Gestational hyperglycaemia and insulin release by the fetal rat pancreas in vitro: effect of amino acids and glyceraldehyde

M. T. Bihoreau, A. Ktorza and L. Picon

Laboratoire de Physiologie du Développement associé au CNRS n° 307, Université Paris 7, Paris, France

Summary. Unrestrained pregnant rats were infused with glucose during the last week of pregnancy to produce slight or high gestational hyperglycaemia. Control rats were infused with distilled water. Insulin secretion of the fetuses at term was studied in vitro using a perfusion system. Compared with controls, perfused pancreases of slightly hyperglycaemic fetuses showed a similar pattern of insulin secretion in response to 10 mmol/l leucine. Arginine-induced insulin secretion at 20 mmol/l was higher than in controls. In both groups, 10 mmol/l α -ketoisocaproate had a poor stimulatory effect on insulin release, and 5 mmol/l D-glyceraldehyde was ineffective in eliciting insulin secretion. In highly hyperglycaemic fetuses all the secretagogues, with the exception of arginine,

which induced a sustained monophasic insulin secretory response, had no effect on insulin release. These data show that long-term exposure of fetal B cells to high plasma glucose levels in utero suppresses or alters further insulin secretory response not only to glucose but also to other nutrient secretagogues. The partially spared insulin secretory response to arginine suggests that the defect may concern stimulus-secretion coupling rather than insulin releasing machinery.

Key words: Slight gestational hyperglycaemia, high gestational hyperglycaemia, fetus, insulin release, perfused pancreas, leucine, arginine.

Many investigators have reported that insulin secretion in response to glucose is, when detected, lower in fetal and neonatal than adult stages in man [1, 2] and the rat [3–5]. Despite immaturity of fetal islets compared with adult islets, evidence indicates that insulin release by fetal pancreases is sensitive to long-term plasma glucose changes in late pregnancy. This is clearly demonstrated in the infants of diabetic women [1, 6, 7] and in fetuses at term from rats with streptozotocin-induced diabetes [5]. However, quite different effects on the fetal pancreatic function have been observed according to the severity of maternal diabetes [5, 8]. In a previous study, we showed that the level of gestational hyperglycaemia, independent of other metabolic disorders usually associated with diabetes, was of crucial importance in the insulin secretory response by the fetal pancreas; moderate gestational hyperglycaemia led to an increase of insulinaemia which was not observed in highly hyperglycaemic fetuses [9].

It seems of interest to assess (a) whether impaired glucose-induced insulin secretion in severely hyperglycaemic fetuses may be ascribed to altered glucose metabolism in the islet; and (b) whether differential effects of gestational hyperglycaemia on insulin secretion are

specific to glucose and glucose metabolism or can be extended to other secretagogues. We have studied insulin release by pancreases of slightly or highly hyperglycaemic fetuses in response to the following secretagogues in vitro: D-glyceraldehyde, which enters glycolysis distal to glucose at the triose phosphate stage; amino acids, which either are metabolized (leucine) [10] or are not metabolized (arginine) by the islet [11].

This study was performed using a perfusion system, which allows the evaluation of insulin secretory dynamics induced by a secretagogue free of environmental interferences. The perfusion system is more convenient than static incubation systems, which have usually failed to reveal a fetal B cell response to glucose or other stimulatory factors [3].

Materials and methods

Animals

Three-month-old virgin female Sherman rats weighing about 250 g were fed ad libitum with laboratory chow (U.A.R. B-03), and caged overnight with a male (17.00–09.00 hours). Pregnancy was detected by abdominal palpation 13 days later. In our colony, birth occurred

Table 1. Weight gain, plasma glucose and insulin concentrations in control and glucose-infused pregnant rats

	Weight gain during the period of infusion (g)	Plasma glucose concentration (g/l)	Plasma insulin concentration (μ U/ml)
Control rats (n=14)	45.3 $\pm$ 11.1	0.79 $\pm$ 0.02	117 $\pm$ 11
Slightly hyperglycaemic rats (n=12)	36.8 $\pm$ 7.1	1.45 $\pm$ 0.05 ^a	353 $\pm$ 83 ^a
Highly hyperglycaemic rats (n=14)	34.1 $\pm$ 10.2	3.52 $\pm$ 0.30 ^{a,b}	603 $\pm$ 112 ^{a,c}

Values are means $\pm$ SEM. The number of observations is indicated in parentheses. Significant differences between control and glucose-infused rats, ^a $p < 0.001$; between slightly and highly hyperglycaemic rats, ^b $p < 0.001$, ^c $p < 0.01$

22 days after mating. Infusion of glucose was performed using the flexible technique for long-term infusions in unrestrained rats developed by Nicolaidis et al. [12].

