Proinsulin processing in the diabetic Goto–Kakizaki rat

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Abstract

The biosynthesis and processing of proinsulin was investigated in the diabetic Goto–Kakizaki (GK) rat. Immunofluorescence microscopy comparing GK and Wistar control rat pancreata revealed marked changes in the distribution of α -cells and pronounced β -cell heterogeneity in the expression patterns of insulin, prohormone convertases PC1, PC2, carboxypeptidase E (CPE) and the PC-binding proteins 7B2 and ProSAAS. Western blot analyses of isolated islets revealed little difference in PC1 and CPE expression but PC2 immunoreactivity was markedly lower in the GK islets. The processing of the PC2-dependent substrate chromogranin A was reduced as evidenced by the appearance of intermediates. No differ-

ences were seen in the biosynthesis and post-translational modification of PC1, PC2 or CPE following incubation of islets in 16.7 mM glucose, but incubation in 3.3 mM glucose resulted in decreased PC2 biosynthesis in the GK islets. The rates of biosynthesis, processing and secretion of newly synthesized (pro)insulin were comparable. Circulating insulin immunoreactivity in both Wistar and GK rats was predominantly insulin 1 and 2 in the expected ratios with no (pro)insulin evident. Thus, the marked changes in islet morphology and PC2 expression did not impact the rate or extent of proinsulin processing either *in vitro* or *in vivo* in this experimental model.

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Introduction

Proinsulin and its conversion intermediates (des 31,32 proinsulin and des 64,65 proinsulin) make up around 10–15% of the total insulin immunoreactivity in the circulation of healthy humans (Ward *et al.* 1987, Clark *et al.* 1992, Reaven *et al.* 1993). They are secreted in a regulated manner from the pancreatic β -cells of islets where they make up around 1–2% islet insulin immunoreactivity (Hou *et al.* 1997). They accumulate in the circulation due to their longer half-lives ($t_{1/2}$) relative to insulin, but have only weak hypoglycemic activity (Creemers *et al.* 1998). Elevation of circulating proinsulin and the processing intermediates is observed in a number of pathophysiological situations including the prodrome of type 1 diabetes (Heaton *et al.* 1988), mild type 2 diabetes (Clark *et al.* 1992), glucose-intolerant states (Davies *et al.* 1993), pancreatic duct obstruction, thyroid disease (Beer *et al.* 1989) and insulin resistance induced by corticosteroids (Ward *et al.* 1987). More pronounced proinsulinemia is associated with genetic mutations in the proinsulin molecule (Nanjo *et al.* 1987, Roder *et al.* 1996),

proinsulin-processing enzyme defects (Naggert *et al.* 1995, Jackson *et al.* 1997, Furuta *et al.* 1998) and pancreatic adenomas (Cohen *et al.* 1986). (Proinsulinemia is defined here as conditions where either the proinsulin molecule or any of the common processing intermediates (split 32,33 proinsulin, des 31,32 proinsulin, split 65,66 proinsulin, des 64,65 proinsulin) are elevated more than 2-fold.) The origin of hyperproinsulinemia in diabetes has variously been attributed to deficiency in β -cell prohormone convertase (PC) activity (Rhodes & Alarcon 1994), changes in the intragranular processing environment (pH and Ca changes) (Furukawa *et al.* 1999) or asynchrony between stimulation of secretion and biosynthesis (Bollheimer *et al.* 1998). Incomplete processing could equally arise from increased secretory granule turnover as a direct consequence of the increased demands imposed by peripheral insulin resistance or decreased functional β -cell mass (Alarcon *et al.* 1995, Gadot *et al.* 1995, Seaquist *et al.* 1996, Laedtke *et al.* 2000). Another possibility could be regulation of the prohormone endopeptidases PC1 and PC2 by endogenous chaperonins or protease inhibitors in the pancreatic β -cell such as PC-derived propeptides (Fugere

et al. 2002) or PC inhibitors 7B2 and ProSAAS (Benjannet *et al.* 1995, Feng *et al.* 2001).

