Appearance of glucose-induced insulin release in fetal rat β -cells

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Abstract

Fetal rat pancreatic cells were isolated from pancreatic primordia on days 12–14 of pregnancy and cultured for 48 h in the presence of 5 mmol/l glucose. Insulin accumulation in the medium over the next 24 h was measured. Cultured cells from day 12 fetuses secreted about 1 fmol insulin per pancreas in response to 5 or 15 mmol/l glucose irrespective of whether 1 mmol/l tolbutamide, 400 μ mol/l diazoxide, 5 mmol/l theophylline or 10 mmol/l mannoheptulose was present. In contrast, insulin released from day 13 cultured cells increased significantly from 3.0 ± 0.6 to 6.2 ± 2.2 fmol per pancreas, when the glucose concentration was raised. Tolbutamide increased, diazoxide and mannoheptulose decreased and theophylline had no effect on insulin release. Even more pronounced effects were found on insulin release from day 14 cultured cells, in which theophylline also increased the release. In addition, insulin

release from cells from pregnancy day 14 was 75 ± 16 amol/min per pancreas when the cells were perfused for 15–20 min in the presence of 5 mmol/l glucose within 3 h of isolation. Increasing the glucose concentration to 15 mmol/l or adding tolbutamide increased, whereas diazoxide decreased, insulin release in the freshly isolated cells. The insulin content of rat pancreata from pregnancy day 13 was 0.06 ± 0.01 pmol per pancreas and increased approximately 10-fold every second day up to 6.7 ± 0.9 pmol on day 17 of pregnancy. Between day 17 and 19 the pancreatic insulin content increased about fivefold to 39 ± 2 pmol. The present data suggest that critical components of the insulin-secretory machinery, including ATP-regulated K^+ channels, glucokinase and adenylate cyclase activities, are present in the developing β -cell earlier than hitherto thought.

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Introduction

In adult life the plasma insulin response to an abrupt increase in blood glucose concentration is characterized by a biphasic pattern. However, this characteristic pattern does not manifest itself until after birth, and a low and sluggish response to glucose has been reported on days 19.5–20 of pregnancy in the rat (Asplund *et al.* 1969, Asplund 1973, Sodoyez-Goffaux *et al.* 1979, Rhoten 1980, Grill *et al.* 1981, Freinkel *et al.* 1984, Hole *et al.* 1988, Hughes 1994, Bliss & Sharp 1994). Recently we demonstrated that rat β -cells cultured for 3 days are already responsive to glucose when explanted on day 16 of pregnancy (Aoyagi *et al.* 1997). Furthermore, rat pancreatic β -cells can be identified on pregnancy day 12 (Rall *et al.* 1973, Yoshinari & Daikoku 1982). The regulation of fetal insulin release at this early stage of β -cell development has not been studied previously, largely because of insufficient sensitivity of the insulin assays. With the aid of a very sensitive insulin assay (Bergsten & Hellman 1993) and our newly developed technique for maintenance of early fetal pancreatic cells in culture (Aoyagi *et al.* 1997), we have now been able to measure

the insulin-secretory response to glucose of rat fetal β -cells explanted as early as day 12 of pregnancy and cultured for 3 days. To confirm that the glucose sensitivity was not induced by the tissue culture, freshly isolated rat fetal β -cells from pregnancy day 14 were studied with respect to glucose-stimulated insulin release. We also investigated to what extent this early secretory response of the fetal rat β -cells is affected by two non-glucose modulators of the ATP-sensitive K^+ channel, namely tolbutamide and diazoxide, by the phosphodiesterase inhibitor theophylline and by the glucokinase inhibitor mannoheptulose. Accumulation of insulin in the fetal rat pancreas was followed from day 13 to day 19 of pregnancy.

Materials and Methods

Chemicals

RPMI 1640 culture medium without glucose, Hanks balanced salts solution (with and without calcium and magnesium), penicillin and streptomycin solution, 0.5% trypsin (containing 0.2% EDTA) solution, 0.5% trypan blue solution and fetal calf serum were supplied by

HyClone (Cramlington, Northumberland, UK); BSA was from Miles (Slough, Bucks, UK); D-glucose, D-mannoheptulose, Hepes, tetramethylbenzidine, theophylline and insulin-peroxidase came from Sigma Chemicals (St Louis, MO, USA); ^{125}I -labeled insulin was from Euro-Diagnostica AB (Malmö, Sweden); guinea-pig insulin antibody for the RIA was purchased from Miles-Yeda (Rehovot, Israel); insulin standard was obtained from Novo Nordisk (Bagsvaerd, Denmark); IgG-certified microtiter plates came from Nunc (Roskilde, Denmark). The mouse insulin antibodies used in the ELISA were raised in our laboratory from guinea-pigs. Diazoxide was a gift from Schering Corp. (Kenilworth, NJ, USA) and tolbutamide from Hoechst AG (Frankfurt/Main, Germany).