Rats were fitted with an intracardiac catheter on day 13 of pregnancy. The infusion period began on day 15 of pregnancy and lasted until the removal of the fetuses. Hypertonic (30%) sterile glucose (Chaix et Dumarais, Paris, France) was infused at an initial rate of 30 μ l/min to produce slight hyperglycaemia or at a rate of 50 μ l/min to produce marked hyperglycaemia. When necessary, infusion rates were slightly modulated throughout the period of infusion to maintain glycaemia in the desired ranges. Controls were infused with sterile distilled water (Chaix et Dumarais). Distilled water was chosen rather than isotonic saline to avoid sodium overload in controls. Since the rate of infusion was very slow compared with the blood flow rate in the superior vena cava, haemolysis was unlikely and not observed.

Samples

A semi-quantitative method (Haemoglukotest, Boehringer, Mannheim, FRG) was used twice daily to measure plasma glucose of infused pregnant rats on blood samples collected from the tail vein.

On day 21.5 of pregnancy, the pregnant rats were killed by cervical dislocation and the fetuses immediately exteriorised after Caesarean section. Blood was collected rapidly from the axillary vessels, centrifuged and plasma glucose concentration determined. Pancreases were dissected out and minced in pieces of approximately 0.5 mm³ using a tissue chopper (McIlwain, Gomshall, UK) and washed in a gassed Krebs-Ringer bicarbonate buffer (in mmol/l: Cl⁻, 123; Na⁺, 121; K⁺, 5.2; Ca⁺⁺, 2.5; Mg⁺⁺, 1.2; H₂PO₄⁻, 1.2; HCO₃⁻, 25; SO₄⁻, 1.2) containing 2.2 mmol/l glucose and 0.5% bovine serum albumin (Albumin, bovine, fraction V, fatty acid free, Sigma, St. Louis, Mo. USA).

Experimental procedure

The perfusion was performed according to the method of Kikuchi et al. [13] as described previously [5, 9]. Briefly, minced tissues from 2 or 3 fetuses of the same litter were placed into the perfusion chambers. The KRB buffer continuously gassed with 95% O₂ and 5% CO₂ (pH=7.4; 37°C) containing bovine serum albumin and 2.2 mmol/l glucose was driven at a flow rate of 0.8 ml/min to the perfusion chambers. A fraction collector was used for the collection of the effluent. The temperature of the chambers and of the flasks was kept at 37°C in a water bath.

A stabilization period of 108 min with the basal medium preceded the stimulatory period (40 min). The effluent of the last 12 min of the stabilization period was collected every 2 min, and the samples obtained during this period were used for the determination of the basal rate of insulin release.

The rate of insulin secretion from the pancreatic pieces was expressed as μ U insulin: min⁻¹ · mg pancreas⁻¹.

After the perfusion procedure, the pieces of pancreas were recovered, weighed and insulin extracted in 1 ml of acid-alcohol solution (ethanol 75%, distilled water 23.5%, 13 N hydrochloric acid 1.5%) by ultrasonic desintegration at 4°C during 30 s. Extracts were centrifuged and the supernatant kept at -20°C until assay.

Assays

Plasma glucose was determined by the glucose oxidase technique using a glucose analyzer (Beckman, Palo Alto, Calif. USA).

Immunoreactive insulin (IRI) was estimated using purified rat insulin as standard (R 171, Novo, Copenhagen, Denmark), antibody to a mixture of porcine and bovine insulin [15] and porcine moniodinated ¹²⁵I-insulin [16] Silicate was used to separate free from bound hormone [15]. The method allowed the determination of 6 μ U/ml (0.25 ng/ml) with a within and between assay coefficient of variation of 10%.

