Effect of forty-eight-hour glucose infusion into rats on islet ion fluxes, ATP/ADP ratio and redox ratios of pyridine nucleotides

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Abstract

Glucose infusion into rats has been shown to sensitize/desensitize insulin secretion in response to glucose. In pancreatic islets from glucose-infused rats (GIR) (48 h, 50%, 2 ml/h) basal insulin release (2·8 mmol/l glucose) was more than fourfold compared with islets from saline-infused controls and the concentration–response curve for glucose was shifted to the left with a maximum at 11·1 mmol/l. The concentration–response curve for 45Ca²⁺ uptake was also shifted to the left in islets from GIR with a maximum at 11·1 mmol/l glucose. Starting from a high basal level at 2·8 mmol/l glucose KCl produced no insulin release or 45Ca²⁺ uptake in islets from GIR. Islets from GIR exhibited a higher ATP/ADP ratio in the presence of 2·8 mmol/l glucose and marked inhibition of ⁸⁶Rb⁺ efflux occurred even at 3 mmol/l glucose. Moreover, in islets from GIR the redox ratios of pyridine nucleotides were increased. On the other hand insulin content was reduced to about 20%.

The data suggest that a 48-h glucose infusion sensitizes glucose-induced insulin release in vitro in concentrations below 11·1 mmol/l. This may, at least in part, be due to enhanced glucose metabolism providing increased availability of critical metabolic factors including ATP which, in turn, decrease the threshold for depolarization and therefore calcium uptake. Calcium uptake may then be further augmented by elevation of the redox state of pyridine nucleotides.


Introduction


Glucose-induced insulin secretion is closely related to the metabolism of glucose through the formation of critical metabolic products including ATP (Cook & Hales 1984, Malaise & Sener 1987) and reduced pyridine nucleotides (Ammon & Wahl 1994). Both are thought to be responsible for initiation and/or modulation of insulin secretion by acting on K_ATP and/or calcium channels (Henquin & Meissner 1984, Boyd 1992, Ammon & Wahl 1994). Since long term exposure of pancreatic islets has been reported to enhance islets glucose metabolism (Bedoya & Jeanrenaud 1991a, Purello et al. 1992, Chen et al. 1994) it was aimed to study whether or not sensitization of insulin release to glucose and KCl caused by a 48-h glucose infusion into rats may be explained by respective changes in critical metabolic products including the ATP/ADP ratio and the redox ratios of pyridine nucleotides and subsequent alterations in K⁺ efflux (measured as ⁸⁶Rb efflux) and ⁴⁵Ca²⁺ uptake.

Materials and Methods

Animals

Female Wistar rats weighing between 250 and 300 g were used. The rats had free access to tap water and a standard pellet diet (Altromin 1324, Altromin-Futterwerk, Lage, Germany) and were kept at 22 °C with a 12 h light-darkness cycle during the whole experimental period.
Glucose infusion procedure

For implantation of chronic catheters, the rats were anesthetized with Ketamin (Ketanest, Parke-Davis, Freiburg, Germany; 45 mg/kg i.p.) plus Rompun (Bayer, Leverkusen, Germany; 12 mg/kg i.p.). Silastic medical-grade tubing (Dow Corning, Midland, MI, USA) was filled with a solution of 0·9% NaCl supplemented with 5000 IU/ml heparin (Liquemine, Roche, Grenzach, Switzerland), inserted via the right jugular vein into the right atrium. The free end was routed subcutaneously and externalized between the shoulders through a teflon anchoring device which was sewn to the skin. The catheter was then routed through a bite-proof stainless-steel spiral fixed to an oscillating arm connected to a swivel allowing the rat to move freely. Infusion of 0·9% NaCl (0·5 ml/h) was administered for a recovery period of 48 h. Then glucose solution (50% glucose, Fresenius, Bad Homburg, Germany) supplemented with 0·45% NaCl or 0·45% saline (control) was continuously infused at a rate of 2·0 ml/h for 48 h with a Perifusor syringe pump (Braun, Melsungen, Germany). Complete infusions over a period of 48 h were achieved in more than 95% of cases with this technique. Blood samples were obtained from the tail vein.

