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Mutant proinsulin proteins associated with neonatal diabetes are retained in the endoplasmic reticulum and not efficiently secreted

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Abstract

Mutations in the preproinsulin protein that affect processing of preproinsulin to proinsulin or lead to misfolding of proinsulin are associated with diabetes. We examined the subcellular localization and secretion of 13 neonatal diabetes-associated human proinsulin proteins (A24D, G32R, G32S, L35P, C43G, G47V, F48C, G84R, R89C, G90C, C96Y, S101C and Y108C) in rat INS-1 insulinoma cells. These mutant proinsulin proteins accumulate in the endoplasmic reticulum (ER) and are poorly secreted except for G84R and in contrast to wild-type and hyperproinsulinemia-associated mutant proteins (H34D and R89H) which were sorted to secretory granules and efficiently secreted. We also examined the effect of C96Y mutant proinsulin on the synthesis and secretion of wild-type insulin and observed a dominant-negative effect of the mutant proinsulin on the synthesis and secretion of wild-type insulin due to induction of the unfolded protein response and resulting attenuation of overall translation.

Keywords

Insulin; Diabetes; Misfolded protein; Unfolded protein response

INTRODUCTION

Recent studies have shown that mutations in the preproinsulin molecule can cause diabetes with onset at birth or shortly thereafter as well as later in life including adulthood [1–8]. They are also the cause of diabetes in the Akita and Munich mouse models of diabetes [9,10]. The diabetes-associated mutations are located in critical regions of the preproinsulin molecule and prevent normal processing of preproinsulin to proinsulin as well as folding and subsequent processing of proinsulin to insulin. They differ from the mutations in insulin and proinsulin associated with familial hyperinsulinemia and familial hyperproinsulinemia which result in the synthesis of an insulin molecule with reduced biological activity or a proinsulin molecule that cannot be processed to insulin [11]. The mutations associated with familial hyperinsulinemia and familial hyperproinsulinemia rarely cause diabetes but rather because of their reduced biological activity lead to accumulation of the mutant proteins in the serum.

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Here, we examine the subcellular localization and secretion of 13 neonatal diabetes-associated mutant preproinsulin molecules (A24D, G32R, G32S, L35P, C43G, G47V, F48C, G84R, R89C, G90C, C96Y, S101C and Y108C) and compare the results with wild-type preproinsulin and mutant preproinsulin proteins associated with hyperproinsulinemia (H34D and R89H).

Material and methods

Human insulin cDNA constructs

The human insulin cDNA [12] was cloned in pcDNA3.1(+). Mutations were introduced by overlap extension PCR mutagenesis and the sequence confirmed by DNA sequencing.

Cell culture, measurement of RNA levels and Western blotting

These methods are described in the Supplementary Material.

Subcellular localization of wild-type and mutant human proinsulin

INS-1 (1×10^6), HEK 293 (1×10^5) and AtT20 (1×10^5) cells cultured on glass cover slips in 6 well plates were transfected with 3 μ g of pcDNA3.1 (vector), WT or mutant human preproinsulin cDNA together with 0.5 μ g of constructs expressing ER-localized monomeric red fluorescent protein (ER-mRFP) [13] or pECFP-Golgi (Clontech, Mountain View, CA), an enhanced cyan fluorescent protein (ECFP) marker for the Golgi apparatus. The cells were cultured for 24 h, fixed with 4% paraformaldehyde for 20 min and then washed with PBS. The fixed cells were incubated with mouse monoclonal anti-human proinsulin C-peptide antibody (Millipore, St. Charles, MO; CBL94) at 1/1,000 dilution in PBS for 1 h at room temperature) or guinea pig polyclonal anti-human insulin antibody (Millipore; 1/1,000 dilution). The antigen was visualized using an anti-mouse secondary antibody conjugated with Alexa Fluor 488 (Invitrogen, Carlsbad, CA; A21202; 1/500 dilution) for human C-peptide or CyTM2-conjugated AffiniPure donkey anti-guinea pig IgG (Jackson ImmunoResearch, West Grove, PA; 1:300 dilution) for human insulin. After immunostaining, the cells were treated with Vectashield® mounting medium with DAPI (Vector, Burlingame, CA) and examined using an Olympus IX80 DSU Spinning Disk confocal microscope with excitation/emission wavelengths of 490/525 for Alexa Fluor 488, 490/528 for Cy2, 565/620 for RFP, 436/470 for ECFP and 350/457 for DAPI. We analyzed overlap of C-peptide or insulin and ER markers using Manders' overlap coefficient with ImageJ Software (NIH, Bethesda, MD) and the JACoP plug-in [14].