Animals and preparation of pancreatic cells

Sprague-Dawley rats bred at our department were used. The rats were fed a commercial pelleted diet (AB Analysen, Lidköping, Sweden) and had free access to food and tap water. They were maintained at an ambient temperature of 22 °C with a 12 h light/12 h darkness cycle. Female and male rats were caged together during the night. The appearance of spermatozoa in the vaginal smear on the following morning defined day 0 of gestation. Isolation and culture of fetal pancreatic cells were performed as recently described (Aoyagi *et al.* 1997). Briefly, pancreata were dissected from fetuses on pregnancy days 12–14. In 12–13-day-old fetuses, pancreatic buds were dissected together with adjacent gut and surrounding mesenchyme. After trypsinization the dispersed cells were washed, counted and viability of the cells determined by trypan blue exclusion.

Culture of fetal pancreatic cells

Pancreatic cells from fetuses removed on pregnancy days 12–14 were resuspended in culture medium containing 5 mmol/l glucose and plated in 24-well tissue culture plates (Multiwell; Falcon, Lincoln Park, NJ, USA). Cells from fetuses of the same day of pregnancy were pooled, and the average number added to each well was 75 000 (day 12), 94 000 (day 13) and 100 000 (day 14). After an initial 48 h culture period, new medium (500 µl) was added containing 5 mmol/l glucose in the presence or absence of 1 mmol/l tolbutamide or 15 mmol/l glucose in the presence or absence of 5 mmol/l theophylline, 400 µmol/l diazoxide or 10 mmol/l mannoheptulose and incubation continued for an additional 24 h period.

Perfusion of freshly isolated fetal pancreatic cells

Pancreatic cells from fetuses removed on pregnancy day 14 were resuspended in medium containing (mmol/l) 125 NaCl, 5.9 KCl, 1.2 MgCl_2 , 1.28 CaCl_2 , 25 Hepes,

5 glucose and 0.5 mg/ml BSA, titrated to pH 7.4 with NaOH. The cells were allowed to attach to a glass coverslip used as the bottom of an open chamber (Sykes & Moore 1959). The number of cells added to the coverslips was $186\,000 \pm 29\,000$. To ensure attachment of the cells, the coverslips were coated with poly-L-lysine, and an incubation period of 2 h preceded the subsequent perfusion of the cells with the same medium. After 60 min of perfusion, sampling of the perfusate for insulin determination began before the glucose concentration was raised from 5 to 15 mmol/l. Before sampling in the presence of 1 mmol/l tolbutamide and 5 mmol/l glucose, the cells were perfused in the presence of 5 mmol/l glucose for 15 min. Similarly, the cells were perfused in the presence of 15 mmol/l glucose alone before sampling in the presence of 400 µmol/l diazoxide and 15 mmol/l glucose. The sampling period was 10–20 min for each condition.

Measurements of insulin release and accumulation and of pancreatic insulin content

Insulin release in the perfusate and accumulation in aliquots of the culture medium taken after the 24 h culture period was measured with a competitive ELISA with the insulin-capturing antibody immobilized directly in the solid phase essentially as described previously (Bergsten & Hellman 1993). Amounts of insulin were calculated from linear standard curves in semilogarithmic plots with a detection limit of 100 amol. Rates of insulin release or insulin accumulation were expressed as amol insulin secreted/min per pancreas or fmol insulin/24 h per pancreas for the perfused and cultured cells respectively. For determination of insulin content in fetal pancreata of pregnancy days 13–19, the dissected tissue was placed in acid alcohol (0.18 mol/l HCl in 70% (v/v) ethanol), disrupted ultrasonically for extraction of insulin overnight at 4 °C, centrifuged and stored at –20 °C. Insulin content was determined by RIA (Heding 1972) and expressed as pmol insulin per pancreas.

Data computation and statistical analysis

Data are expressed as mean $\pm$ S.E.M. Each individual observation represents a separate experiment. Probabilities (*P*) of chance differences between the experimental groups were calculated by the two-tailed Student's *t*-test for paired observations.