There are few experimental data that shed light upon these possible molecular and cell biological defects. Rodent islets, on which most insulin secretion studies are performed, have two non-allelic insulin gene products that are expressed at different levels and processed at different rates. The determination of proinsulin and the major intermediates in humans is facilitated by the availability of two-site immunometric assays (Heaton *et al.* 1988, Davies *et al.* 1993). However, these cannot be applied to rodent (pro)insulins and most data have been obtained by laborious RIA of samples separated by HPLC (Leahy *et al.* 1991, Gadot *et al.* 1995).

In the present study, we have used the Goto-Kakizaki (GK) rat model of type 2 diabetes (Goto *et al.* 1988) to investigate the expression of the prohormone convertases PC1, PC2 and carboxypeptidase E (CPE) and the PC inhibitors 7B2 and ProSAAS and co-related this to proinsulin processing *in vitro* and *in vivo*. We found changes in islet morphology consistent with β -cell degranulation and marked alterations in the steady-state level and biosynthesis of PC2. Such changes, however, appear to have little impact on the processing of proinsulin in the rat.

Materials and Methods

Animals and tissue preparation

Guidelines for the use and care of laboratory animals were followed at the institutions where the animals were housed. GK and Wistar control animals of 9–10 weeks of age were used to model the early stages of diabetes when body weight is normal and β -cell mass and density is largely preserved (Ostenson *et al.* 1993). Animals were fed freely and food withdrawn 4 h prior to killing. Islets were prepared by collagenase digestion (Guest *et al.* 1989) and cultured overnight in Dulbecco's modified Eagle's medium (Gibco) containing 8.3 mM glucose.

Histology

Pancreas pieces from 8- and 12-week-old animals were fixed in 10% formalin, paraffin embedded and prepared for immunofluorescence microscopy by conventional procedures (Pow & Clark 1990). Sections were incubated with a guinea pig polyclonal antibody to insulin (1:200) or mouse monoclonal antibody to glucagon (Sigma, 1:500) in combination with a polyclonal rabbit antibody to one of the following: PC1 (Lindberg (Louisiana State University) NH₂-terminal 1:200), PC2 (Creemers (University of Leuven) 1:200), CPE (Hutton 1:100) or ProSAAS (Lindberg recombinant 1:50). 7B2 (Creemers 102 1:200) antibody was combined with either the above insulin antisera or rabbit anti-glucagon (Hutton 1:200) (Guest *et al.* 1989, 1992, Creemers *et al.* 1996, Sayah *et al.* 2001).

Binding of the primary antibodies was detected with appropriate CY-2-, CY-3- or rhodamine-conjugated species-specific anti-IgGs (Jackson Laboratories, Bar Harbor, ME, USA) in the second layer. All procedures on GK and Wistar sections were performed in parallel. Preimmune sera and preabsorption controls were performed in each case (data not shown). Images were captured with a Coolsnap HQ digital camera (Photometrics, New York, NY, USA) on a Nikon FXA microphot fluorescence microscope using Intelligent Imaging System (Denver, CO, USA) software.

Western blot analysis

For analysis of PC1, PC2 and CPE, groups of 100 freshly isolated islets were collected into SDS-PAGE loading buffer containing 65 mM dithiothreitol, heated for 5 min at 100 °C and electrophoresed on gels polymerized from 12.5% acrylamide 0.08% N, N'-bisacrylamide in an SDS-Tris-glycine buffer system (Hutton *et al.* 1990). Proteins were transferred electrophoretically onto PVDF membranes (Amersham) and subjected to Western blot analysis using antisera to PC1 (rabbit 1:1000), PC2 (rabbit 1:1000), CPE (rabbit 1:1000) and chromogranin A (1:2000) and visualized by chemiluminescence (Amersham kit). All primary antibodies were generated in-house to recombinant proteins.