Blood glucose and plasma insulin assays

Blood glucose was measured by the hexokinase method (Glucoquant, Boehringer, Mannheim, Germany) and plasma insulin by radioimmunoassay (double antibody method, Linco Research, St Louis, MO, USA) using rat insulin (Novo Research, Bagsvaerd, Denmark) as a standard. 125I-insulin was a kind gift from Hoechst AG (Frankfurt, Germany).

Preparation of islets

After a 48-h infusion of glucose (glucose-infused rats (GIR)) or saline (saline-infused rats (SIR)) islets were isolated by the collagenase method (Lacy & Kostianovsky 1967). Since islets were exposed to different blood glucose concentrations during the 48-h infusion period, i.e. approximately 11·1 mmol/l in GIR and 5·6 mmol/l in SIR, we performed islet preparation steps, including preincubation, for islets of GIR in the presence of 11·1 mmol/l glucose and for islets of SIR in the presence of 5·6 mmol/l glucose in order to keep them in the same glucose environment until testing for various parameters. In a further control experiment we also prepared islets from SIR in the presence of 11·1 mmol/l glucose.

The pancreas was distended with Krebs-Ringer-HEPES (N-2-hydroxyethyl-piperazine-N’-2-ethanesulfonic acid) (Roth, Karlsruhe, Germany) buffer, supplemented with glucose (see below) at room temperature and the tissue was cut into small pieces and digested with collagenase (Worthington, Freehold, USA; 30 mg, 213 U/mg) for 10–12 min at 37 °C in a shaking water bath. The islets were collected with siliconized constriction pipettes under a stereomicroscope. The average yield of the isolation procedure was about 400 islets/pancreas. One hundred and fifty islets of the best visual quality and medium size were used for the experiments.

Insulin secretion

Insulin secretion was tested using the batch incubation method with five islets in 1 ml medium per batch. The incubation time was 60 min. Radioimmunoreactive insulin was determined with the double antibody method using rat insulin as a standard (Morgan & Lazarow 1963, Soeldner & Slone 1965).

45Ca2+ net uptake

Net uptake of 45Ca2+ was studied according to the method described by Henquin (1980). Batches of 10 islets were placed in polyethylene microfuge tubes together with 0·1 ml Krebs-Ringer-HEPES buffer. At time zero, 0·05 ml medium with 45CaCl2 (Amersham-Buchler, Braunschweig, Germany) (0·80 MBq/ml) and substances to be tested were added. Both tubes and media were prewarmed before starting the incubation. After 5 min, islets were separated from the surrounding medium by centrifugation through a layer of 0·1 ml silicone oil into 0·01 ml 3 mol/l KOH both being already present during the incubation. The bottom of the tube with the islets was cut off and transferred into scintillation vials. 45Ca2+ content was determined by liquid scintillation counting after disappearance of luminescence. Results were calculated as pmol total Ca2+ after having subtracted blanks and corrected for label in the extracellular [3H]sucrose space.

86Rb+ efflux

86Rb+ is a tracer in studies of K+ efflux. 86Rb+ efflux, now a standard technique, was measured as previously described by our group (Ammon & others 1998) and by others (Henquin 1977). Briefly, groups of 40 islets were first incubated for 90 min in Krebs-Ringer-bicarbonate buffer containing 0·2 mmol/l 86RbCl (Amersham-Buchler) (16·6–21·1 GBq/mmol specific activity) in the presence of 5·6 (controls) and 11·1 mmol/l glucose. After brief washing, islets were placed into perifusion chambers (0·3 ml) to which the perfusate was conveyed at 37 °C and a flow rate of 1·1 ml/min. 86Rb+ appearing in the effluent fractions (collected at 2-min intervals) and remaining in the islets at the end of the experiment was counted by liquid scintillation. For each collection interval, the fractional efflux of 86Rb+ was calculated.

