Altered proinsulin conversion in rat pancreatic islets exposed long-term to various glucose concentrations or interleukin-1β

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Abstract

In order to elucidate a possible relationship between β-cell function and conversion of proinsulin to insulin, isolated rat pancreatic islets were maintained in tissue culture for 1 week at various glucose concentrations (5.6–56 mM). Studies were also conducted on islets cultured for 48 h with interleukin-1β (IL-1β). By pulse-chase labelling and immunoprecipitation, the relative contents of newly synthesized proinsulin and insulin were determined. ELISA was used to analyse insulin and proinsulin content in medium and within islets. Using real-time PCR, the mRNA levels of proinsulin converting enzymes (PC1 and PC2) were studied. Islets cultured at 56 mM glucose had an increased proportion of newly synthesized proinsulin when compared with islets cultured at 5.6 mM glucose after a 90-min chase periods, however, no difference was observed after culture at 11 and 28 mM glucose. ELISA measurements revealed that culture at increased glucose concentrations as well as islet exposure to IL-1β increased proinsulin accumulation in the culture media. The mRNA expression of PC1 was increased after culture at 11 and 28 mM glucose. Treatment for 48 h with IL-1β increased the proportion of proinsulin both at 45 and 90 min when compared with control islets. These islets also displayed a decreased mRNA level of PC1 as well as PC2. Calculations of the half-time for proinsulin demonstrated a significant prolongation after treatment with IL-1β. We conclude that a sustained functional stimulation by glucose of islets is coupled to a decreased conversion of proinsulin which is also true for islets treated with IL-1β. This may contribute to the elevated levels of proinsulin found both at the onset of type 1 diabetes as well as in type 2 diabetes.


Introduction

High concentrations of proinsulin and abnormal proinsulin conversion have been associated with type II diabetes (T2D) as well as subjects at risk for T2D (Gordon et al. 1974, Mako et al. 1977, Heding et al. 1981, Ward et al. 1987, Yoshioka et al. 1988, Temple et al. 1989, Saad et al. 1990, Haffner et al. 1994, Kahn et al. 1995, Mykkänen et al. 1995, Roeder et al. 1995). Increased levels of proinsulin have also been found at the onset of type 1 diabetes (T1D) and in relatives of T1D patients (Ludvigsson & Heding 1982, Hartling et al. 1989, Snorgaard et al. 1990). In rat models of hyperglycaemia, increased ratios of circulating proinsulin to insulin have been determined (Leahy et al. 1991, Leahy 1993, Alarcon et al. 1995, Gadot et al. 1995).

Biosynthesis of proinsulin in β-cells is stimulated by glucose and this imposes an increased demand on the mechanism for proinsulin to insulin conversion. The endoproteolytic processing of proinsulin to insulin is mediated by proinsulin convertases 1 and 2 (PC1 and PC2) (Bailey et al. 1992, Bennett et al. 1992, Steiner et al. 1996). The conversion of proinsulin to insulin is regulated by glucose, acutely, partly through effects on the biosynthesis of PC1 and PC2 (Alarcon et al. 1993, Martin et al. 1994, Schuppin & Rhodes 1996).

Most studies of glucose regulation of the proinsulin conversion in β-cells have been performed over relatively short-time periods. In addition, previous studies of proinsulin conversion have been studied using isolated pancreatic β-cells or cell lines. Herein, we investigated the effects of long-time glucose culture on proinsulin conversion in isolated rat pancreatic islets.

The proinflammatory cytokine interleukin-1β (IL-1β) is a central mediator of β-cell destruction (Bendtzen et al. 1986). For instance, rat islets cultured for 48 h with IL-1β has decreased insulin secretion, insulin biosynthesis and oxidative metabolism (Sandler et al. 1987).

