BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **248,** 186–189 (1998)
ARTICLE NO. RC988924

Effect of High Glucose Concentration on Proinsulin Biosynthesis and Conversion by Human Islets

Elena F. Usac, Betlem Nadal, Rosa Gasa, Josefa Fernandez-Alvarez, and Ramon Gomis[1]
Servei d'Endocrinologia i Diabetis, IDIBAPS, Hospital Clínic, Facultat Medicina, Universitat de Barcelona, C/Villarroel 170, 08036 Barcelona, Spain

Received June 5, 1998

In the present study we investigate whether glucose concentration could have an effect on proinsulin biosynthesis and processing. We cultured control human islets under chronic high and low glucose concentrations. After the culture period, islets were pulse-labeled and chased for different periods of time. Proteins from islets were collected, insulin immunoprecipitated, and analyzed by alkaline–urea gel electrophoresis. We have found an accelerated rate of proinsulin conversion by those islets exposed to high glucose concentration (at 24.4mM of glucose), but not by those islets cultured at low glucose concentration (at 5.5mM of glucose). However, we do not observe any decrease or increase on newly proinsulin synthesis in any of these conditions. © 1998 Academic Press

Key Words: **human islets; proinsulin conversion; high glucose concentration.**

Insulin is initially synthesized in pancreatic islet β cells as an inactive precursor molecule, proinsulin, which is then converted to insulin and C-peptide in the secretory granules [1–3]. Complete conversion involves cleavage at the B-chain/C-peptide junction by a type 1 endopeptidase activity [4] which has been proposed to correspond to PC1/3 endoprotease [5-6] and at the C-peptide/A-chain junction by a type 2 activity [4–5,7], which corresponds to the PC2 endoprotease [5,7]. Proinsulin biosynthesis has been studied using several cell lines and rat or mouse pancreatic islets [8-14] but little data is available from human beta cells [15]. The bulk of evidence for human proinsulin processing supports a sequential mechanism whereby PC3 cleaves intact proinsulin to des 31,32 proinsulin and thereafter PC2 exerts its action to obtain final products, insulin and C-peptide [16-17]. Cleavage only by type 1 or type 2 activity and subsequent trimming of C-terminal basic amino acids by carboxypeptidase H/E leaves two inactive conversion intermediates (des-31,32-split proinsulin and des-64,65-split proinsulin, respectively).

It has been suggested that chronic elevated glucose levels may alter normal proinsulin biosynthesis and conversion [3]. The aim of the present study was to analyze proinsulin biosynthesis and conversion by human pancreatic islets exposed to high and low glucose concentration.

We demonstrate, here, an accelerate conversion of proinsulin to mature insulin by those islets chronically exposed to high levels of glucose concentration.

MATERIALS AND METHODS

Human pancreatic islet isolation and culture. Pancreases for the study were obtained, after informed consent of their families and approval of the Hospital Ethics Committee, from three human cadaveric organ donors, [two males and a female age matched (69, 59, and 74; 67.3 ± 7.63 years old). Islets were isolated by collagenase (Boehringer Mannheim, Heidelberg, Germany) digestion of the pancreas as previously described [18], and separated from exocrine tissue by bovine serum albumin (Advanced Protein Products LTD, Brierley Hill, UK) density gradient. Isolated islets were washed several times in Hanks-balanced salt solution (HBSS) and then transferred to RPMI-1640 medium (Gibco BRL, Pisley, U.K.) supplemented with fetal calf serum 10%, L-gluthamine 2mM, D-glucose 5.5 mM, fungizone 10 μg/ml and antibiotics (penicillin 100U/ml, gentamycine 50 μg/ml), and maintained overnight at 37°C in humidified atmosphere of 95% air-5% CO_2. Islets were picked and cultured for 24h or 8 days in RPMI-1640 medium supplemented with either 5.5 mM or 24.4 mM of glucose. One sample of islets, from every batch used, was stained with Acridine orange and ditizone to control their viability and integrity.

