Long-term *in vitro* exposure to high glucose increases proinsulin-like-molecules release by isolated human islets

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Abstract

The aim of this study was to determine the effect of long-term *in vitro* exposure to high glucose on the release and content of proinsulin and insulin in human islets. After 48 h culture in CMRL medium at 5.5 mM (control islets) and 16.7 mM glucose (experimental islets), islets were perfused and acutely stimulated with 16.7 mM glucose, followed by 3.3 mM glucose. Compared with control islets, experimental islets showed a higher basal release of true insulin and proinsulin-like-molecules (PLM), with no increase of true insulin and PLM release in response to 16.7 mM glucose, and a paradoxical true insulin release in response to 3.3 mM glucose; the PLM/total insulin ratio

increased significantly after 16.7 mM glucose. Moreover these islets showed a decreased true insulin content and an increased PLM/total insulin ratio. Quantitative ultrastructural analysis of granules, supported by double gold immunostaining with monoclonal antibodies against proinsulin and insulin, showed an increased proinsulin to insulin ratio in β -cells from experimental islets. These data support *in vitro* what was recently shown *in vivo*, and further confirm that culture in high glucose is a useful tool to mimic the effect of *in vivo* chronic hyperglycemia on human β -cell function.

Journal of Endocrinology (1998) **158**, 205–211

Introduction

Proinsulin-like-molecules (PLM) consist of several molecules on the pathway of production of insulin from proinsulin (C-peptide and intact and partially processed proinsulin) with a low insulin-like biological activity (Temple *et al.* 1989). PLM have been found elevated in type II diabetic patients (NIDDM) (Polonsky & Rubenstein 1989, Temple *et al.* 1989), in newly diagnosed type I diabetic patients (IDDM) (Snorgaard *et al.* 1990), in healthy siblings (Lindgren *et al.* 1993) or twins of IDDM patients (Heaton *et al.* 1988), as well as in elderly subjects (Shimizu *et al.* 1996). Different hypotheses have been proposed to understand the etiology of high PLM levels: (1) a primary dysfunction in insulin secretion (Porte & Kahn 1989); (2) a consequence of chronic hyperglycemia; and (3) a decreased proinsulin clearance (Polonsky & Rubenstein 1989, Porte 1991, Rhodes & Alarcon 1994).

Primary islet dysfunction as the cause of high PLM is supported by the finding of hyperproinsulinemia without hyperglycemia in identical relatives of IDDM patients (Heaton *et al.* 1988), or by clinical situations of increased metabolic demand without hyperproinsulinemia such as in patients with pharmacologically induced insulin resistance (Kahn *et al.* 1989a), in partial pancreatectomized dogs

(Ward *et al.* 1988) or in obese Pima Indians (Kahn *et al.* 1989b).

High serum levels of PLM could be related to an increased secretory demand placed on β -cells, as it has recently been shown to occur after hemipancreatectomy (Sequist *et al.* 1996), in the presence of glucose intolerance (Shiraishi *et al.* 1991), in cystic fibrosis (Hartling *et al.* 1988), or after prolonged intravenous glucose administration (Davis *et al.* 1993). Cross-sectional data in NIDDM have shown that increased PLM occurs after the onset of hyperglycemia (Birkeland *et al.* 1994). Finally, many but not all studies have found partial to full normalization of the PLM in patients with hypoglycemic therapies (Yoshioka *et al.* 1989). This hypothesis was also confirmed in animal models of NIDDM in which hyperproinsulinemia has been described as the result of the premature release of proinsulin before it could be fully processed (Alarcon *et al.* 1995, Gadot *et al.* 1995). An immunocytochemical study showed an impaired processing of proinsulin due to a chronic secretory state (Bendayan *et al.* 1995).

We have previously shown that *in vitro* culture of isolated human islets in the presence of high glucose is a suitable model to mimic the effects of *in vivo* chronic hyperglycemia (Davalli *et al.* 1991, 1992). The aim of the

Table 1 True insulin and PLM release and content of islets cultured in 5.5 and 16.7 mM glucose. True insulin and PLM release are expressed as pg/islet per min during basal release and as pg/islet per 20 min (AUC and Δ-AUC) after glucose stimulation or inhibition (n=12; four replicates from three pancreata). True insulin and PLM content are expressed as ng/islet (n=33; 11 replicates from three pancreata). Means ± s.e.

