, is susceptible to genetic and environmental influences due to the inherent aggregation-prone nature and low relative folding rate of insulin precursor [21]. It is observed that PIHO disorders are thus induced in several diabetic models such as the *Ins2*$^{+/Akita}$ [21], NOD, and db/db mice at pre-diabetic stages (as that mentioned early in Section 1). These recent findings collectively suggest that the PIHO disorder is a defect of β-cells that contributes to the occurrence of disproportionate hyperproinsulinemia in diabetes. To further investigate the molecules/pathways participating in PIHO disorders and consequences will help develop novel therapeutic means to alleviate the hyperproinsulinemia abnormality for curing diabetes. The investigations are underway.

Acknowledgments

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