The present study examines the possibility that rat pancreatic islets may adapt to change functional demands or the cytokines by altering the rate of conversion of proinsulin to insulin. In order to elucidate this issue, the intracellular ratio of newly synthesized proinsulin/insulin was assessed, and proinsulin/insulin accumulation and content were measured as well as the levels of mRNAs encoding for PC1 and PC2, in isolated rat islets maintained in tissue culture at high glucose concentrations or after exposure to the cytokine IL-1β.
Materials and Methods

Islet isolation and culture

Pancreatic islets were isolated from adult Sprague-Dawley rats by collagenase digestion and hand-picked with a braking pipette. The use of animals was in accordance with international guidelines (National Institutes of Health publication 85-23) and approved by the regional laboratory animal ethics committee in Tierp, Sweden. Preculture of islets was performed for 3–4 days at 37 °C on non-attachment Sterilin dishes (Bibby Sterilin Ltd, Stone, Staffs, UK) using Roswell Park memorial institute (RPMI) 1640 medium containing 10% fetal calf serum, 2 mM L-glutamine, benzyl penicillin (100 U/ml), streptomycin (0.1 mg/ml) and 11 mM glucose, in an atmosphere of air +5% CO₂.

Groups of 50–60 islets were then treated with IL-1β as described below or cultured at different glucose concentrations (5, 11, 28 or 56 mM) for 7 days. The medium was changed every second day.

Treatment of islets with IL-1β

Treatment with human IL-1β (PeproTech, London, UK) was performed by the addition of the cytokine at an activity of 25 U/ml. Culture was continued for 48 h in medium RPMI 1640 supplemented with 11 mM glucose.

Proinsulin and insulin accumulation and islet content

During the experiments, samples of culture medium were taken and analysed for insulin using a rat insulin ELISA according to the manufacturer’s instructions (Mercodia, Uppsala, Sweden). To analyse insulin content, islets were ultrasonically disrupted in 0.5 ml acid ethanol and the insulin extracted overnight at 4 °C. For analysis of proinsulin accumulation in medium and islet content, we used a rat proinsulin ELISA according to the manufacturer’s instructions (Mercodia).

Radioactive labelling of newly synthesized proinsulin and insulin

Pulse–chase labelling of islet proteins was achieved by incubating in each experiment three groups of 50 islets, with 100 μCi l-[4,5-3H]leucine in 100 μl bicarbonate buffer supplemented with 10 mM Heps (Kreb’s–Ringer bicarbonate buffer supplemented 10 mM Heps, KRBH), 50 μg/ml BSA and 16.7 mM glucose for 30 min at 37 °C. The islets were subsequently washed with KRBH and one sample of islets immediately frozen, whilst two of the samples were further incubated for 45 or 90 min after addition of KRBH supplemented with 16.7 mM glucose. This incubation in non-radioactive medium was designated as the chase period. The samples were then washed with KRBH and frozen at −70 °C before further processing as described below.

Immunoprecipitation of insulin

Labelled islets were sonicated in 50 μl of a 50 mM glycine buffer (pH 8.8) supplemented with 2.5% BSA and 0.1% Triton X-100. After addition of 70 μl solution of antibovine insulin antisera (Biomakor, Rehovot, Israel), the samples were shaken and left for 1 h at room temperature. Subsequently, 100 μl protein A-Sepharose CL-4B suspended in glycine buffer, was added and the samples gently shaken for 15 min. After sedimentation by gentle centrifugation, the pellet was rinsed twice with the glycine buffer and frozen at −70 °C.

Separation by PAGE

The antibody precipitated proteins were dissociated from the protein A complex and separated on a gradient gel of 10–15% acrylamide/0.5–0.75% methylenebisacrylamide with 6 M urea. For this purpose, the Sepharose pellet was resuspended in 30 μl sample buffer (62.5 mM Tris–Cl (pH 6.8), 2% SDS, 5% β-mercaptoethanol and 6 M urea), briefly, boiled and the supernatant loaded on the gel. The electrophoresis was run in a Tris–glycine buffer (25 mM Tris-base and 0.2 M glycine) supplemented with 0.1% SDS at 12 mA for 6–7 h. The gel was subsequently soaked in amplifier (Amplify) for 20–30 min and dried overnight. The dried gel was exposed at −70 °C to Hyperfilm-MP with an intensifying screen. The intensities of the spots were determined by densitometry (DU-62 spectrophotometer, Beckman Instruments, Fullerton, CA, USA). A protein standard (Rainbow Markers) with molecular weight ranging from 2350 to 46 000 Da was run in parallel with the samples. Two distinct protein bands were found in the gel, one around 9000 Da and the other at 3500 Da. These were regarded as proinsulin and the B chain of insulin. The insulin A chain migrated with the protein front and was not measured.