	5.5 mM glucose	16.7 mM glucose
Islet secretion (n=12)		
True insulin		
Basal	4.6 ± 0.5	9.0 ± 1.0**
After glucose stimulus (AUC)	438.3 ± 91.3	160.5 ± 16.9*
After glucose stimulus (Δ-AUC)	346.6 ± 91.5	− 18.9 ± 19.0**
After glucose inhibition (AUC)	192.4 ± 25.7	264.4 ± 26.3
After glucose inhibition (Δ-AUC)	− 168.9 ± 22.8	64.9 ± 40.0**
PLM		
Basal	2.9 ± 0.3	4.4 ± 0.6*
After glucose stimulus (AUC)	112.8 ± 40.2	73.6 ± 15.2
After glucose stimulus (Δ-AUC)	54.4 ± 41.4	− 15.3 ± 16.7
After glucose inhibition (AUC)	64.6 ± 13.6	121.6 ± 30.9
After glucose inhibition (Δ-AUC)	− 26.4 ± 31.3	60.3 ± 21.2*
PLM/total insulin		
Basal	0.39 ± 0.03	0.33 ± 0.03
After glucose stimulus	0.18 ± 0.03	0.29 ± 0.04*
After glucose inhibition	0.23 ± 0.04	0.27 ± 0.04
Islet content (n=33)		
True insulin	12.82 ± 1.39	5.53 ± 0.61**
PLM	4.02 ± 0.59	2.90 ± 0.37
PLM/total insulin	0.22 ± 0.02	0.34 ± 0.02**

*P<0.05, **P<0.001 vs 5.5 mM glucose islets.

present study was to verify, by the same model, the effects of high glucose on proinsulin content and PLM release of human islets.

Materials and Methods

Islets isolation

Islets were isolated from human pancreata from multiorgan donors using a modification of the automated method and purified by centrifugation on a Euroficol discontinuous gradient (Sigma Chemical Co., St Louis, MO, USA) (Socci *et al.* 1991). The mean age of the donors was 35 ± 10 years. Ethical Committee approval had been obtained.

In vitro culture

After a 12 h overnight culture at 37 °C in CMRL 1066 (10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin sulfate) in 95% O₂:5% CO₂, groups of 100 islets (150–200 µm in diameter) were hand picked and transferred to CMRL 1066 with 5.5 mM glucose (control islets), or 16.7 mM glucose (experimental islets).

Perifusions

After 48 h culture in low or high glucose concentration, two experiments were performed with each batch of islets. Aliquots of 100 hand picked islets from three pancreata were put overnight in tubes containing 1 ml acid–alcohol for insulin and PLM extraction. The following day, tubes were centrifuged for 10 min at 2000 r.p.m. at 4 °C, and diluted samples of the supernatant were stored at − 20 °C for assay of the insulin and PLM content. Insulin and PLM release were measured by perifusion experiments as follows. After a 40 min equilibration period at 3.3 mM glucose, islets were acutely stimulated with 16.7 mM glucose for 20 min, followed by 3.3 mM glucose for 20 min.

Assays

Total insulin (true insulin plus PLM) was assayed by RIA, using commercial kits (RIA Kits, Incstar, Stillwater, MN, USA). In our laboratory this RIA has the following characteristics: intra-assay coefficient of variation (CV) 3%, interassay 5%, recovery of added doses 98–100%, minimum sensitivity 12 pmol/l.

True insulin was assayed by Micro-particle Enzyme Immunoassay (MEIA insulin, IMX System, Abbott

Laboratories, Abbot Park, IL, USA). In our laboratory, MEIA has the following characteristics: intra-assay CV 3%, interassay 5%, recovery of added doses 98–100%, minimum sensitivity 6.0 pmol/l.