Pyridine nucleotides

The contents of total islet NADPH and NADP were assayed by a fluorometric technique according to the
principles described by Passonneau and Lowry (1974). At the end of the incubation period, the glass tubes were placed in an ice bath, and medium around the islets was immediately removed by aspiration. For the determination of NADPH and NADH, islets were then immediately disintegrated in 20 µl of a mixture of 0.5 mmol/l cysteine and 40 mmol/l NaOH and heated for 15 min at 60 °C. For the determination of NADP or NAD only 5 µl of this mixture was used, and 50 µl of a mixture of H₂SO₄ (39 mmol/l) and Na₂SO₄ (0.15 mmol/l) were added and heated for 30 min at 60 °C. The pyridine nucleotides were determined from calibration curves prepared from authentic NADPH, NADP, NADH and NAD (Boehringer) taken through the same procedures. Disintegration of islets was assessed by microscopy. Recovery of NADPH added in various amounts to disintegrated islets was 97 ± 4% (mean ± s.e.m., n=6). Since NAD, NADH, NADP and NADPH were determined in different batches of islets it was not possible to calculate s.e.m. for totals and ratios of pyridine nucleotides.

**ATP/ADP ratio**

ATP/ADP was assayed by measuring ATP and ADP in the same batch of islets with a modified method according to Johnson et al. (1970). At the end of the incubation period the medium was immediately removed by suction. Islets were disintegrated with NaOH (40 mmol/l)/cysteine solution (0.5 mmol/l) and stored at −20 °C.

Each sample was divided into two parts. One part was incubated with a buffer containing creatine phosphate (20 mmol/l)/glycine (100 mmol/l)/MgSO₄ (1 mmol/l) at pH 9.0 plus creatine kinase (20 µg/ml) for 10 min at room temperature before neutralization. The second part of the sample was incubated with the same buffer but without creatine kinase and neutralized at once with 3.3 mmol/l HCl to pH 7.65. Luminescence was measured after 20 s using an ATP assay kit 1243 (Bio-orbit, Turku, Finland) in a luminescence biometer 1253 (Bio-orbit). ATP standard curves were performed in the same buffers as described for the probes. (ATP and ADP were from Boehringer). ADP was determined in the same samples as used for the ATP assay by adding creatine phosphate/creatine kinase (Gruber et al. 1974) and creating additional ATP by phosphorylation of ADP. The ATP was measured as described above with the bioluminescent assay. The ADP content was calculated as the difference between total ATP and ATP in each individual sample.

**Protein, DNA and insulin content**

For determination of protein, DNA and insulin content islets were stored at −20 °C after the isolation procedure.

**Protein** The protein content of the pancreatic islets, solubilized with 0.1 mol/l NaOH, was measured using bovine serum albumin as standard (Bradford 1976).

**DNA** DNA quantification was performed according to Beckmann et al. (1981). Pancreatic islets were extracted with cysteine (0.5 mmol/l)/NaOH (40 mmol/l). For determination of DNA 50 µl dianaminobenzoic acid dichloride solution were added to the samples and standards. After 40-min incubation in a 60 °C waterbath the reaction was stopped with 1 mol/l HCl and fluorescence was measured at 405 nm and 500 nm in a filter fluorimeter.

**Insulin** Extraction of insulin was performed according to the acid ethanol technique (Steinke & Driscoll 1965), employing 50 µl acid ethanol for each group of three islets. The islets were homogenized, frozen and neutralized with 0.1 mol/l NaOH. After dilution with Krebs-Ringer-HEPES buffer aliquots were taken and stored at −20 °C until determination of insulin by RIA.

**Statistical analysis**

All values are given as means ± s.e.m. for a certain number of separate experiments. The Student’s t-test of unpaired data was used for statistical evaluation.

**Results**

**In vivo experiments**

In order to characterize the in vivo situation existing when pancreatic islets were exposed to chronic glucose infusion at the time of pancreas removal it was necessary to measure blood glucose and plasma insulin and to calculate the insulinogenic index over the period of infusion (not shown).