Pulse chase-protocol. Islets were washed 3 times in Krebs Ringer Bicarbonate Buffer (KRBB) containing 20mM Hepes, 0.1% BSA, pH 7.4 and 16.4 mM glucose. After 30 min preincubation in this same buffer at 37°C, 150 islets per condition were labeled for 20 min in 200μl KRBB-16.4mM glucose containing 2μCi ^{35}S methionine, cysteine/islet ([^{35}S]methionine, cysteine [specific activity >1000 Ci/mM] Amersham, Life Science, Little Chalfont, UK). The labeled islets were washed 3 times in ice-cold KRBB and then chase incubated at

[1] Correspondence and reprint requests should be addressed to Dr. Ramon Gomis, Department of Endocrinology and Diabetes, Hospital Clinic, Villarroel 170, 08036 Barcelona, Spain. Fax: (34-3) 227 54 54/ (34-3) 4515272; E-mail: Gomis@medicina.ub.es.

Abbreviations: BSA, bovine serum albumin; KRBB, Krebs–Ringer bicarbonate buffer; RIA, radioimmunoassay; FCS, fetal calf serum.

0006-291X/98 $25.00

37°C with warm KRBB 2.8 or 16.7 mM glucose. After the period of time indicated, each tube was centrifuged at 1,000 g and washed islet pellets were immediately frozen with dry ice.

Immunoprecipitation. Islet pellets were resuspended in 400μl lysis buffer containing 25 mM Na_2 B_4 O_7 (pH 9), 0.1% NaN_3, 1% Tween20, 3% (w/v) BSA and protease inhibitors [1mM phenylmethane sulphonyl fluoride (PMSF), 0.1mM trans-epoxysuccinyl-L-Leucyl-amido (4-guanidino) butane (E-64), 1mM EDTA, 0.1mM-N-tosyl-L-phenylalanine chloromethyl ketone (TPCK) and 10μM Pepstatin A]. They were sonicated (25W for 20s) and centrifuged at 10,000 g for 5 min, and the resultant supernatant was stored at −20°C. Cell proteins were then precleared with 50μl Cowan (100mg/ml on lysis buffer) for 1h at 25°C and then subjected to immunoprecipitation with specific immunoadsorbent to insulin as previously described [19]. Immunoprecipitates were eluted in 400 μl of 25% acetic acid and dried on the Speed Vac Concentrator (SVC200H, Savant). Proinsulin related peptides were separated through an alkaline-urea gel electrophoresis and treated for a fluorography. Densitometric scanning of the bands obtained were used to quantify data (Bio Image Whole band Analyzer. Sun Microsystems, Inc. California).

Morphometric analysis of the beta cells. Serial sections were cut from frozen pancreas blocks and immunostained for insulin using the indirect peroxidase technique. Antiserum to insulin (DAKO, Glostrup, Denmark) was used in a dilution of 1/10 and, as a second antibody a peroxidase conjugated antiguinea pig (SIGMA, St. Louis, MO). Morphometry was done as described previously [20], using a manual optical analyzer (Model MOP-1, Olympus, Tokyo) on a projected image of the histological sections of the pancreas.

Islet insulin content. To determine the insulin content, batches of 10 islets were disintegrated by sonication at 4°C in 0.5 ml acid-ethanol solution (75% ethanol, 0.15 N HCl). Peptide extraction were performed after an overnight precipitation at 4°C, and subsequent centrifugation for 15 min at 2600 g. Supernatants were dried down and kept at −20°C until their use. Samples were reconstituted in 0.5 ml of a buffer composed of 150mM NaCl with 50mM Na_2HPO_4, 0.1% NaN_3 and 0.5% BSA and insulin assayed by RIA (CIS, Biointernational, Gif-Sur-Yvette, France).

Western blotting. Denatured proteins from lysate islets were separated by SDS-PAGE and transferred to nitrocellulose by electroblotting. After transfer, the membranes were blocked with TBS buffer (Tris-base 20mM, 150mM NaCl; pH 7.4) containing 6% (w/v) of non fat dried milk for 1 h. Subsequently they were incubated for 3 h with the first antibody, to PC2 or PC3 diluted to 1/3000 with the blocking buffer containing 0.1% of tween-20 (v/v). Nitrocellulose membranes were washed with the dilution buffer before incubating them with goat peroxidase-conjugated anti-rabbit IgG (Sigma, St. Louis, MO) for 1h at room temperature. Visualization of immunocomplexes was performed by ECL (Amersham Life Science, Little Chalfont, UK).