PLM were obtained by subtracting values of MEIA from insulin RIA values (Monti *et al.* 1995). This method has the following characteristics: intra-assay CV <1.6% and 2.5% at low and at medium-high values, recovery of added doses 87–99.7%, minimum sensitivity 4.0 pmol/l. Our procedure to evaluate PLM was compared with a commercial kit for proinsulin assay by ELISA (Total Proinsulin, Dako, Milan, Italy). Even if a 20% overestimation was found with our procedure, a highly significant correlation was demonstrated between the two assays ($r=0.99$; $P<0.001$).

Electron microscopy and quantitative evaluation

Islets of Langerhans from six pancreata were processed for electron microscopy as previously described (Saccomanno *et al.* 1993). Briefly, the islets were fixed for 2 h in 2.5% glutaraldehyde in 0.1 M cacodylate buffer. They were then post-fixed in 1% OsO_4 in cacodylate buffer at 4 °C for 1 h, dehydrated in graded ethanol up to propylene oxide and finally embedded in an Epon–Araldite mixture. Well preserved areas rich in granules were identified by light microscopy of semi-thin sections (0.5 μm). Subsequently, serial ultra-thin sections (80 nm) were mounted on 200-mesh copper grids, stained with uranyl acetate and lead citrate, and finally examined with a Zeiss CEM 902 electron microscope.

Each preparation was thoroughly scanned and well preserved areas composed of β -cells were photographed and analyzed at a final magnification of $\times 15\,750$. Insulin and proinsulin granules were identified by morphologic characteristics supported by immunolocalization results. Insulin granules were characterized by a paracrystalline electron dense core and a well delimited membrane, proinsulin granules by ovoid or rounded slightly electron dense core delimited by a membrane. The number of insulin and proinsulin granules was counted over a median area of 1126 μm^2 (ranging from 421 to 2257 μm^2) for each preparation and expressed per 500 μm^2 . Granules with morphology of equivocal interpretation were considered mixed.

Immunolocalization

Immunoelectron microscopy was carried out using the double gold labeling for the detection of both insulin and proinsulin on three out of six pancreatic islet preparations fixed in paraformaldehyde 4% and LR-White embedded, according to the procedure previously described, with some modifications. Briefly, islet sections were incubated with normal goat serum in order to avoid non-specific labeling and then incubated with monoclonal anti-human

Figure 1 (A) True-insulin release of islets cultured in 5.5 mM glucose (■) and in 16.7 mM glucose (○). After a 40 min equilibration period in 3.3 mM glucose, islets were acutely stimulated for 20 min with 16.7 mM glucose, followed by 3.3 mM glucose for 20 min. Means $\pm$ S.E. ($n=12$, four replicates from three pancreata). (B) PLM release in islets cultured in 5.5 mM glucose (■) and in 16.7 mM glucose (○). Means $\pm$ S.E. ($n=12$, four replicates from three pancreata).

proinsulin sera (Novo Nordisk Biolabs, Bagsvaerd, Denmark) diluted 1:10 in PBS+1% BSA. After 1 h at 37 °C islets were labeled with 5 nm colloidal gold particles coated with goat anti-mouse IgG (YLEM s.r.l., Avezzano, Italy). After several washes, the sections were incubated with monoclonal anti-human insulin sera (Immunotech, Marseille, France) diluted 1:10 in PBS+1% BSA and labeled with 10 nm colloidal gold particles coated with goat anti-mouse IgG. At the end the sections were rinsed in PBS several times and in twice-distilled water, fixed in 2.5% glutaraldehyde and stained with uranyl acetate. No cross reactivity has been described for anti-human proinsulin sera with human insulin and human C-peptide. The specificity of the immunostaining was tested by

Figure 2 Electron microscopy of a glutaraldehyde-fixed and Epon-embedded β -cell after a 48 h incubation in 5.5 mM glucose. The cell appears full of dense paracrystalline core granules (g), which are positive for insulin as shown by immunogold labeling (inset); only a small number of round granules without a crystalline core are recognizable as proinsulin granules (arrows). N: nucleus.

substituting normal mouse serum for anti-insulin and proinsulin antisera, and by absorption of antisera with their respective purified antigens (10 nmol antigen/ml diluted antiserum). Granules with a double immunostaining were considered mixed.