Chronic infusion of saline (0.45%, 2 ml/h) over a period of 48 h had no significant effect on plasma glucose, plasma insulin and the insulinogenic index. When glucose (50% in 0.45% saline, 2 ml/h) was infused, the plasma glucose level was about 16 mmol/l after 24 h. Despite further continuous infusion of the same quantity of glucose, the plasma glucose level declined to about 11 mmol/l after 48 h. A 24-h infusion of glucose produced a plasma insulin level of 500 µU/ml which declined to about 450 µU/ml after 48 h of infusion. The insulinogenic index increased from 0.45 to about 1.7 after 24 h, and a further increase to 2.0 was seen after 48 h.

**In vitro studies**

**DNA, protein and insulin content** Table 1 shows DNA, protein and insulin contents of pancreatic islets from SIR and GIR. The data indicate that in these islets DNA content was not significantly changed by glucose infusion whereas protein was increased significantly. However, insulin content of islets from GIR dropped to as little as
20% compared with SIR. The data are in agreement with observations of others (Bedoya & Jeanrenaud 1991a).

For the following in vitro studies islets of SIR, which served as controls, were prepared and preincubated either in the presence of 5·6 or 11·1 mmol/l glucose (see Materials and Methods) in order to test whether the glucose concentration present in the preparation and incubation media would affect insulin release, calcium uptake and metabolic parameters.

There being no significant difference between the two groups (data not shown) all islets in the following SIR experiments were prepared and preincubated at 5·6 mmol/l glucose - the physiological environment during the infusion period.

### Table 1

Pancreatic islet content of DNA, protein and insulin after a 48-h infusion of saline or glucose. Values are means ± S.E.M.

<table>
<thead>
<tr>
<th></th>
<th>DNA (µg/10 islets)</th>
<th>Protein (µg/10 islets)</th>
<th>Insulin (ng/10 islets)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>0·34 ± 0·04</td>
<td>3·87 ± 0·40</td>
<td>528 ± 41</td>
</tr>
<tr>
<td>(n=16)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>0·40 ± 0·04*</td>
<td>5·75 ± 0·61*</td>
<td>104 ± 23***</td>
</tr>
<tr>
<td>(n=10)</td>
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*P<0·05, ***P<0·001 compared with saline infusion. ns, not significant.

Insulin secretion (Fig. 1) Islets from SIR exhibited the typical sigmoid curve of insulin release in response to glucose. When islets from GIR were used insulin secretion in the presence of 2·8 mmol/l glucose was more than fourfold higher compared with SIR. Again, glucose increased insulin secretion in a concentration-dependent manner. However, distinct to islets from SIR, the maximal secretion rate was already achieved at 11·1 mmol/l glucose and further elevation of the glucose concentration did not produce additional discharge of insulin from islets of GIR.

In islets from SIR in the presence of 2·8 mmol/l glucose, KCl produced a concentration-dependent secretion of insulin. This was not the case when islets from GIR were employed, where secretion of insulin was already increased in the presence of 2·8 mmol/l glucose at the lowest concentration of KCl tested.

**45Ca**\(^{2+}\) net uptake (Fig. 2) Islets from SIR exhibited a concentration-dependent increase of **45Ca**\(^{2+}\) net uptake in response to glucose. When islets from GIR were used basal **45Ca**\(^{2+}\) net uptake at 2·8 mmol/l glucose was nearly twofold higher compared with islets from SIR. Increasing the glucose concentration produced a concentration-dependent elevation of **45Ca**\(^{2+}\) net uptake, the maximum being achieved at 11·1 mmol/l glucose. Similar to the experiments on insulin secretion the concentration–response curve was shifted to the left. Further increase in
the glucose concentration did not cause additional uptake of calcium.

In islets from SIR in the presence of 2.8 mmol/l glucose, KCl enhanced $^{45}$Ca$^{2+}$ net uptake in a concentration-dependent manner. This was not the case when islets from GIR were employed where $^{45}$Ca$^{2+}$ net uptake was already increased even at the lowest concentration of KCl tested in the presence of 2.8 mmol/l glucose.