Statistical analysis

Results are expressed as means $\pm$ SEM. Significance of differences between means as evaluated by Student's unpaired t-test.

Results

The different flow rates of glucose infusion allowed a clear separation of the three groups of pregnant rats. Plasma glucose concentration in slightly hyperglycaemic rats was maintained between 1.4 g/l and 2 g/l throughout the period of infusion, while it reached much higher values (more than 3 g/l) in highly hyperglycaemic rats. Blood glucose concentrations in controls were always maintained between 0.8 g/l and 1 g/l. The precise measure of plasma glucose concentration in pregnant rats on the day of delivery confirmed these data (Table 1).

Hyperglycaemia led to increased plasma insulin levels in both groups of glucose-infused rats; the levels were more pronounced in highly hyperglycaemic rats (Table 1). Infusion with glucose did not induce a significant change in weight gain of pregnant rats (Table 1). As expected, plasma glucose concentration was significantly higher in fetuses from slightly (1.11 $\pm$ 0.09 g/l) or highly (2.86 $\pm$ 0.15 g/l) hyperglycaemic rats than in controls (0.49 $\pm$ 0.01 g/l).

Insulin release by perfused fetal pancreases in vitro

In the three experimental groups, insulin release by perfused fetal pancreases was initially very high. It fell rapidly and continuously until 64 min. Thereafter, a stabilized baseline was obtained. In fetuses from highly hyperglycaemic mothers, insulin secretion rates stabilized more rapidly than in the two other groups; a pla-

Fig. 1. Insulin release by minced pancreases from control (○—○), slightly hyperglycaemic (●·····●), or highly hyperglycaemic (▲-----▲) fetuses during the prestimulatory period with KRB-2.2 mmol/l glucose. Results represent the mean of 38 (control), 36 (slightly hyperglycaemic fetuses), 40 (highly hyperglycaemic) fetus observations

Fig. 2. Effect of 10 mmol/l leucine added to the KRB-2.2 mmol/l glucose medium on insulin release by minced pancreases from control (○—○), slightly hyperglycaemic (●·····●), or highly hyperglycaemic (▲-----▲) fetuses. The stimulation period runs from 0 to 40 min. Number of observations: 7 for control fetuses, 8 for slightly hyperglycaemic fetuses, 8 for highly hyperglycaemic fetuses

teau was observed from 16 min (Fig. 1). After reaching a stable baseline, insulin release during the prestimulatory period was similar in all groups of fetuses (Figs. 2-6; Table 2).

Fig. 3. Effect of 5 mmol/l glyceraldehyde on insulin release by pieces of pancreas from control (○—○) ($n=11$), slightly (●-----●) ($n=6$), or highly hyperglycaemic (▲-----▲) ($n=6$) fetuses. n =number of cases. The experimental procedure was identical to that used for leucine

Fig. 4. Insulin secretory response to 10 mmol/l α -keto-isocaproic acid of pieces of pancreas from control (○—○) ($n=8$), slightly hyperglycaemic (●·····●) ($n=6$), or highly hyperglycaemic fetuses (▲-----▲) ($n=6$). n =number of observations

Insulin secretory response to 10 mmol/l leucine was similar in fetuses from control and slightly hyperglycaemic rats; i.e. a two-fold increase of insulin release was observed in the presence of this secretagogue. It was un-

able to induce any increase above the basal insulin secretion in fetuses from highly hyperglycaemic rats (Fig. 2; Table 2).

Regardless of experimental group, neither 5 mmol/l glyceraldehyde nor 10 mmol/l KIC noticeably stimulated insulin secretion by pieces of fetal pancreas (Fig. 3; Table 2) ($p < 0.2$).

The medium containing 20 mmol/l arginine had a potent stimulatory effect on insulin release by pancreases from control and slightly hyperglycaemic fe-

tuses. This effect was even more marked in the latter group, in which the rate of insulin secretion in response to arginine was significantly higher than in controls ($p < 0.01$). In highly hyperglycaemic fetuses, 20 mmol/l arginine elicited a sustained increase of insulin secretion, although it was lower than in the other two groups. A clear-cut early phase of insulin secretion, could be observed the magnitude of which was similar to that of controls (Fig. 5; Table 2).