Results

Number of fetal pancreatic cells

Dispersed cells used for perfusion or monolayer culture were prepared from pancreata obtained from rat fetuses on

Table 1 Insulin accumulation in the culture medium of dispersed fetal rat pancreatic cells from pregnancy days 12, 13 and 14 over 24 h in the presence of 5 or 15 mmol/l glucose. Results are means $\pm$ S.E.M. from four (day 12), eight (day 13) and ten (day 14) separate experiments

Glucose (mmol/l)	Addition	Insulin release (fmol/24 h per pancreas)		
		12	13	14
5	—	1.0 $\pm$ 0.6	3.0 $\pm$ 0.6	6.9 $\pm$ 1.3
15	—	1.0 $\pm$ 0.2	6.2 $\pm$ 2.2 ^a	17.2 $\pm$ 3.1 ^b
5	Tolbutamide	1.2 $\pm$ 0.6	6.4 $\pm$ 1.6 ^c	13.5 $\pm$ 2.8 ^c
15	Diazoxide	0.9 $\pm$ 0.2	4.0 $\pm$ 1.5 ^c	6.5 $\pm$ 1.6 ^d
15	Theophylline	1.0 $\pm$ 0.2	7.1 $\pm$ 2.6	45.1 $\pm$ 8.6 ^d
15	Mannoheptulose	0.6 $\pm$ 0.2	3.0 $\pm$ 0.6 ^c	7.2 $\pm$ 1.4 ^d

Tolbutamide (1 mmol/l), diazoxide (400 μ mol/l), theophylline (5 mmol/l), or mannoheptulose (10 mmol/l) were added as indicated. The cells were initially cultured for 2 days in a medium containing 5 mmol/l glucose. Statistical comparisons were performed by the two-tailed Student's *t*-test for paired observations. ^a*P*<0.05 and ^b*P*<0.01 denote the effect of increasing the glucose concentration. ^c*P*<0.05 and ^d*P*<0.01 denote the effect of the additions.

pregnancy days 12–14. The number of fetuses used were 31 (day 12), 43 (day 13) and 45 (day 14) for four, eight and ten separate experiments respectively. The total numbers of dispersed cells per fetus were 75 300 $\pm$ 2900 (day 12), 96 600 $\pm$ 5600 (day 13) and 149 200 $\pm$ 5500 (day 14). More than 95% of the cells were viable after trypsinization as evidenced by trypan blue exclusion.

Insulin release and accumulation

Cultured pancreatic cells explanted on pregnancy day 12 and maintained in culture for 3 days accumulated about 1 fmol insulin in the culture medium per pancreas during the preceding 24 h in the presence of 5 mmol/l glucose (Table 1). Neither an increase in glucose concentration to 15 mmol/l, addition of 1 mmol/l tolbutamide in the presence of 5 mmol/l glucose or 400 μ mol/l diazoxide in the presence of 15 mmol/l glucose, nor supplementation with 5 mmol/l theophylline or 10 mmol/l mannoheptulose in the presence of 15 mmol/l glucose during the last 24 h affected the rate of insulin accumulation. In contrast, during the last 24 h of culture, pancreatic cells explanted on pregnancy day 13 increased the insulin accumulation from 3.0 $\pm$ 0.6 to 6.2 $\pm$ 2.2 fmol per pancreas in response to a rise in the glucose concentration from 5 to 15 mmol/l. Tolbutamide stimulated insulin accumulation in the presence of 5 mmol/l glucose as effectively as 15 mmol/l glucose alone. Diazoxide inhibited insulin accumulation in the presence of 15 mmol/l glucose almost to the level observed in the presence of 5 mmol/l glucose. Whereas addition of theophylline to the medium containing 15 mmol/l glucose did not affect insulin accumulation, mannoheptulose inhibited the rate of accumulation in the presence of 15 mmol/l glucose to that recorded in the presence of 5 mmol/l

glucose. When cultured cells explanted on pregnancy day 14 were used, increasing the glucose concentration from 5 to 15 mmol/l enhanced the insulin accumulation about 2.5 times during the last 24 h in culture. Tolbutamide stimulated and diazoxide strongly inhibited insulin accumulation. In contrast, with cells from day 13, theophylline more than doubled the insulin accumulation observed in the presence of 15 mmol/l glucose. Mannoheptulose inhibited the glucose-stimulated insulin accumulation.