Measurement of C-peptide, proinsulin and insulin in media and cell lysates

Twenty-four hours post-transfection, cells were washed with PBS and then cultured for 4 h in the media described above but with 0.5% bovine serum albumin instead of 10% FBS. The media was collected and the cells were harvested. The cells were washed with PBS and harvested with a cell scraper in lysis buffer at 4° C (50 mM HEPES, 1% Triton X-100 and Complete Protease Inhibitor Cocktail (Roche). The lysate was sonicated for 10 sec (output 2 with microprobe) using a Sonicator W-380 (Heat Systems-Ultrasonics) and then centrifuged at 12,000 rpm for 20 min at 4 °C in an Eppendorf 5415D centrifuge. The supernatant was removed and assayed for human C-peptide (Immulite 2000 C-peptide; Siemens, Los Angeles, CA) and human proinsulin (Human Total Proinsulin ELISA Kit, Millipore, St. Louis, MO; EZHPI-15K).

Statistical analysis

Data are presented as mean $\pm$ SEM. Differences between groups were assessed using an unpaired *t*-test. *P* < 0.05 was considered significant.

Results

Transient expression of wild-type and mutant human proinsulin in cultured cells

Previous studies indicated that transient expression of mutant proinsulin proteins in INS-1 or MIN6 insulinoma cells can lead to cell death in 48–96 h [5,15,16]. In order to avoid possible confounding effects of ongoing apoptosis, we examined the time-course of expression of WT and mutant (H34D, G84R and C96Y) proinsulin mRNAs in INS-1 cells. The time-course was similar for each with insulin mRNA levels peaking at 24 h and then declining (Supplementary data Fig. 1). Thus, we studied the cells 24 h post-transfection.

Western blotting of INS-1 cell extracts using a human C-peptide antibody showed a band with the mobility of the recombinant human proinsulin standard in cells expressing WT and mutant proinsulin proteins, except for the signal peptide cleavage site mutant proinsulin A24D (Fig. 1). In cells expressing A24D proinsulin, the prominent band was of a larger size consistent with a lack of cleavage of the signal peptide. There was also a second/minor band with the mobility of proinsulin representing less than 5% of the total immunoreactive protein suggesting that INS-1 cells were able to cleave the mutant signal peptide, albeit not efficiently. The site of this cleavage is presently unknown. The human C-peptide antibody used in these studies recognized all the mutant proinsulin proteins except G84R proinsulin. This mutation is located in the C-peptide four residues from the C-terminus and may disrupt the epitope recognized by this particular human C-peptide antibody (Gly does not have a side chain in contrast to Arg and the presence of the side chain of Arg may block binding of the C-peptide antibody by steric hindrance).

Subcellular localization of wild-type and mutant proinsulin

We examined the subcellular localization of the WT and mutant proteins in INS-1 and AtT20 cells by immunohistochemistry using an antibody to human C-peptide to identify human preproinsulin, proinsulin or C-peptide (Fig. 2). This antibody does not cross-react with rodent C-peptide and thus allows us to study the biosynthesis and processing of human proinsulin in rat INS-1 insulinoma cells. The cells were co-transfected with constructs encoding ER-localized RFP that allowed us to visualize the ER and Golgi-localized ECFP that allowed us to visualize the Golgi apparatus.