TABLE 1

Ratios of Newly Synthesized Insulin/Proinsulin at 180 min Chase Time

	24 h		8 days	
	5.5 mM	24.4 mM	5.5 mM	24.4 mM
2.8 mM	6.03 ± 0.17	118.50 ± 5.20	6.03 ± 0.21	65.20 ± 2.01
16.7 mM	13.16 ± 0.21	72.86 ± 2.44	9.80 ± 0.46	75.60 ± 2.80

Note. Data from the pulse-chase studies of control human islets cultured for 24 h or 8 days at 5.5 and 24.4 mM of glucose concentration was quantified by densitometric scanner. The media ± SEM is shown from three independent experiments. Both chase conditions studied (2.8 and 16.7 mM of glucose concentration) are indicated.

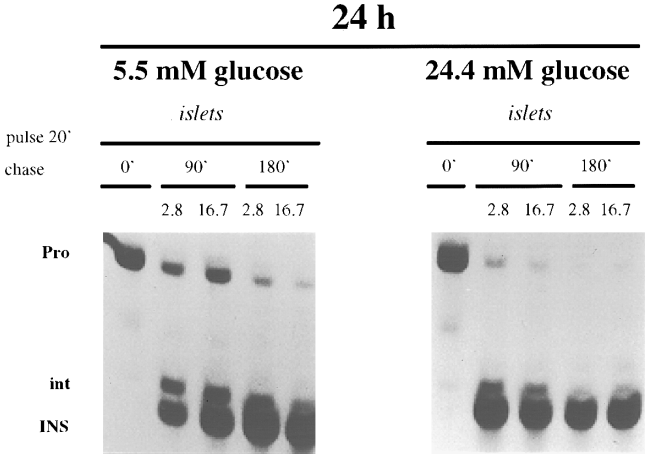

FIG. 1. Proinsulin processing by control human islets exposed to high glucose concentration. After being incubated for 24h under 5.5 and 24.4 mM glucose, islets were pulse-radiolabeled for 20 min at 16.7mM glucose and chased at 2.8 and 16.7mM glucose for the indicated times. Insulin related proteins were immunoprecipitated from islet lysates, analyzed by alkaline-urea gel electrophoresis and the gel was fluorographied. The migration of insulin related proteins is indicated: Pro (intact proinsulin), int (intermediates) and INS (insulin). A representative fluorography is shown. This experiment was reproduced with three different control pancreases with no significative differences.

RESULTS

No increase or decrease in newly proinsulin synthesis were observed in human islets cultured at high glucose concentration (24.4mM) compared to those islets exposed at 5.5mM of glucose concentration after 20 minutes of labeling regardless of the period of time they were exposed. However, what one can see is that the ratio of newly synthesized insulin/proinsulin at 180min chase time is higher on those islets chronically exposed at 24.4mM of glucose compared with those exposed at 5.5mM of glucose concentration. This observation was evident either after being only cultured for 24h (Fig. 1, Table 1) or after 8 days at high glucose concentration (Fig. 2, Table 1). However a decrease of the signal of newly proinsulin synthesis after 20 minutes of pulse is seen on those islets cultured for 8 days, compared to those cultured for 24h, whatever glucose concentration was in the culture medium (Fig. 1 and 2).

Western blot results, with antiPC2 and antiPC3 antiserum, did not show differences between the islet content of these enzymes. No changes can be observed between islets that have been cultured at high glucose concentration and the ones that have been under low glucose concentration (Fig. 3).

Results of the morphometric analysis have showed similar beta cell mass on each of the three organs processed (Table 2). Insulin content from these islets was measured, before any culture condition and we have