RNA isolation and cDNA synthesis

Total RNA from 50 to 60 islets in each experimental group was isolated with RNeasy Mini Kit (Qiagen) and digested with RNase free DNase (Qiagen). RNA was concentrated by sodium acetate/ethanol precipitation (0.3 M sodium acetate in 66% ethanol (vol%),) dried and dissolved in 10 μl RNase free water and stored at −70 °C. Synthesis of cDNA was performed with Reversed Transcription System (Promega) using 9 μl total RNA per 20 μl cDNA synthesis reaction and 5 mmol/1MgCl₂, 1X reverse transcription buffer, 1 mmol/l of each dNTP, 1 U/μl Recombinant RNasin ribonuclease inhibitor, 15 U/μl Avarian myeloblastosis virus (AMV) Reverse transcriptase and 1 μg (dT)₁₅ primer. The reactions were incubated for 60 min at 42 °C followed by 5 min at 99 °C and stored at −20 °C.

RT-PCR

Light Cycler Instrument (Roche) combined with sequence independent detection with SYBR Green I was used to amplify and analyse generated cDNA. Primers for
glucose-6-phosphate dehydrogenase (G6PDH), PC1, PC2 and porphobilinogen deaminase haem biosynthetic enzyme (PBG) were as follows (TIB Molbiol Syntheselabor, Berlin, Germany): G6PDH f 5'-ATTGACCACTACCTGGGCAA-3', G6PDH r 5'-GAGATACACTTCAACACTTTGACCT-3', PC1 f 5'-TGAATGTTGTGGAGAAGCGG-3', PC1 r 5'-GCACCTGAGACTTCTTTGGTG-3', PC2 f 5'-CTGAGGCTGTGTGGCTAC-3', PC2 r 5'-AGCTGGC GTTCTGATTA-3', PBG f 5'-CCTGGCATACAGTTTGAAATCAT-3', PBG r 5'-TTTCCCTAAAAAC AACAGCAT-3'.

PCRs were performed in a total volume of 10 µl, containing 1 µl cDNA, 1 µM of each primer, 1 X LightCycler Fast Start reaction mixture (Roche) and 5-0 mmol/l MgCl2 (Roche). Before PCR amplification, the Taq polymerase was added and activated by incubating the samples for 10 min at 95 °C. Amplification was performed by denaturing for 15 s at 95 °C, annealing for 10 s at 60 °C and elongation for 15 s at 72 °C for 45 cycles. In our RT-PCR experiments we used G6PDH as the housekeeping gene. However, increasing glucose concentrations seemed to induce the transcription of this gene. Therefore, we set up our glucose experiments with the gene for PBG as the housekeeping gene. Transcription of this gene was not affected by culture of the islets at different glucose concentrations.

Data computation
When calculating the half-time ($T_{1/2}$) for conversion, time 0 was arbitrarily determined as 15 min after the labelling started. For RT-PCR experiments, the Ct (cycle threshold) values were used to calculate the amount of amplified PCR product when compared with the housekeeping gene. Relative amount of mRNA was calculated as $2^{-\Delta Ct}$. The results are expressed as means ± S.E.M. Statistical analysis was performed with Student's t-test for paired samples. A probability ($P$) for a chance difference $<$ 0·05 was regarded as significant.

Results

The effect of increasing glucose concentrations on proinsulin conversion
The accumulation of insulin in culture medium from rat islets kept in the 1-week culture at different glucose concentrations is shown in Fig. 1a. The insulin accumulation in medium from islets cultured at 5·6 mM glucose was decreased when compared with the medium from islets cultured at 11 mM. At the end of the culture period, the insulin accumulation was elevated in the 28 and 56 mM glucose medium.

During the culture period, the proinsulin accumulation in media from islets kept at 11 mM glucose was 0·5 ng proinsulin per 10 islets and hour (Fig. 1b). This corresponded to 2–3% of secreted insulin·proinsulin (molecular weights were used for calculation).