When freshly isolated pancreatic cells from pregnancy day 14 were perfused at 5 mmol/l glucose, they released 75 $\pm$ 16 amol insulin/min per pancreas (Table 2). Increasing the glucose concentration to 15 mmol/l increased the

Table 2 Insulin release of freshly isolated fetal rat pancreatic cells from pregnancy day 14. Results are means $\pm$ S.E.M. from five separate experiments

Glucose (mmol/l)	Addition	Insulin release (fmol/24 h per pancreas)
5	—	75 $\pm$ 16
15	—	113 $\pm$ 19 ^a
5	Tolbutamide	93 $\pm$ 18 ^b
15	Diazoxide	61 $\pm$ 12 ^b

Dispersed cells were allowed to attach to the bottom of the perfusion chamber for 2 h in medium containing 5 mmol/l glucose before they were perfused at the same glucose concentration for 60 min. The perfusate in the presence of 5 and 15 mmol/l glucose was sampled for 15–20 min each. Before the cells were exposed to 1 mmol/l tolbutamide or 400 μ mol/l diazoxide, they were perfused in the presence of 5 or 15 mmol/l glucose alone for 10–15 min. Statistical comparisons were performed by the two-tailed Student's *t*-test for paired observations. ^a*P*<0.005 denotes the effect of increasing the glucose concentration. ^b*P*<0.05 denotes the effect of the additions.

Table 3 Insulin content of freshly isolated fetal pancreatic glands from pregnancy days 13–19. Extracted insulin is given as mean ± S.E.M. for the indicated number of experiments

Day of pregnancy	Insulin content (pmol/pancreas)	No. of experiments
13	0.06 ± 0.01	3
14	0.13 ± 0.03	6
15	0.51 ± 0.03	10
16	0.9 ± 0.1	13
17	6.7 ± 0.9	14
18	23 ± 2	15
19	39 ± 2	13

insulin release by 51%. After the glucose concentration in the perfusion medium had been returned to 5 mmol/l, 1 mmol/l tolbutamide caused a 24% increase in insulin release. In the presence of 15 mmol/l glucose, 400 µmol/l diazoxide decreased the rate of insulin release to 34% below that recorded in the presence of 5 mmol/l glucose.

Insulin content

The insulin content of freshly isolated fetal pancreatic glands from pregnancy day 13–19 is shown in Table 3. The hormonal content of day 13 pancreata, 0.06 ± 0.01 pmol/pancreas, increased approximately 10-fold every second day until pregnancy day 17. There was a fivefold increase in the pancreatic insulin content between days 17 and 19.

Discussion

In the present study we show that rat pancreatic β-cells from the early fetal period respond to an increase in glucose concentration with release of insulin, although to a much smaller extent than adult cells. An inherent difficulty in the study of the early functional development of the fetal rat β-cell is the small size of the developing pancreas and the low proportion of insulin-containing cells, requiring a highly sensitive assay method for the measurement of the release and content of the hormone. By pooling pancreatic cells from fetuses of the same litter, it was possible to accumulate enough β-cells to obtain detectable amounts of insulin in response to different agents affecting the secretory rate of insulin. In the present study the tissue specimens obtained from the 12 to 13-day-old fetuses comprised not only the pancreatic primordia but also some of the surrounding mesenchymal and endodermal cells, which precluded meaningful calculations of insulin release on a per cell basis. However, care was taken to include the entire pancreatic primordium, which justifies the expression of both insulin release and content per individual pancreas. By the utilization of

monolayer cultures of the fetal pancreatic cells (Aoyagi *et al.* 1997) and an insulin assay with a sensitivity of 100 amol (Bergsten & Hellman 1993), it became possible to assess the function of the developing β-cells.

The present experimental approach using a 3-day culture period of the pancreatic cells raises the question as to what extent the observed effects of the test substances reflected changes in the rates of differentiation and/or proliferation of β-cells rather than in the rates of insulin release from the individual β-cell. Assuming a cell cycle time of 15 h (Swenne 1992) for fetal rat β-cells, it is unlikely that proliferation of preformed β-cells in response to glucose could alone account for more than a fraction of the observed changes in insulin content of the culture medium. Accelerated neogenesis by differentiation of progenitor cells to β-cells is an alternative explanation, which cannot be entirely excluded. However, this would presuppose a rapid and marked induction of insulin gene transcription in a large number of progenitor cells affected by glucose, tolbutamide or theophylline with a very marked effect of the latter on pancreatic cells obtained on pregnancy day 14. Although at later stages of development glucose is known to contribute to β-cell differentiation, there is little evidence of such an effect by either tolbutamide or theophylline (Swenne 1992). Nevertheless, it seemed important to confirm the occurrence of glucose-stimulated insulin release in freshly prepared cells from the early fetal pancreas. Indeed, within a perfusion period of only 20 min, the rate of insulin secretion from cells obtained after 14 days of gestation increased significantly in response to glucose. The finding that the release was increased by tolbutamide and decreased by diazoxide extended these observations to include a sensitivity also to sulfonylureas. Although these observations do not exclude the possibility that in the culture system differentiation and proliferation of β-cells may have contributed to insulin accumulation in the medium, they strongly support the view that the increased accumulation in the presence of high glucose reflected insulin release from glucose-sensitive β-cells rather than changes in β-cell mass. It is nevertheless of note that the rate of insulin release at the basal glucose concentration (5 mmol/l) increased almost fivefold between pregnancy days 12 and 14. It is not clear if this was due to an increased β-cell mass alone or also to increased secretion by the individual β-cells.