Biosynthetic radiolabeling

Groups of 100 islets were preincubated for 30 min in 100 μ l Krebs bicarbonate buffer containing 3.3 or 16.7 mM glucose at 37 °C in 1.5 ml capacity microcentrifuge tubes. They were then recovered by centrifugation for 10 s at 800 g (Microcentaur microcentrifuge; MSE) and resuspended in 100 μ l of the same pre-warmed medium containing 150 μ Ci [³⁵S]methionine (Amersham) and incubated for 30 min at 37 °C (Hutton *et al.* 1990). Pulse-chase experiments were performed using a 30 min radiolabeling in 16.7 mM glucose followed by resuspension of the islets for 45 min or 3 h in pre-warmed medium without radioisotope but containing 2 mM methionine and either 3.3 or 16.7 mM glucose. The islets and their media were then separated and the islets sonicated for 15 s at 25 W (MSE Sonifier, Crawley, UK) in 200 μ l lysis buffer (25 mM Na₂B₄O₇ (pH 9), 3% BSA, 1% Tween-20, 1 mM phenylmethanesulfonyl fluoride, 0.1 mM E-64, 1 mM EDTA and 0.1% NaN₃). The lysates were centrifuged for 5 min at 13 000 g and the supernatants retained for immunoprecipitation.

Immunoprecipitation

Islet lysates or media were incubated for 1 h at room temperature with 20 μ l 100 mg/ml suspension of Cowan-strain *Staphylococcus aureus* cells and then centrifuged for

5 min at 13 000 *g*. The supernatants were subjected to serial immunoprecipitation with a monoclonal insulin antibody (3B7) and then antisera against PC1, PC2 and CPE using established methodology (Hutton *et al.* 1990). The eluted proteins were electrophoresed on gels polymerized from either 7.5% acrylamide 0.08% *N*, *N'*-bisacrylamide in a Tris–HCl–urea buffer system (insulin) (Hutton *et al.* 1990) or by standard Laemmli SDS–PAGE on 12.5% acrylamide 0.08% *N*, *N'*-bisacrylamide gels in a Tris–glycine buffer system (PC1, PC2 and CPE) followed by fluorography and densitometric analysis (Guest *et al.* 1989).

Circulating proinsulin

Sera were pooled from three or four overnight-fasted rats and insulin-related peptides partially purified using C18 Sep–Pak cartridges (Waters & Associates, Milford, MA, USA) (Cohen *et al.* 1986). The eluted peptides were resolved by reverse-phase HPLC using a C-18 (0.5 μ m) 250 $\times$ 4.6 mm Synchropac column (Hewlett-Packard, Boeblingen, Germany) on a 1050 Hewlett-Packard HPLC system. The column was eluted at 1 ml/min under isocratic conditions for 25 min with 30.5% acetonitrile in 0.2% trifluoroacetic acid and then with a gradient of acetonitrile to 35.5% over an additional 55 min. Fractions (1 ml) were collected into tubes containing 0.1 ml 0.1% BSA, freeze-dried and reconstituted in 1 ml RIA buffer (PBS with 0.1% BSA). The elution positions of the rat 1 and 2 insulin-related peptides were determined with L-[4,5-³H]leucine- and [³⁵S]methionine-radiolabeled standards. Insulin-related peptides in the eluate were measured by RIA using rat insulin (Novo–Nordisk, Bagsvaerd, Denmark) as a standard. Porcine ¹²⁵I-insulin was obtained from Diagnostic Products Corp. (Los Angeles, CA, USA) and guinea-pig anti-porcine insulin from Linco Research Inc. (St Charles, MO, USA). Separation of bound and free hormone was performed using goat anti-guinea pig IgG serum and precipitation of the immune complex using 4% (w/v) polyethylene glycol.