Figure 1 Insulin (a) and proinsulin (b) accumulation from rat pancreatic islets cultured at different glucose concentrations 5-6 (dark grey bars), 11 (grey bars), 28 (striped bars) or 56 mM (black bars) for 1 week. The percentage of proinsulin is shown in Fig. 1c. Values are means ± S.E.M. for four experiments. *$P<0·05$ using Student’s paired t-test when compared with islets cultured at 11 mM glucose.

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Cultures of islets at increased glucose concentrations (28 and 56 mM) resulted in elevated levels of proinsulin in the culture medium (Fig. 1b and c). The proinsulin accumulation in media of the 5·6 mM group was decreased (Fig. 1b and c).

There was a tendency toward decreased proinsulin content in islets cultured at 56 mM glucose as well as in islets cultured at 5·6 mM when compared with islets cultured at 11 mM glucose (Table 1).

Exposure of islets for 1 week to increasing glucose concentrations consistently revealed that after a chase period of 45 min about 30–40% of the immunoprecipitated proteins migrated with molecules of the same size as proinsulin (Fig. 2). Decreased PC1 mRNA levels were observed in rat islets incubated for 7 days at 5·6 mM glucose when compared with islets cultured at 11 mM glucose (Table 2). After a 90-min chase period, the proportion of proinsulin was less but the same (about 20%) in islets cultured at 5·6, 11 or 28 mM glucose, whereas a significant increase was found after culture at 56 mM glucose (55%, Table 2). The $T_{1/2}$ of conversion was similar at all glucose concentrations (43–52 min). Decreased PC1 mRNA levels were observed in rat islets incubated for 7 days at 5·6 mM glucose when compared with islets cultured at 11 mM glucose. After culture at 56 mM glucose, a tendency to increased levels was seen, however, this was not statistically significant (Fig. 2a). No alterations in the levels of PC2 were detected when islets were cultured at 5·6, 11 or 28 mM glucose, whereas a significant increase was found after culture at 56 mM glucose (55%, Table 2). The $T_{1/2}$ of conversion was similar at all glucose concentrations (43–52 min). Decreased PC1 mRNA levels were observed in rat islets incubated for 7 days at 5·6 mM glucose when compared with islets cultured at 11 mM glucose. After culture at 56 mM glucose, a tendency to increased levels was seen, however, this was not statistically significant (Fig. 2a). No alterations in the levels of PC2 were detected when islets were cultured at various glucose concentrations (Fig. 2b).

Interestingly, the RT-PCR results reveal considerably higher mRNA levels of PC2 when compared with PC1 (1000-fold).

The effect of IL-1β on proinsulin conversion

Exposure to IL-1β for 48 h tended to result both in decreased insulin content (Table 3) and in insulin accumulation (Table 4). The proinsulin content within islets exposed to IL-1β was decreased when compared with control islets (Table 3). Islet exposure to IL-1β resulted in an increase in the proinsulin ratio in the culture media (Table 4). Islets treated

### Table 1

<table>
<thead>
<tr>
<th>Medium glucose (mM)</th>
<th>Insulin content (ng/10 islets)</th>
<th>Proinsulin content (ng/10 islets)</th>
<th>PI/PI +1 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5·6</td>
<td>600 ± 35</td>
<td>9·3 ± 1·0*</td>
<td>1·0 ± 0·15*</td>
</tr>
<tr>
<td>11</td>
<td>620 ± 22</td>
<td>27± 2·7</td>
<td>2·9 ± 0·43</td>
</tr>
<tr>
<td>28</td>
<td>430 ± 11</td>
<td>32 ± 5·2</td>
<td>4·6 ± 0·59</td>
</tr>
<tr>
<td>56</td>
<td>320 ± 9·6*</td>
<td>19 ± 1·5</td>
<td>3·8 ± 0·29</td>
</tr>
</tbody>
</table>

Insulin and proinsulin content in rat pancreatic islets cultured at different glucose concentrations (5·6, 11, 28 or 56 mM) for 1 week. *P<0·05 using Student’s paired t-test when compared with islets cultured at 11 mM glucose.