The staining in cells expressing WT proinsulin and the hyperproinsulinemia mutation R89H showed a punctate pattern at the periphery of the cell suggesting that the protein is localized in secretory granules (Fig. 2A). The hyperproinsulinemia mutation H34D showed a unique pattern with C-peptide staining of secretory granules at the periphery of the cell, as well as perinuclear Golgi staining. The H34D mutation affects hexamer formation and sorting into dense core granules of the regulated secretory pathway [17]. This proinsulin also has 4–5-fold higher receptor binding activity than WT and may bind to insulin receptors in the trans-Golgi and be secreted constitutively or degraded in lysosomes [17,18].

Cells expressing mutant proinsulin proteins associated with diabetes (A24D, G32R, G32S, L35P, C43G, G47V, F48C, R89C, G90C, C96Y, S101C and Y108C) show C-peptide immunostaining that is localized within an expanded ER. The Golgi in these cells appears very similar in size and shape to that in cells expressing WT proinsulin. The G32R and Y108C mutant proteins show some localization to secretory granules suggesting they can enter the secretory pathway although not efficiently.

The human C-peptide antibody did not recognize the diabetes-associated G84R mutant protein in INS-1 cells (Fig. 2A). Since human insulin antibodies cross-react with rat insulin, we cannot use human insulin antibodies to study the localization of G84R proinsulin/insulin in INS-1 cells. We thus studied the subcellular localization of WT and G84R proinsulin in AtT20 cells

using a human insulin antibody (Fig. 2B). The pattern of human insulin staining in G84R proinsulin expressing cells indicates that G84R proinsulin is able to exit the ER and enter the secretory pathway in contrast to the other diabetes-associated mutant proinsulin proteins.

We quantified the overlap between C-peptide (or insulin) and ER-marker staining using Manders' correlation coefficient to estimate the extent of colocalization of C-peptide and ER [14] (Supplementary data Table 1). There was no significant difference in the overlap between C-peptide and ER between WT and H34D or R89H expressing cells. However, there is increased overlap in cells expressing the diabetes-associated mutations A24D, G32R, G32S, L35P, C43G, G47V, F48C, R89C, G90C, C96Y, S101C and Y108C indicating that these mutant proteins are retained in the ER. We carried out similar analyses comparing the overlap between WT and G84R proinsulin in AtT20 cells but using insulin staining to localize proinsulin/insulin. There was no significant difference in the overlap between insulin and ER in these cells suggesting that the G84R mutant protein can exit the ER.

Secretion of wild-type and mutant proinsulin

We examined the secretion of WT and mutant proteins by static incubation (4 h) using a human C-peptide Immunoassay (Immulite 2000 C-peptide, Siemens) to measure the amount of C-peptide immunoreactive material in the media and cell extracts (intracellular). There were readily detectable levels of C-peptide immunoreactive protein in cell extracts of INS-1 cells expressing WT and mutant proinsulin proteins (Table 1). There were, however, differences in immunoreactive C-peptide levels between the WT and various mutant proteins. It is unknown if these are due to differences in cross-reactivity with the C-peptide antibody or to other mechanism(s) (e.g. the specific effect of the mutant protein on translation or other mechanisms).

We observed efficient secretion of WT proinsulin/C-peptide and the hyperproinsulinemia mutant proteins H34D and R89H as well as G84R proinsulin from INS-1 cells (Table 1). The diabetes-associated mutant proinsulins were poorly secreted. We detected low levels of secretion of G32R and Y108C consistent with the localization of some C-peptide immunoreactivity in secretory granule-like structures in these cells. The amounts of C-peptide secreted by the other diabetes-associated mutant proinsulin proteins in INS-1 cells were below the sensitivity of the assay. We also examined secretion by transfected HEK 293 with quantitatively similar results (Supplementary data Table 2). Thus, secretion of the diabetes-associated mutant proteins is impaired whether by the regulated (INS-1 cells) or constitutive pathway (HEK 293 cells).