Results

Immunohistochemistry

At the point of killing, the GK rats were comparable to controls in body weight (Table 1) and they showed no signs of obesity or dehydration. They were markedly hyperglycemic but had comparable circulating insulin levels (total insulin immunoreactivity).

The yield and size of isolated islets was comparable for each group (100–150/rat) and the protein content was comparable (data not shown). The borders of GK islets, however, were irregular and they appeared more vascularized as revealed by their visible content of red blood cells. Immunofluorescence microscopy (Fig. 1) revealed

Table 1 Animal data (means $\pm$ S.E.M.)

	Wistar (<i>n</i> = 13)	GK (<i>n</i> = 16)
Body weight (g)	221 $\pm$ 2	231 $\pm$ 4
Blood glucose (mmol/l)	8.7 $\pm$ 0.7	18.6 $\pm$ 1.6
Serum insulin (μ U/ml)	64 $\pm$ 5	65 $\pm$ 6

that the proportion of β -cells to non- β -cells appeared normal in islets of GK animals but that the insulin immunoreactivity in the β -cell population was more variable than in control Wistar islets. Glucagon-positive cells were as prominent in GK islets as in Wistar islets; however, their spatial distribution was markedly altered in the GK animals. The peripheral localization of glucagon-positive cells in Wistar islets was replaced in GK animals with a more random distribution throughout the core of GK islets.

PC1 immunoreactivity was expressed at a higher level in β -cells than non- β -cells in both Wistar control and GK rats and there was little, if any, overlap in PC1 and glucagon immunoreactivity (Fig. 1A). There was an uneven distribution of PC1 among insulin-positive cells of the GK rat and an apparent correlation between the level of expression of insulin and PC1 within the same cell across the entire population of β -cells.

PC2 was broadly distributed in β - and α -cells in both GK and Wistar islets and double-labeling experiments again showed a general correlation between the intensity of PC2 and insulin immunofluorescence in β -cells and a similar correlation between PC2 and glucagon immunofluorescence in α -cells (Fig. 1A).

CPE was found in β - and α -cells (Fig. 1B, double labeling with insulin not shown) with no obvious difference in distribution between α - and β -cells between GK and Wistar animals. The chaperonin/PC2 inhibitor 7B2 was present in both α - and β -cells and the immunofluorescence intensity was similar in both cells with no obvious changes in cellular association in Wistar and GK animals. The ProSAAS molecule, which is a putative regulator of PC1 activity, was found in both α - and β -cells in both Wistar and GK rats. Within the β -cell population there was again a variable distribution of CPE, 7B2 and ProSAAS from cell to cell and, as in the case of PC1 and PC2, a general correlation between the expression of each marker and insulin (data not shown).

Western blot analysis of PCs and chromogranin A

The steady-state levels and maturation of the proprotein converting enzymes was assessed by Western blot analyses using equal numbers of islets per lane (Fig. 2). The endopeptidase PC1 appeared as the processed COOH-truncated 66 kDa form in both GK and Wistar islets with

A.

B.

Figure 2 Western blot analyses of prohormone convertases. Western blot analyses of PC1, PC2, CPE and chromogranin A (CgA) were performed on SDS-Laemmli gels with batches of 100 islets. Similar results were obtained in three separate experiments using islets pooled from four animals.

no significant difference in the overall level of expression in GK animals. The PC2 endoprotease appeared as the mature 65 kDa form in islets from both GK and control rats but was present at lower levels in the GK islets. CPE expression was similar in both animal groups although there was proportionately more of the unprocessed 55 kDa precursor form in GK islets. Chromogranin A, which is converted in the pancreatic β -cell by PC2 to the 21 kDa peptide betagranin (Arden *et al.* 1994), was found in the GK rat islets to include larger molecular forms, presumably processing intermediates (Fig. 2). Western blot analyses with anti-insulin antibodies detected abundant insulin but negligible proinsulin or proinsulin intermediates in either GK or Wistar animals (data not shown).