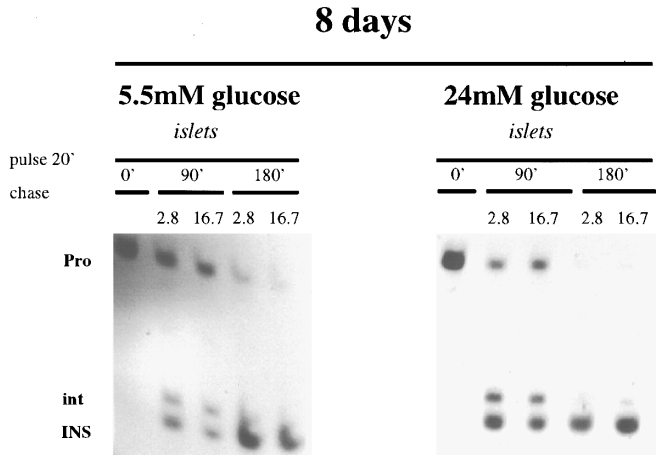

FIG. 2. Proinsulin processing by control human islets exposed to high glucose concentration. After being cultured for 8 days under 5.5 and 24.4 mM glucose, islets were pulse-radiolabeled for 20 min at 16.7mM glucose and chased at 2.8 and 16.7mM glucose for the indicated times. Insulin related proteins were immunoprecipitated from islet lysates, analyzed by alkaline-urea gel electrophoresis and the gel was fluorographied. The migration of insulin related proteins is indicated: Pro (intact proinsulin), int (intermediates) and INS (insulin). A representative fluorography is shown. This experiment was reproduced with three different control pancreases with no significative differences.

not found significative changes on the insulin content of the islets obtained from each of the three pancreases (Table 2).

DISCUSSION

In order to study whether high glucose concentration could impair proinsulin synthesis and conversion, isolated islets from control donor pancreases were exposed to severe chronic high glucose concentration for 24 hours and 8 days (Fig. 1 and 2, respectively), during

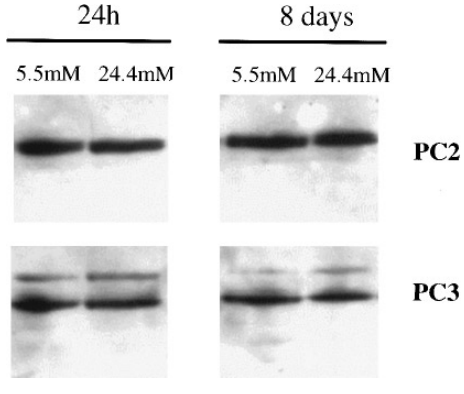

FIG. 3. Representative Western blot analysis of PC2 and PC3 expression in islets cultured for 24h and 8 days at 5.5mM and 24.4mM of glucose concentration. 50 and 60 μg of islet protein per lane were loaded, respectively. This experiment was performed three times with no significative differences.

TABLE 2

Morphometric Analysis of Beta Cells and Islet Insulin Content

	% β cell	Islet insulin content
Control 1:	2.40 ± 0.74% (n = 4)	5.06 ± 0.10 pmols/islet (n = 3)
Control 2:	2.86 ± 0.10% (n = 4)	5.70 ± 0.14 pmols/islet (n = 3)
Control 3:	2.52 ± 0.20% (n = 4)	5.50 ± 0.24 pmols/islet (n = 3)

Note. Four sections of each pancreas (n = 4) were analyzed, by immunofluorescence, for insulin staining. Data are shown as percentage with respect whole pancreas area ± SEM. Insulin extraction and precipitation was performed per triplicate (n = 3), as described in 'Methods', over batches of 10 islets from each pancreas processed. Results are expressed as the mean ± SEM.

which, an increased secretory demand would be placed on the beta cells.

The present study shows that human islets that have been exposed to high glucose concentration have striking acceleration of proinsulin conversion to insulin. Earlier works have shown efficient conversion of proinsulin to insulin when human proinsulin was expressed in AtT20 cells, a cell line which expresses high levels of PC3 but very low levels of PC2 [9]. Two more recent works [13-14] suggest that PC3 or PC2 alone when overexpressed can cleave proinsulin at both its junctions. However expressing both enzymes together ensures more efficient cleavages than with either enzyme alone and can even accelerate proinsulin conversion [13-14]. With regard to this later point, we suggested that possible adaptive changes, in PC2 and PC3 expression in response to high glucose environment, could explain the different proinsulin conversion pattern seen by islets under 24.4mM glucose exposure. However, we have not been able to detect any changes in the expression of PC2 or PC3. The possibility still remains, that although no higher expression is seen, their intrinsic enzyme activity may be favored in a high glucose environment.