Effect of C96Y proinsulin on expression and secretion of wild-type proinsulin

We examined the effect of co-expression of increasing amounts of C96Y proinsulin cDNA (0–1.5 μ g) with a constant amount of WT proinsulin cDNA (1 μ g) on C-peptide immunoreactivity in cell extracts and media. There was a linear increase in human proinsulin mRNA levels with increasing amounts of cDNA input (WT and C96Y) (Fig. 3A).

The levels of C-peptide immunoreactivity in the cell extracts (representing both WT proinsulin/C-peptide and C96Y proinsulin) and media (WT only since as described above C96Y proinsulin is not secreted) were significantly decreased with increasing C96Y proinsulin cDNA input (Fig. 3B). This was accompanied by increased levels of phospho-eIF2 α suggesting that the cells co-expressing C96Y proinsulin had activated the unfolded protein response (UPR) leading to attenuation of translation (Fig. 3C and D) [20]. This effect on levels of C-peptide immunoreactivity was not observed when we added increasing amounts of WT proinsulin cDNA (Fig. 3B).

Discussion

In these studies, we have followed the lead of others who have shown that WT and mutant forms of human proinsulin can be studied using *in vitro* cell culture systems, including cell lines that do not normally synthesize insulin such as HEK 293 and AtT20 cells as well as those that do, such as INS-1 or other insulinoma cell lines [5,15,21–28]. There are advantages to studying heterologous expression of human proinsulin in multiple types of cells as parallel studies allow comparison of secretion via constitutive (HEK 293 cells) and regulated pathways (INS-1 cells). We chose to express “native” human preproinsulin in these systems. Others have used fluorescent protein (GFP, Venus, mCherry)-tagged preproinsulin, either integrated within the C-peptide region of the proinsulin molecule [5,21] or appended to the C-terminus [15,29,30] to monitor proinsulin and insulin biosynthesis and secretion *in vitro*. We chose not to do so because of the possibility that the presence of the GFP moiety might perturb the folding of the mutant proinsulins or conversely that GFP folding and fluorescence could be negatively affected by proinsulin folding [31]. Comparison of the results of studies using fluorescent protein-tagged preproinsulin [5,21,32] or native preproinsulin showed no apparent differences, at least with respect to the mutations examined to date.

Mutations in the preproinsulin protein that affect processing to proinsulin or normal folding of proinsulin result in retention of the mutant protein in the ER and impaired secretion in agreement with studies of others [5,9,21,32]. The severity of the effect of individual mutations on folding is reflected in the amount of C-peptide reactive material found within the cells (see Table 1); e.g. the C43G mutant which cannot form the B19-A20 disulfide bond which is the first to form and may serve as a template for further folding shows the greatest depression in intracellular levels [33]. The mutant protein also leads to attenuation of synthesis and secretion of the WT protein (and presumably other cellular proteins) (Fig. 3). Thus, the misfolded proinsulin protein compromises beta-cell function, decreases insulin secretion and sets in place a pathological process whereby the functionally beta-cells are stimulated to produce more insulin which ultimately leads to beta-cell failure and diabetes.

The results presented here suggest we will be able to classify diabetes-associated mutations based on their effect on proinsulin biosynthesis. For example, the proinsulin mutations (G32R and Y108C) result in the synthesis of a protein that can be targeted to the secretory pathway and secreted although inefficiently. If the efficiency of this process could be improved, it may be possible to delay or prevent the development of diabetes in patients with these mutations. The preproinsulin mutant protein A24D may constitute another class and it seems likely that if the efficiency of cleavage of the signal peptide from this protein could be increased that it would enter the secretory pathway and be secreted. Further studies of other mutant insulin proteins may reveal yet other classes.

The results of the studies of the G84R mutant protein suggest that it is not retained in the ER as are the other diabetes-associated mutations and that it can exit the ER and be sorted to secretory granules. This mutation was identified in a Vietnamese patient diagnosed with diabetes on the first day of life [2]. In addition to diabetes, he has developmental delay and anal atresia. The non-diabetic mother does not carry the mutation and the father is not available for testing. The functional studies presented here suggest that the G84R mutation may not affect proinsulin biosynthesis and thus may not be the cause of this patient's diabetes.