It may be noted that the pattern of insulin release by pieces of fetal pancreas appeared to be different according to the potency of secretagogues: 20 mmol/l arginine which had a powerful effect on insulin secretion-induced biphasic insulin release, while less effective secretagogues such as 10 mmol/l leucine elicited insulin secretory responses which could not be clearly separated into two phases. Hence in order to homogenize the expression of the results, the rate of insulin release was estimated during the whole stimulatory period, regardless of insulin secretion pattern.

Insulin content of the pancreases, as measured at the end of the perfusion period, was significantly higher in slightly hyperglycaemic fetuses (3.62 ± 0.25 mU/mg pancreas) than in control (2.32 ± 0.35 mU/mg pancreas) and highly hyperglycaemic fetuses (2.63 ± 0.29 mU/mg pancreas) ($p < 0.01$). No significant difference was observed between the two latter groups.

Fig. 5. Effect of 20 mmol/l arginine on insulin release by pieces of pancreas from control (○—○) ($n=12$), slightly hyperglycaemic (●·····●) ($n=13$), or highly hyperglycaemic fetuses (▲----▲) ($n=20$). Arginine was added to the basal medium (KRB 2.2 mmol/l glucose) at 0 min. n =number of cases

Discussion

The high initial insulin release we observed in all groups of fetuses (but to a lesser degree in highly hyperglycaemic fetuses) has also been reported by other investigators using the same technique [13, 14]. It is usually considered to be largely artifactual and related to non-specific insulin release by B cells injured by the section of the pancreas. Pancreatic insulin concentration at the

Table 2. Insulin release by perfused pancreases of control, slightly hyperglycaemic, or highly hyperglycaemic fetuses in response to 5 mmol/l glyceraldehyde, 20 mmol/l arginine, 10 mmol/l leucine, or 10 mmol/l α -keto-isocaproic acid (KIC)

	Insulin release ($\mu\text{U} \cdot \text{min}^{-1} \cdot \text{mg of pancreas}^{-1}$)						% of basal rate		
	Prestimulatory phase (min)			Stimulatory phase (min)			Controls SH HH		
	(-12-0)			(0-40)					
	Controls	SH	HH	Controls	SH	HH			
Glyceraldehyde (5 mmol/l)	1.45 ± 0.13 (11)	2.16 ± 0.44 (6)	1.69 ± 0.34 (6)	1.79 ± 0.21 (11)	2.58 ± 0.33 (6)	1.95 ± 0.23 (6)	123 (11)	120 (6)	120 (6)
Arginine (20 mmol/l)	2.75 ± 0.51 (12)	2.84 ± 0.58 (13)	2.38 ± 0.30 (20)	5.26 ± 0.69 (12) c	7.75 ± 1.00 (13) b, d	3.61 ± 0.62 (18) a, c	191 (12)	274 (13)	151 (18)
Leucine (10 mmol/l)	2.15 ± 0.51 (5)	1.59 ± 0.62 (5)	1.99 ± 0.33 (6)	4.05 ± 0.68 (5) c	3.93 ± 0.94 (5) c	1.94 ± 0.26 (6) b	188 (5)	202 (5)	102 (6)
KIC (10 mmol/l)	1.95 ± 0.27 (8)	1.57 ± 0.37 (6)	2.35 ± 0.48 (5)	3.01 ± 0.47 (8)	2.63 ± 0.77 (6)	2.19 ± 0.46 (5)	154 (8)	167 (6)	—

SH: slightly hyperglycaemic fetuses; HH: highly hyperglycaemic fetuses; number of cases in parentheses. Values are means $\pm$ SEM; the average rates of insulin release were calculated from data shown in Figures 2–5. Significant differences between control and SH or HH fetuses, ^a $p < 0.05$; ^b $p < 0.01$. Significant differences between basal and stimulated insulin release, ^c $p < 0.05$; ^d $p < 0.001$

end of the perfusion, which was much higher than insulin release during the perfusion, was not decreased in highly hyperglycaemic fetuses compared with controls. Thus, lowered insulin secretion in the prestationary phase by pancreases of highly hyperglycaemic fetuses compared with the two other groups can hardly be supposed to reflect a decrease of insulin storage.