Calculations and statistical analysis

Data are always expressed as means $\pm$ s.e. Integrated insulin release during perfusions was calculated by the linear trapezoidal method (area under the curve, AUC, and the variation of area under the curve, Δ -AUC) and was expressed as pg/islet per 20 min; an insulin secretion index was also calculated as the ratio between peak and basal insulin values. Comparisons were performed by Student's *t*-test for unpaired data. *P* values <0.05 were considered statistically significant.

Results

True insulin release

Islets cultured in 16.7 mM glucose released more true insulin under basal conditions (3.3 mM glucose) than

control islets (Table 1) and showed a decreased insulin response to an acute glucose stimulus, both in terms of the AUC and Δ -AUC (Table 1), and in secretion index (1.1 ± 0.1 vs 11.2 ± 3.5 ; $n=12$; $P<0.05$); further, these islets showed a paradoxical insulin release in response to a low glucose concentration (Table 1, Fig. 1A).

PLM release

Islets cultured in high glucose released more PLM during basal conditions and showed a paradoxical PLM release after low glucose (Table 1, Fig. 1B), with no difference compared with control islets during the acute glucose stimulus (Table 1).

The PLM/total insulin ratio was higher in islets cultured in high glucose than in control islets, after high glucose challenge, with no differences during basal conditions and after low glucose (Table 1).

True insulin and PLM content

Islets cultured in high glucose showed a decreased true insulin content; PLM content was unchanged as an

Figure 3 Electron microscopy of a glutaraldehyde-fixed and Epon-embedded β -cell after a 48 h incubation in 16.7 mM glucose. Note the prevalence of round or ovoid granules without crystalline cores (g) positive for proinsulin as shown by immunogold labeling (inset); only few granules show a more dense paracrystalline core (insulin-rich). N: nucleus; M: mitochondria.

absolute value, and increased as a PLM/total insulin ratio (Table 1).

Electron microscopy and immunolocalization

By electron microscopy, β -cells cultured in control medium were characterized by a large amount of secretory granules with a dense crystalline core, surrounded by a clear halo (Fig. 2); β -cells cultured in high glucose contained in particular round or ovoid secretory granules, without a crystalline halo (Fig. 3). Golgi apparatus and rough endoplasmic reticulum were well developed in both cases. A prevalence of mitochondria, lysosomes and vacuolization of the cytoplasm was observed in islets cultured in high glucose.

Immunolocalization of insulin and proinsulin by double immunogold technique localized insulin in mature granules, as exemplified in Fig. 2 for control islets, and proinsulin in immature granules from islets cultured in high glucose (Fig. 3). Absorption of anti-proinsulin sera with an excess of purified proinsulin produced a drastic decrease in labeling intensity; nevertheless absorption with insulin did not result in any variation in the proinsulin labeling pattern.

The morphometric analysis per $500\ \mu\text{m}^2$ revealed, in islets cultured in high glucose, a decrease in insulin-containing granules as absolute number, although not statistically significant ($P=0.08$), and as percentage of total granule number, while proinsulin granules, unchanged as absolute number, increased in percentage of total granule number (Table 2).

Discussion

We have studied the effects of long-term exposure to high glucose on human islets content and release of insulin and PLM. We confirmed that human islets cultured for 48 h in 16.7 mM glucose lose the capability to release insulin after an acute glucose stimulus, show a paradoxical response to a glucose inhibition, and secrete significantly more insulin than control islets under basal conditions (Davalli *et al.* 1991, Ling & Pipeleers 1996). In this study we show that islets cultured in high glucose secrete more PLM at 3.3 mM glucose than control islets, and show a relative increase in the PLM/total insulin ratio after glucose stimulation. Hormone extraction showed that β -cells exposed to high glucose contain less true insulin than