We must remark here that the isolation process did not alter the integrity and viability of the islets from the three processed pancreases. Cell viability was higher than 80% in all samples, as well as their insulin content, which was equal on each of the islet batches of the three pancreases measured before initiation of any culture condition. The reduction of proinsulin synthesis observed after 8 days of culture on both glucose concentration conditions compared to those islets cultured for only 24h, could be attributed to the lose of beta cells after long culture exposition, as it has been reported [21]. However high glucose does not seem to influence the newly proinsulin synthesis. No changes (any decrease or increase) on the synthesis of this precursor molecule can be seen, at least after 20 minutes of labeling, between islets exposed to either glucose concentration used.

In summary, islets under high glucose concentration seem to try to accelerate the process of proinsulin conversion, probably, for the need of active insulin with the intention to compensate the supraphysiologic glucose condition.

ACKNOWLEDGMENTS

We thank Dr. J.C. Hutton (Denver, Colorado) for providing insulin immunoadsorbent and the PC2 and PC3 antiserum. We also thank the organ transplant coordination group from the Hospital Clínic of Barcelona and all members from our laboratory involved on the human islet isolation. We are also grateful to Jordi Ferrer for critical review of the manuscript. This work was supported by FIS grant 96/0164.

REFERENCES

1. Orci, L. (1985) *Diabetologia* **28,** 528–546.
2. Orci, L., Ravazzola, M., Storch, M. J., Anderson, R. G. W., Vassalli, J. D., and Perrelet, A. (1987) *Cell* **49,** 865–868.
3. Rhodes, C. J., and Alarcon, C. (1994) *Diabetes* **43,** 511–517.
4. Davidson, H. W., Rhodes, C. J., and Hutton, J. C. (1988) *Nature* **333,** 93–96.
5. Smeekens, S. P., Montag, A. G., Thomas, G., et al. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 8822–8826.
6. Baylies, E. M., and Hutton, J. C. (1992) *Biochem. J.* **286,** 223–229.
7. Bennet, D. L., Baylies, E. M., Nielsen, E., et al. (1992) *J. Biol. Chem.* **267,** 15229–15236.
8. Neerman-Arbez, M., Sizonenko, S. V., and Halban, P. A. (1993) *J. Biol. Chem.* **268,** 16098–16100.
9. Irminger, J. C., Wollenweider, F. M., Neerman-Arbez, H., and Halban, P. A. (1994) *J. Biol. Chem.* **269,** 1756–1762.
10. Alarcón, C., Leahy, J. L., Schuppin, G. T., and Rhodes, C. J. (1995) *J. Clin. Invest.* **95,** 1032–1039.
11. Wollenweider, F., Kaufmann, J., Irminger, J. C., and Halban, P. A. (1995) *Diabetes* **44,** 1075–1080.
12. Skelly, R. H., Schoppin, G. T., Ishihara, H., Oka, Y., and Rhodes, C. J. (1996) *Diabetes* **45,** 37–43.
13. Irminger, J. C., Meyer, K., and Halban, P. (1996) *Biochem. J.* **320,** 11–15.
14. Kaufmann, J. E., Irminger, J. C., Mungall, J., and Halban, P. A. (1997) *Diabetes* **46,** 978–982.
15. Sizonenko, S., Irminger, J. C., Buhler, L., Deng, S., Morel, P., and Halban, P. A. (1993) *Diabetes* **42,** 933–936.
16. Given, B. D., Cohen, R. M., Shoelson, S. E., Frank, B. U., Rubenstein, A. H., and Tager, H. S. (1985) *J. Clin. Invest.* **76,** 1398–1405.
17. Sobey, W. J., Beer, S. F., Carrington, C. A., et al. (1989) *Biochem. J.* **260,** 535–541.
18. Vives, M., Sarri, Y., Conget, I., et al. (1992) *Transplantation* **53,** 243–245.
19. Hutton, J. C., Bailyes, E. M., Rhodes, C. J., and Guest, P. C. (1990) *in* Peptide Hormone Secretion: A Practical Approach, pp. 309–336, IRL press.
20. Clark, A., Wells, C. A., Buley, I. D., et al. (1988) *Diabetes Research* **9,** 151–154.
21. Andersson, A., Bark, H., Groth, C. G., et al. (1976) *J. Clin. Invest.* **57,** 1295–1301.