### Table 2

<table>
<thead>
<tr>
<th>Chase periods (min)</th>
<th>45</th>
<th>90</th>
<th>$T_{1/2}$ (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium glucose (mM)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5·6</td>
<td>32 ± 1·6</td>
<td>20 ± 1·8</td>
<td>49 ± 6·4</td>
</tr>
<tr>
<td>11</td>
<td>39 ± 2·4</td>
<td>19 ± 2·7</td>
<td>46 ± 3·8</td>
</tr>
<tr>
<td>28</td>
<td>42 ± 3·5</td>
<td>24 ± 2·5</td>
<td>52 ± 5·9</td>
</tr>
<tr>
<td>56</td>
<td>38 ± 1·4</td>
<td>55 ± 2·0*</td>
<td>43 ± 4·4</td>
</tr>
</tbody>
</table>

The islets were labelled by a 30 min pulse of [1-4-5-3]leucine and then chased at non-radioactive 16·7 mM glucose for 45 or 90 min. $T_{1/2}$ was calculated using 15 min after the start of labelling as time 0. *P<0·01 using Student’s paired t-test when compared with islets cultured at 5·6 mM glucose.

### Table 3

<table>
<thead>
<tr>
<th>Exposure</th>
<th>Insulin content (ng/10 islets)</th>
<th>Proinsulin content (ng/10 islets)</th>
<th>PI/PI +1 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>720 ± 130</td>
<td>17 ± 2·4</td>
<td>1·6 ± 0·11</td>
</tr>
<tr>
<td>IL-1β</td>
<td>550 ± 120</td>
<td>7·3 ± 0·92*</td>
<td>0·93 ± 0·13</td>
</tr>
</tbody>
</table>

*P<0·05 using Student’s paired t-test.
with IL-1β (25 U/ml) for 48 h had a marked increased proportion of labelled proinsulin when compared with control islets (Fig. 3). There was a notable increase in the $T_{1/2}$ in islets treated with IL-1β to 68±7.8 min, which should be compared with 37±10 min in the controls ($n=6$, $P<0.05$). A modest decrease in PC1 mRNA levels and a 70% decrease of PC2 mRNA expression were detected in pancreatic islets incubated with IL-1β (Fig. 4a and b).

Discussion

Our results from the 1-week culture at various glucose concentrations revealed that increasing glucose concentrations lead to an increased proinsulin secretion to the culture medium. We believe that the increase in proinsulin secretion could be explained by a sustained state of cellular activation.

The half-time of conversion of newly synthesized proinsulin from the intracellular pool was determined using three time points. In all experiments, except those in which islets were treated with IL-1β, the $T_{1/2}$ was found to be 40–50 min when 15 min after the start of radioactive labelling was set as time 0. This agrees well with previously published studies (Steiner et al. 1972, Rhodes & Halban 1987 Nagamatsu et al. 1987) indicating an intracellular $T_{1/2}$ of proinsulin of about 60 min in freshly isolated islets. In the present study, this rate of conversion was maintained after culture in glucose concentrations in the range 5.6–28 mM, whereas a decreased rate was measured only in islets which had been cultured for 7 days at 56 mM glucose and incubated for 90 min after the labelling period. The increase in proinsulin to insulin indicates unresponsiveness of the islets after the treatment at high glucose (56 mM). These data are in agreement with previous work on human isolated β-cells (Hostens et al. 1999).

A general observation in this study was that the mRNA levels of PC2 were considerably higher than those of PC1. Using the Ct values, taking into consideration the relative comparison, we could estimate that the mRNA levels of PC2 were up to a thousand times higher than the PC1 mRNA levels. This observation is also in agreement with the results of Hostens et al. (1999), showing higher protein levels of PC2 than PC1 in human β-cell preparations.

Table 4  Insulin and proinsulin accumulation in medium from rat pancreatic in islets exposed to IL-1β for 48 h. Values are means±S.E.M. for three experiments

<table>
<thead>
<tr>
<th>Exposure</th>
<th>Insulin accumulation (ng/10 islets h)</th>
<th>Proinsulin accumulation (ng/10 islets h)</th>
<th>PI/PI+I (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>17±3.4</td>
<td>0.24±0.062</td>
<td>0.91±0.063</td>
</tr>
<tr>
<td>IL-1β</td>
<td>5.5±0.30</td>
<td>0.23±0.015</td>
<td>2.7±0.038*</td>
</tr>
</tbody>
</table>

* $P<0.05$ using Student’s paired t-test.