Table 2 Number of insulin, proinsulin and mixed granules per 500 μm^2 in β -cells of islets cultured in 5.5 and 16.7 mM glucose. Percentage of total number of granules is in parentheses. Means $\pm$ S.E., $n=6$ (mean from six pancreata)

Granule type	5.5 mM glucose	16.7 mM glucose
Insulin	349 $\pm$ 74 (57 $\pm$ 1%)	184 $\pm$ 42 (39 $\pm$ 3%)**
Proinsulin	148 $\pm$ 38 (24 $\pm$ 2%)	192 $\pm$ 33 (42 $\pm$ 3%)**
Mixed	103 $\pm$ 20 (18 $\pm$ 2%)	91 $\pm$ 19 (19 $\pm$ 2%)
Total	601 $\pm$ 121	467 $\pm$ 91

** $P<0.001$ vs 5.5 mM glucose islets.

control islets. This is not surprising, in fact β -cell degranulation is expected to occur after chronic stimulation and has been found in several experimental situations of increased metabolic demand (Curry 1986, Leahy 1993). Immunocytochemical staining for proinsulin and insulin granules demonstrated an increased proinsulin to insulin ratio in β -cells kept in high glucose, similar to that already reported in animal models of NIDDM (Bendayan *et al.* 1995). The paradoxical release of insulin to a glucose inhibition as well as high insulin release in basal conditions could be a consequence of a glucose hypersensitivity due to an impaired translocation of glucokinase (Noma *et al.* 1996), an up-regulated hexokinase (Hosokawa *et al.* 1995), or a subcellular shift of the enzyme to mitochondria (Rabuaazzo *et al.* 1997).

The increase of PLM release and relative proinsulin content suggests that chronic glucose stimulation unbalances the rates of biosynthesis, intracellular degradation and release of insulin from β -cells, or simply that insulin is released before its maturation is complete. However, human islets transplanted into diabetic nude mice and maintained in hyperglycemia for 4–6 weeks, showed an impairment in glucose-induced insulin release accompanied by normal proinsulin biosynthesis (Eizirik *et al.* 1997); in rat islets, insulin biosynthesis and proinsulin conversion enzyme (PC2/PC3) activity have been shown to be unaffected by a long-term *in vitro* exposure to high glucose; the decreased PC2–PC3 content appears to be due to an increased release and not to a change in biosynthesis (Alarcon *et al.* 1995). A variation in neither proinsulin conversion nor intra-islet degradation was observed (Gadot *et al.* 1995).

In NIDDM patients an increased proinsulin/insulin ratio has been observed under basal conditions, as well as in response to an acute glucose stimulus (Ward *et al.* 1987, Saad *et al.* 1990, Davis *et al.* 1993, Leahy 1993). During an oral glucose tolerance test, the ratio between serum proinsulin and total insulin is 42% in diabetic patients compared with 16% in control patients (Leahy *et al.* 1992). These data are similar to those observed by us in the experimental islets after an acute glucose stimulation. In contrast, data on the release under basal condition are not

in agreement with data *in vivo*; it should be pointed out, however, that basal insulin and proinsulin secretion are largely influenced *in vivo* by hormone clearance, glycemia, insulin resistance, body composition, and nervous and hormonal stimuli (Saad *et al.* 1990, Capito *et al.* 1992).

Moreover, the isolation procedure itself could be an islet stress situation and could impair the insulin secretion process, increasing proinsulin release (Pai *et al.* 1993).

In conclusion, this study demonstrates that human β -cells respond to a chronic glucose stimulation by increasing the relative proinsulin to insulin content and by releasing more PLM than control β -cells; this is similar to what happens *in vivo* in animal models of NIDDM and in humans (Sequist *et al.* 1996). This confirms that culture in high glucose is a useful tool to mimic the effect of *in vivo* chronic hyperglycemia on human β -cell function.

Acknowledgements

This work was partially supported by the Italian Ministry of Health, the National Research Council (CNR, grant #90.00034.PF70), Telethon (grant #C.11; #E.443), and Regione Lombardia (grant #879).

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Received 12 December 1997

Accepted 19 March 1998