Biosynthetic radiolabeling

Pulse-chase radiolabeling experiments coupled with immunoprecipitation analysis showed that the biosynthesis of PC1 in GK islets followed a similar pattern in the Wistar controls (Fig. 3A). Incorporation of radioactivity was stimulated markedly by glucose compared with the response of total proteins (2- to 3-fold increase in trichloroacetic acid-precipitable radioactivity (Guest *et al.* 1989)). PC1 underwent post-translational modification including

early glycosylation and carbohydrate trimming (increase from 83 kDa to 87 kDa and back) and subsequent COOH-terminal proteolysis (decrease from 83 kDa to 66 kDa) with similar kinetics. CPE labeling and post-translational processing were similar in islets of both animal strains but, in contrast, the synthesis of PC2, which is normally glucose-insensitive, appeared to be responsive to glucose in GK rats but not in Wistar controls. The incorporation of radioactivity into PC2 was similar in both animal strains at 16.7 mM glucose but at 3.3 mM GK islets show considerably less incorporation of [35 S]methionine into PC2 than Wistar islets. The post-translational processing of the 75 kDa precursor to the 65 kDa mature form over the pulse-chase protocol proceeded with similar kinetics and no major differences were detectable over the 3 h time course. The observed changes in molecular sizes are in accord with previous studies (Creemers *et al.* 1998).

No major differences were observed in the biosynthesis and processing of proinsulin between the GK and Wistar islets (Fig. 3B). Islets incorporated similar levels of [35 S]methionine under basal conditions, elevation of glucose increased incorporation to a similar extent and the conversion of proinsulin to insulin proceeded with similar kinetics via the conversion intermediates. Under the conditions used, only rat proinsulin 2 is labeled as

Figure 1 Immunohistochemistry of insulin in isolated islets and PC1 and PC2 in pancreatic sections of GK and Wistar animals. Indirect immunofluorescence microscopy was performed on paraffin-embedded pancreas sections using combinations of an antibody to either insulin or glucagon alongside antibodies from another species directed to other antigens. Secondary antibodies conjugated to CY-2 (green channel) and either CY-3 or rhodamine (red channel) were used in the second layer. Images of each fluorophore are displayed in pseudocolor and the merged images are shown in each case. (A) The most striking differences between the GK and Wistar animals were the heterogeneity of expression of insulin, PC1 and PC2 and more dispersed distribution of α -cells in the GK islets. The exclusion of PC1 from α -cells was apparent in both control and diabetic animals. (B) CPE, 7B2 and ProSAAS were expressed in both α - and β -cells. Like insulin (A), they showed a more heterogeneous expression in β -cells in the GK animals.

Figure 3 Biosynthetic radiolabeling of PCs and proinsulin. Islets in batches of 100 were preincubated for 30 min and then labeled with [³⁵S]methionine for 30 min in the presence of 3.3 mM glucose (track 1) or 16.7 mM glucose (track 2) to assess the affects of glucose on biosynthesis (see Methods section). For pulse-chase experiments, islets were radiolabeled as above in 16.7 mM glucose and then chased in medium containing 2 mM methionine (no radiolabel) and either 3.3 or 16.7 mM glucose for either 45 or 180 min. (A) Immunoprecipitates of the cellular forms of the PCs (100 islet equivalents/lane) were analyzed on SDS-Laemmli gels. (B) Immunoprecipitates of the cellular (20 islet equivalents/lane) and medium (100 islet equivalents/lane) forms of labeled (pro)insulin were analyzed on Tris–glycine–urea gels. Similar results were obtained in three separate experiments using batches of islets pooled from four animals.

proinsulin 1 does not contain a methionine residue. Labeled insulin peptides accumulated in the medium under conditions of glucose stimulation in both GK and Wistar rat islets. Previous studies have shown that the release of newly synthesized insulin peptides can be triggered approximately 30 min after labeling when they are first delivered to the secretory granule (Creemers *et al.* 1998). The composition of secreted insulin-related peptides is initially dominated by proinsulin and at later times by insulin, a reflection of the proteolytic maturation of the granule cargo. The composition of the peptides in the medium determined in these experiments thus effectively integrates the changes in cellular composition of the peptides over the 180 min chase period. The observation that the composition of the immunoprecipitated peptides was similar in both GK and Wistar islets suggests that both the intracellular conversion of proinsulin and release of the insulin-related peptides followed similar kinetics.