Continued studies of mutant insulin proteins that cause diabetes could identify the specific proteins and pathways that allow the beta-cell to maintain ER homeostasis and lead to new approaches for preserving beta-cell function in patients expressing a mutant insulin as well as in those that have type 2 diabetes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix A. Supplementary Material

Supplementary Material associated with this article can be found, in the online version, at [insert information].

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Fig. 1.

Expression of human proinsulin in INS-1 cells. Cells were transfected with vector (pcDNA3.1) or WT and mutant human proinsulin cDNAs. Cell lysates were prepared 24 h post-transfection and 15 μ g of total protein separated by 15% SDS-PAGE and transferred to a PVDF membrane. The membrane was probed with an anti-human proinsulin C-peptide antibody. The lane labeled "Proinsulin" is the recombinant human proinsulin standard.

Fig. 2.

Subcellular localization of wild-type and mutant proinsulin. A. INS-1 cells. B. AtT20 cells.

The localization of human C-peptide (INS-1) or human insulin (AtT20) immunoreactive protein is shown in green, ER is shown in red and the Golgi marker in cyan. Nuclei were stained with DAPI (blue). Data shown are representative of three independent experiments. Scale bar: 5 μ m.

Fig. 3.

Dominant-negative effect of C96Y proinsulin on wild-type proinsulin biosynthesis. INS-1 cells were transfected with 1 μ g of WT insulin cDNA and increasing amounts of C96Y insulin cDNA (0, 0.5, 1.0 and 1.5 μ g). Human insulin mRNA and C-peptide levels and rat total and phospho-eIF2 α levels were determined 24 h post-transfection by real-time RT-PCR, immunoassay and Western blotting (15 μ g of cell lysate), respectively. The results are the mean $\pm$ SEM of three independent experiments. A. Relative levels of human mRNA level (normalized to rat TBP mRNA). B. Total human C-peptide levels in cell extracts and media. The C-peptide levels are significantly decreased ($P < 0.01$) in cells transfected with C96Y insulin cDNA compared to cells transfected with 1 μ g of WT insulin cDNA. C. Induction of phospho-eIF2 α in cells transfected with pcDNA3.1 (2 μ g), WT (2 μ g) or C96Y (1 μ g) and WT (1 μ g) cDNAs. D. Fold induction of phospho-eIF2 α .

Table 1

Total C-peptide and proinsulin immunoreactive material in cell extracts and media of INS-1 cells expressing wild-type and mutant proinsulin/insulin proteins (4 h static incubation)

Mutation	Intracellular	Media	% of total in media
pcDNA3	ND	ND	-
WT	0.87 ± 0.01	0.25 ± 0.002	22 ± 0.3
A24D	0.27 ± 0.01	ND	-
G32R	0.36 ± 0.01	0.07 ± 0.002	16 ± 0.8
G32S	0.34 ± 0.004	ND	-
H34D	0.24 ± 0.01	0.17 ± 0.001	42 ± 0.6
L35P	0.24 ± 0.002	ND	-
C43G	0.07 ± 0.003	ND	-
G47V	0.44 ± 0.03	ND	-
F48C	0.15 ± 0.004	ND	-
G84R	0.85 ± 0.03	0.17 ± 0.003	17 ± 0.6
R89C	0.23 ± 0.000	ND	-
R89H	1.52 ± 0.06	0.5 ± 0.05	25 ± 1
G90C	0.32 ± 0.001	ND	-
C96Y	0.15 ± 0.001	ND	-
S101C	0.39 ± 0.01	ND	-
Y108C	0.33 ± 0.004	0.09 ± 0.001	21 ± 0.01

ND: Not-detectable; i.e. C-peptide in sample < 33.1 pmol/L (< 0.0661 pmol of total C-peptide)