Figure 4  Relative mRNA expression of PC1 (a) and PC2 (b) in rat pancreatic islets cultured without (black bars) or with 25 U/ml IL-1β (grey bars) for 48 h. Relative mRNA expression ($2^{-\Delta\Delta C_t}$) was calculated by subtracting Ct values for G6PDH from the Ct values for PC1 or PC2. Values are means±S.E.M. for five experiments. * $P<0.05$ using Student’s paired t-test.

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It has been suggested that in rat islets, PC1 biosynthesis, but not PC2, appears to be acutely regulated by glucose (Alarcon et al. 1993). Both these studies indicate that PC1 is the rate limiting enzyme in proinsulin conversion, acutely as well as long-term. This is in line with our observations with increased levels of PC1 mRNA but no differences in the expression of PC2 after culture at different glucose concentrations. That is, in a situation with a sustained hypersecretion of insulin, the β-cells therefore seem to be unable to sufficiently enhance the conversion of proinsulin to maintain a normal ratio in the secretory granule. This is, in line with clinical observations where increased proportion of proinsulin in the plasma of diabetic patients as well as in glucose intolerant subjects was published (Duckworth & Kitabchi 1972, Gordon et al. 1974, Mako et al. 1977, Heding et al. 1981, Ludvigsson & Heding 1982, Rhodes & Halban 1987, Hardling et al. 1989, Snorgaard et al. 1990).

Our results from islets being exposed to IL-1β for 48 h lead to an increased secretion of proinsulin. IL-1β treatment of pancreatic islets decreased the mRNA levels of both PC1 and PC2, indicating a decreased proinsulin conversion. Indeed, our data show a marked increase in the proportion of newly synthesized proinsulin after 48 h IL-1β treatment. These islets have previously been shown to have decreased insulin release in response to glucose and decreased insulin and DNA content (Bendtzen et al. 1986, Sandler et al. 1987, Spinas et al. 1992). Our results are in line with previously published data, indicating decreased conversion rate in rat islets after 24-h IL-1β (Hansen et al. 1988) and that exposure of β-cells to IL-1β for 24 h suppresses both proinsulin biosynthesis as well as the expression of PC2 by 46% via an NO-mediated pathway (Zambre et al. 2001). These results are in line with ours showing 70% reduction in PC2 transcription after 48 h.

In previous experiments with human β-cell preparations, the cytokine combinations, in particular IL-1β and interferon (IFN)-γ, revealed disproportionally increased medium proinsulin levels (Hostens et al. 1999). In view of the role for IL-1β in the β-cell destruction leading to T1D, it is of note that increased circulating levels of proinsulin have been observed during the period preceding the clinical manifestation of the disease. The possibility that a high local concentration of IL-1β in the islets may contribute to an increased proinsulin release should therefore be considered.

Our observations suggest that a lasting increased functional stimulation of islets causes an increase in proinsulin ratio as well as in converting enzymes. This may reflect an adaptive response of the β-cell to an enhanced turnover of a diminished insulin pool. This adaptation, however, appears insufficient after culture at 56 mM glucose, as shown by increased levels of proinsulin and no adaptation of the converting enzymes. These data are in accordance with previous data indicating slightly impaired effect by slight lowering of the ATP:ADP ratio and glucose oxidation (Sandler et al. 1991). Furthermore, IL-1β stimulation of islets decreases the conversion of proinsulin and is associated with a decrease in conversion enzymes. This is applicable to in vivo findings, showing increased levels of circulating proinsulin levels in hyperglycaemic states (Duckworth & Kitabchi 1972).

We conclude that a sustained functional stimulation by glucose of islets is coupled to a decreased conversion of proinsulin which is also true for islets treated with IL-1β. This may contribute to the elevated levels of proinsulin found both at the onset of type 1 diabetes as well as in type 2 diabetes.

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