It has been suggested that the immature insulin response of the fetal β-cell to glucose reflects the presence of a low level of the glucose transporter protein GLUT2 or a low activity of glucokinase (Hughes 1994, Tiedge & Lenzen 1993). However, the latter proposal is challenged by the present results showing inhibition of insulin release by mannoheptulose and by a recent report in which fetal rat islets obtained on pregnancy day 21 showed amounts and activities of glucokinase similar to those found in adult islets (Tu & Tuch 1996). This indicates that glucokinase already has regulatory significance for insulin release in

early β -cell development. However, there appears to be less glucose oxidation in the fetal than the adult β -cell (Rorsman *et al.* 1989). This could explain the lack of increase in the fetal islet ATP content after 30 min of exposure to high glucose levels (Rorsman *et al.* 1989, Wahl *et al.* 1992). The present observation of glucose-sensitive insulin release already on day 14 of pregnancy nevertheless suggests a role for glucose metabolism in the regulation of fetal insulin release.

A stimulatory effect of glucose on insulin synthesis has been demonstrated in fresh fetal rat pancreas on pregnancy day 15 (Kakita *et al.* 1983) and in isolated islets from 20-day-old rat fetuses (Eriksson *et al.* 1980). However, in monolayer culture of pancreatic cells obtained on pregnancy day 16, glucose failed to stimulate insulin biosynthesis (Aoyagi *et al.* 1997), suggesting that the present glucose-induced increase in insulin release does not reflect an increase in insulin biosynthesis. Likewise it has been shown that theophylline has minimal effects on insulin biosynthesis in fetal β -cells of late gestation (Svenne 1982).

In the adult β -cell, the reduction in the permeability of the ATP-sensitive K^+ channel is an important step in membrane depolarization and opening of the voltage gated L-type Ca^{2+} channels, which leads to an increase in cytoplasmic Ca^{2+} concentration and insulin release (Hellman *et al.* 1988). Functional ATP-sensitive K^+ channels, sensitive to sulfonylureas, and Ca^{2+} channels have been demonstrated in rat fetal β -cells from pregnancy day 16 (Weinhaus *et al.* 1995). Our results with glucose, tolbutamide and diazoxide indicate that the channels are already operational in β -cells explanted on pregnancy day 14. In these cells, tolbutamide in the presence of 5 mmol/l glucose induced an insulin release that was similar to or slightly lower than that produced in the presence of 15 mmol/l glucose. This suggests that the supply of ATP is the rate-limiting factor for insulin secretion, rather than depolarization of rat fetal β -cells, similar to the situation in adult pancreatic islets (Eliasson *et al.* 1997, Westerlund *et al.* 1997).

cAMP formation has been found to be lower in fetal than in adult rat β -cells (Grill *et al.* 1975), analogous to the lower ATP synthesis. When the low fetal cAMP levels were elevated in rat fetal β -cells of pregnancy day 21 by either inhibition of its breakdown or stimulation of its synthesis, glucose-induced insulin release was augmented (Ammon *et al.* 1989, Mourmeaux *et al.* 1989). The results of our study are well in line with these data and suggest that the potentiating effect of cAMP is effective well before day 21 of pregnancy.

The present results show for the first time that freshly isolated fetal rat β -cells from as early as gestational day 14 and cultured rat β -cells from as early as gestational day 13 secrete more insulin when the glucose concentration is increased. Until now the earliest dynamic measurements of insulin release from the fetal rat pancreas have been

from days 19.5–20 of pregnancy and show a similar sustained and shallow response to glucose (Rhoten 1980, Bliss & Sharp 1994). Furthermore, both the freshly isolated and the cultured glucose-responsive cells are also responsive to tolbutamide and diazoxide. Glucokinase activity seems to appear at the same stage, whereas theophylline-sensitivity, indicating increased adenylate cyclase activity, appears somewhat later. Our combined results indicate that critical components of the insulin-secretory machinery, such as ATP-sensitive K^+ channels, glucokinase and adenylate cyclase activities, are present in the developing β -cell earlier than hitherto known.

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