Measurements of circulating (pro)insulin levels

Determination of the composition of circulating insulin peptides by a combined HPLC/RIA technique showed major immunoreactive peaks corresponding to insulin 1 and insulin 2 with lesser amounts of a component that co-elutes with insulin 2 which has undergone oxidation of its single Met residue either *in vivo* or during sample preparation. These molecular forms corresponded to 92% (Wistar) and 93% (GK) of the total immunoreactivity recovered from the HPLC gradient (two assays from seven animals). The levels of proinsulin or proinsulin-processing intermediates in the circulation of both the Wistar and GK rats were less than 2% of the insulin immunoreactivity (Fig. 4), a situation in marked contrast to human where levels in the range of 10–30% are recorded (Jackson *et al.* 1997). The insulin 1 to insulin 2 ratio was similar in both animal groups (2.58 vs 2.56).

Discussion

The GK rat is a non-obese insulin-resistant model (Bisbis *et al.* 1993) of type 2 diabetes involving multiple genetic loci (Galli *et al.* 1996, Gauguier *et al.* 1996) that was produced by selective inbreeding for a hyperglycemic trait (Goto *et al.* 1988). At the level of the islet, changes in glucose metabolism (Tsuura *et al.* 1993, Ling *et al.* 1998) or cAMP generation (Abdel-Halim *et al.* 1998) are associated with a secretory defect that is more pronounced *in vivo* and in perfused pancreas (Portha *et al.* 1991) than in static incubations (Portha *et al.* 1991, Hughes *et al.* 1994). The present studies have used animals from the Stockholm colony (Abdel-Halim *et al.* 1994), which have been shown to have preserved β -cell density (Guenifi *et al.* 1995) and to have less-pronounced morphological and diabetic

phenotypes than the Paris colony (Portha *et al.* 1991, Movassat *et al.* 1997).

We focused on the biosynthesis and post-translational processing of proinsulin and the proinsulin-processing enzymes in these animals, events that are acutely regulated by glucose at the level of protein translation and potentially influenced by a complex series of post-Golgi sorting and membrane-trafficking events (Creemers *et al.* 1998). Initial immunocytochemical analysis provided a survey of the molecular components of the proinsulin-processing machinery that potentially affect the production of the active hormone at the cellular level. Dramatic differences between GK and Wistar rats were observed in the distribution of α -cells throughout the islet and heterogeneity of the β -cell population in the GK animals with respect to their expression of insulin. The heterogeneity in insulin expression appeared to be paralleled by heterogeneity in the expression of the other components of the processing machinery, namely the enzymes PC1, PC2 and their respective inhibitors 7B2 and ProSAAS and the exopeptidase CPE. All the proteins in question are localized to the secretory granule and the observed changes are consistent with cell-to-cell differences in granulation, yet the overall insulin content per islet was not altered in these animals (C-G Östenson, V Poitout & J C Hutton, unpublished observations). The heterogeneity is possibly the result of the differences in the thresholds of individual β -cells for glucose-activated metabolism (Heimberg *et al.* 1993, Bennett *et al.* 1996), granule exocytosis and insulin biosynthesis (Kiekens *et al.* 1992), or a feedback mechanism that interconnects these processes. It is conceivable that changes in paracrine relationships between the β -cell and α -cell could play a role given the marked changes in α -cell distribution that characterize the islets of the GK rat and other diabetic models (Movassat *et al.* 1997).