$^{86}$Rb$^+$ efflux (Fig. 3) As shown in Fig. 3A increasing glucose from 0 to 3 mmol/l produced only a marginal decrease of $^{86}$Rb$^+$ efflux in islets from SIR. When the glucose concentration was increased from 3 mmol/l to 5.6 mmol/l the classical inhibition of $^{86}$Rb$^+$ efflux was obvious and was reversed on return to 3 mmol/l.

In contrast to the results obtained with islets from SIR, islets from GIR (Fig. 3B) showed abrupt significant inhibition of $^{86}$Rb$^+$ efflux when the glucose concentration was increased from 0 to 3 mmol/l, reaching approximately 1.75%/min, the effect being reversible. In a further experiment starting with 3 mmol/l glucose, $^{86}$Rb$^+$ efflux from islets of GIR was lower than when starting with 0 mmol/l. Elevation of glucose to 5.6 mmol/l again produced inhibition of $^{86}$Rb$^+$ efflux declining to approximately 1.25%/min which was reversible on return to 3 mmol/l glucose.
ATP/ADP ratio (Fig. 4) In islets obtained from GIR the ATP/ADP ratio was significantly higher than in islets from SIR, whether in the absence or in the presence of glucose. In both groups elevation of glucose produced a small rise in the ATP/ADP ratio. The total ATP+ADP was not significantly different in the two groups (not shown).

Pyridine nucleotides NAD-NADH (Fig. 5) Islets from GIR exhibited about 100% higher total NAD+NADH than islets from SIR. Increasing the glucose concentration caused no significant change.

In islets from SIR addition of glucose to the incubation medium did not significantly change NAD levels. There was a tendency towards an increase in NADH which was, however, not of statistical significance (not shown). NAD+NADH did not change. Only a small increase in the NADH/NAD ratio was observed.

In islets from GIR increasing the glucose concentration produced no significant change in NAD, but significantly enhanced the NADH levels to a maximum at 16-7 mmol/l glucose (not shown). Elevation of glucose concentrations did not affect total NAD+NADH but increased the NADH/NAD ratio to a greater extent than in islets from SIR.

NADP-NADPH (Fig. 5) Islets from GIR exhibited about 150% higher total NADP+NADPH levels compared with islets from SIR at all glucose concentrations tested. There was no concentration relationship. In islets from SIR glucose produced a slight concentration-dependent decrease in NADP and an increase in NADPH (not shown) which resulted in a marked elevation of the NADPH/NADP ratio. In islets from GIR starting from higher levels of NADP and NADPH at 2·8 mmol/l glucose, elevation of the glucose concentration led to a small decline in NADP and an increase in NADPH (not shown). The NADPH/NADP ratio increased in a manner which paralleled and exceeded that observed in islets from SIR.

Discussion

The results of this study show that a 48-h glucose infusion which caused moderate hyperglycemia (about 11 mmol/l), decreased islets insulin content but sensitized insulin
release in vitro in response to glucose, at least at glucose levels up to 11·1 mmol/L. These observations are in accordance with data of others (Bedoya & Jeanrenaud 1991b, Thams 1991, Purrello et al. 1992, Leahy et al. 1987) suggesting that hyperinsulinemia occurring during glucose infusion is due to increased sensitivity of the secretory mechanism in spite of the dramatic fall in the insulin content.

Sensitization of insulin secretion raises the question whether it is a common phenomenon being valid against any initiator of insulin release which causes depolarization or whether or not sensitization is restricted to depolarization by glucose. The data obtained with KCl showing no concentration-dependent effect on discharge of insulin and calcium uptake indicate that in islets of GIR, sensitization of the secretory mechanism is limited to the initiating action of glucose. This interpretation is supported by observations of others (Bedoya & Jeanrenaud 1991b) who reported that leucine, tolbutamide and Û-ketoisocaproate did not stimulate insulin release in islets from GIR. Since these compounds cause secretion of insulin by mechanisms which are different from that of glucose and do not include glucose metabolism it is obvious that sensitization of the secretory machinery has to do with glucose metabolism.