The finding that proinsulin biosynthesis and processing of proinsulin appeared normal in 9-week-old GK rats was remarkable in the face of such striking morphological changes. The results agree with previous studies (Giroix *et al.* 1993, Nagamatsu *et al.* 1999) and suggest that the depletion of secreted constituents in β -cells does not arise from a failure to recognize glucose as an activator of prohormone biosynthesis and granule biogenesis. Rather it points to an inability of the β -cell population as a whole to meet the demands upon insulin secretion imposed by chronic hyperglycemia *in vivo*. Although circulating insulin levels did not differ between GK rats and their Wistar controls, they were inappropriate for the level of glycemia indicative of insulin resistance or either a secretory defect and/or reduction in functional β -cell mass. The evident degranulation (Fig. 1) and previous studies showing reduced β -cell mass from a very early age (Serradas *et al.* 1998) suggest that the latter may be more important. Given that the defect in the GK β -cell lies at the level of cyclic nucleotide generation and stimulus-secretion coupling (Portha *et al.* 1991, Abdel-Halim *et al.* 1998), it is

Figure 4 HPLC analysis of circulating immunoreactive forms of insulin in Wistar and GK rats. The position of elution of insulin 1 and insulin 2 are indicated. The processing intermediates elute between the insulin and proinsulin molecules. Oxd. INS 2=oxidized insulin 2. Similar results were obtained in two separate experiments using serum pooled from either three or four animals.

likely that there may not be an impairment in stimulus–biosynthesis coupling since it is not thought to depend on this second messenger. Under such circumstances, the extent of proinsulin processing within the β -cell might actually be more complete since immature granules would not be exocytosed. Defective exocytosis, on the other hand, would prevent the positive feedback of insulin or other secreted products on β -cell transcriptional activity and replication/antiapoptosis (Burks & White 2001),

which in the longer term would affect the secretory capacity of individual β -cells and pancreatic β -cell mass.

The major biochemical changes that were observed in the GK rat appeared to be in the prohormone-processing enzyme PC2. Steady-state levels of islet PC2 in GK islets were reduced relative to Wistar islets yet rates of biosynthesis and processing at high glucose *in vitro* appeared normal. It appeared that PC2 biosynthesis at low glucose concentrations was diminished. Since it is evident that

PC2 is more concentrated in α -cells of normal islets (Fig. 1A), the question arises as to whether the changes in the steady-state levels and biosynthesis of PC2 in the GK animals truly reflect changes occurring in the β -cell. Islets from another diabetic model, the *ob/ob* mouse, like the GK rat, show a glucose-stimulated PC2 biosynthetic response that is not evident in control strains (Martin *et al.* 1994). Since cultured Min6 β -cells also show glucose-stimulated PC2 biosynthesis (Skelly *et al.* 1996), it has been argued that glucose-stimulated PC2 biosynthesis is an intrinsic property of the β -cell that is unmasked in *ob/ob* mice by the massive expansion of β -cell mass and the loss of α -cells. The glucagon content of GK islets is not markedly changed, although GK islets appear similar to *ob/ob* islets in that the α -cell distribution is altered (Baetens *et al.* 1978). It appears more likely in the present instance that there is a reduced biosynthesis of PC2 in the diabetic GK rat islets at lower glucose concentrations. The reduced islet content of PC2 revealed by Western blotting could reflect this phenomenon operating *in vivo* and/or an effect of granule depletion. Granule constituents with a slow rate of maturation such as PC2 ($t_{1/2}$ of conversion 2–3 h vs 5–10 min for PC1) are most likely to be affected by degranulation.

From studies in rats rendered hyperglycemic by repeated glucose injection or by 90% pancreatectomy (Leahy *et al.* 1991), it has been inferred that β -cell granule exhaustion is the primary cause of alterations in circulating proinsulin in man. Similar conclusions have been reached by direct measurement of the circulating proinsulin levels in the spontaneously diabetic sand rat *Psammomys obesus* (Gadot *et al.* 1995). In each case, a very marked decrease in insulin content and degranulation was observed and elevated proinsulin secretion was seen to be the consequence of the relative immaturity of the granule population. In humans, a decrease in β -cell mass produced by hemipancreatectomy (Seaquist *et al.* 1996) induces a 5- to 6-fold increase in circulating proinsulin without major excursions in plasma glucose. Likewise low-dose streptozotocin in a non-human primate can cause hyperproinsulinemia without hyperglycemia (Kahn *et al.* 1992). Suppression of endogenous insulin secretion in mildly diabetic humans *in vivo* can restore circulating proinsulin/insulin ratios and pulsatile release within 24 h (Laedtke *et al.* 2000), which again fits with the concept that granule maturity is a key determinant of the release of proinsulin and particularly the major intermediate, des 31,32 proinsulin.

The circulating levels of proinsulin or proinsulin conversion intermediates in the GK diabetic rat did not model the situation in diabetic humans. The failure to observe any proinsulin or processing intermediates in the normal Wistar rat also contrasts with the situation found in healthy human subjects. The simplest explanation for these differences is that proinsulin conversion is more efficient or more extensive in the rodent than in human islets ($t_{1/2}$ of

conversion 40 vs 100 min (Sizonenko *et al.* 1993)). There are important sequence differences at both the B-chain/C-peptide and C-peptide/A-chain cleavage sites between the human and rodent proinsulins (Sizonenko & Halban 1991). The processing of human proinsulin occurs at sites marked by the sequence Lys-Thr-Arg-Arg and Leu-Gln-Lys-Arg, which are cleaved by the endoproteases PC1 and PC2 respectively (Davidson *et al.* 1987). These enzymes work sequentially and thus little cleavage of the C-peptide/A-chain junction by PC2 occurs in the absence of prior cleavage at the B-chain/C-peptide junction by PC1 (Jackson *et al.* 1997). By contrast, processing of rat proinsulin 2 (as studied in the present radiolabeling experiments) occurs at sites marked by Met-Ser-Arg-Arg (B-chain/C-peptide junction) and Arg-Gln-Lys-Arg (C-peptide/A-chain junction), both of which can be cleaved by PC1 alone (Vollenweider *et al.* 1995). The presence of a P-4 Arg at the C-peptide/A-chain junction is critical in this regard and probably also makes rat proinsulin a better substrate for PC2. Changes in the steady-state level and biosynthesis of PC2 in GK diabetic animals of the magnitude observed might therefore have little impact on proinsulin conversion in the rat. In the PC2 knockout mouse, only minor impairment in islet proinsulin processing occurs (identical B-chain/C-peptide and similar C-peptide/A-chain junctions to rat except mouse insulin 2 lacks the P4 Arg) (Furuta *et al.* 1998). The heterozygote (PC2 +/–) mouse, which could have a comparable defect to the GK rat (50% reduction in PC2 expression), is reportedly normal. The changes which were observed nevertheless may still be sufficient to impact rates of proprotein conversion as evidenced by the accumulation of intermediate forms of chromogranin A NH₂-terminal processing, a reaction that is catalyzed by PC2 (Arden *et al.* 1994). The chromogranin A intermediates that appear in the GK islets (Fig. 2) were identical in size to intermediate forms seen in the islets of PC2 null mice (J C Hutton & D F Steiner, unpublished observations).

Equivalent changes in PC2 expression in human diabetic islets might nevertheless impede conversion of des 31,32 proinsulin to insulin and lead to elevation in the circulation of that intermediate (Hou *et al.* 1997). In this context it is notable that it is des 31,32 proinsulin more than proinsulin or the des 64,65 intermediate that contributes to the increase of (pro)insulin immunoreactivity in diabetes (Clark *et al.* 1992). It would be of interest to observe how a GK rat bearing a human proinsulin transgene would behave in this context.

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