It has been reported that islets chronically exposed to high glucose exhibit increased glucose metabolism as indicated by enhanced glucose transporter affinity (Purrello et al. 1992), V_{max} of glucokinase activity (Chen et al. 1994), glucose utilization (Bedoya & Jeanrenaud 1991a) and glycogen production (Marynissen et al. 1990), and thus probably produce metabolic factors related to initiation and/or modulation of insulin secretion. In this context it was suggested that increased glycogen content causes hypersensitivity to glucose (Marynissen et al. 1990).

Critical factors in glucose metabolism are ATP and reduced pyridine nucleotides. In fact, as shown in this study, the ATP/ADP ratio as well as the redox ratios of pyridine nucleotides are increased in islets obtained from GIR. Whereas the ATP/ADP ratio is closely linked to K_{ATP} channels (Cook & Hales 1984), it has been hypothesized that the redox systems of pyridine nucleotides are related to calcium uptake (Ammon & Wahl 1994). Thus the raised ATP/ADP ratio in islets from GIR should decrease the threshold for glucose-inducible depolarization. That this is the case is evident from the data shown in Fig. 3, indicating marked inhibition of ⁸⁶Rb efflux in islets of GIR by as little as 3 mmol/l glucose.

Consequently, in islets from GIR at low (2·8 mmol/l) glucose, influx of calcium is increased when compared with islets from SIR.

Taking into account the fact that in islets from GIR, in the presence of 3 mmol/l glucose potassium efflux is already inhibited and therefore calcium uptake is increased, it is plausible to assume that in these islets addition of KCl to the incubation medium is not able to cause depolarization and subsequent calcium uptake since in this case B-cells are already under depolarization.

It has been suggested that not only changes in the ATP/ADP ratio but also other products deriving from glucose metabolism (Ammon & Verspohl 1979, Ammon & Mark 1985, Ashcroft et al. 1992, Ammon & Wahl 1994), including increased redox state of pyridine nucleotides where the NADPH/NADP ratio is thought to be of special interest (Ammon & Wahl 1994), may modulate calcium entry in response to depolarization. If this is true then the increase in these ratios in islets of GIR may contribute to increased calcium uptake finally resulting in enhanced insulin secretion.

As discussed above, a glucose concentration greater than 11·1 mmol/l possesses no additional secretory action in islets from GIR. It must, therefore, be assumed that these islets have lost sensitivity to high glucose. Desensitization of glucose-mediated insulin release following long term exposure to high glucose has been found to be associated with diminished glucose oxidation (Eizirik et al. 1992), decreased expression of mRNA of α subunit of voltage-dependent calcium channels (Iwashima et al. 1993), decreased calcium uptake and cytosolic calcium in response to glucose (Okamoto et al. 1992, Anello et al. 1995). Whether this is also true for the decreased secretory response at high glucose in this study is not certain. Nevertheless, our observations that calcium uptake in islets of GIR does not follow a concentration-response relationship beyond 11·1 mmol/l glucose is not against such a possibility. Moreover, it is possible that the dramatic fall in the insulin content (Marynissen et al. 1990, Bedoya & Jeanrenaud 1991a) does not allow further discharge of insulin when the glucose concentration exceeds 11·1 mmol/l (Steinke et al. 1972).

In conclusion, in pancreatic islets obtained from GIR glucose-induced insulin release is sensitized to glucose stimulation in vitro in a concentration range up to 11·1 mmol/l whereas this is not the case at higher glucose concentrations. Moreover, insulin secretion is not sensitized to KCl. Sensitization against glucose is, at least in part, due to enhanced glucose metabolism providing increased availability of critical factors including ATP and reduced pyridine nucleotides which in turn decrease the threshold for depolarization and enhance glucose-mediated calcium uptake.

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