Many authors have suggested that insulin secretory response to amino acids is present in the rat fetus at term, but is greatly attenuated compared to the adult one [4, 5, 17]. In the present study, leucine elicited a discrete but significant increase in insulin release in control and slightly hyperglycaemic fetuses; however, the pattern of the response was not clearly biphasic. On the basis of *in vivo* and *in vitro* experiments (static incubation systems), leucine was reported to be poorly effective in insulin release by islets of 1-day-old rats [4]. Increment of insulin secretion in response to the mixture leucine + arginine by fetal rat pancreases was shown by Rothen [3]; however, in this report, the effect of leucine alone was not emphasized.

In the adult rat, D-glyceraldehyde, which enters glycolytic pathway at the triose phosphate step, and α -ketoisocaproic acid, the first catabolic product of leucine, have a potent stimulatory effect on insulin secretion [18, 19]. To our knowledge, the insulin secretory response of the fetal rat pancreas to α -ketoisocaproic acid has not been previously investigated. The effect of glyceraldehyde on this secretion remains controversial. Agreen et al. [20] showed a slight but significant effect of glyceraldehyde on insulin secretion, whereas Grill et al. [4] found no significant effect. We failed to demonstrate any response of insulin release to D-glyceraldehyde in all groups of fetuses; on the other hand, α -ketoisocaproic acid had a poor stimulatory effect on insulin secretion in control and slightly hyperglycaemic fetuses, and was completely ineffective in highly hyperglycaemic fetuses. Although it cannot be excluded that these negative results could be ascribed to blunted permeability of the fetal B cell *in vitro*, these data suggest that the attenuated insulin secretory response to glucose and leucine observed in the fetus may result from the poor metabolism of these nutrients in fetal islets. Recent findings of unchanged $\text{NADP}^+/\text{NADPH}$ ratios [21], potassium efflux and calcium uptake [22] by increased glucose concentration within the fetal islets support this point of view.

Arginine, which is not metabolized within the islet and depolarizes B cells without decreasing the permeability to potassium, in contrast with glucose and leucine [23], sharply stimulated insulin secretion by pancreases of control fetuses. Rhoten [3] also reported a potent stimulatory effect of arginine on insulin secretion by pancreata of 20-day-old fetuses *in vitro*; however, in this study, the response was monophasic. This slight discrepancy with our results may be attributed to the lower dose of arginine used in his study (10 mmol/l) compared to our study (20 mmol/l). On the basis of *in vivo* experiments, it has also been reported that the human fetal pancreas might respond to arginine very early in gestation and that the response might be of a high magnitude in the fetus at term [24, 25]. The arginine-induced insulin release by the pancreas of slightly hyperglycaemic fetuses was significantly enhanced compared with that of controls. Similarly, it has been shown that human newborns from gestationally diabetic mothers responded to arginine infusion with a greater and more sustained rise of plasma insulin than newborns from normal women [25]. The synergistic effects of glucose and arginine upon insulin release has been demonstrated in premature infants [26] and in adult men [27, 28] or rats [29]. Our results show that sustained slight gestational hyperglycaemia potentiates the insulin response to arginine in the fetal rat at term.

Insulin secretion was always lower in highly hyperglycaemic fetuses than in control and slightly hyperglycaemic fetuses. However, differentiated severity of the alteration could be observed according to the nature of the secretagogue. In a previous study, insulin release was insensitive to glucose alone or associated with theophylline in these fetuses [9]. In the present study, inhibited responsiveness to leucine of pancreas from highly hyperglycaemic fetuses demonstrates that altered insulin response is not limited to glucose but can be extended to other nutrient secretagogues. In contrast, although lower than in slightly hyperglycaemic fetuses, a sustained but monophasic insulin secretory response to arginine was observed in highly hyperglycaemic fetuses. Taken together with the finding that pancreatic insulin storage was not different from that of controls, this strongly suggests that the defect may concern the stimulus-secretion coupling rather than the insulin releasing machinery, which is probably in other respects intact.

Our experimental model allows the production of well-controlled hyperglycaemia and avoids other main metabolic disorders associated with diabetes [30]. Therefore, the findings reported here suggest that the level of gestational glycaemia, independent of other environmental factors or toxic injuries, is of crucial importance in the further insulin secretory responsiveness to both glucose and amino acids. It may especially affect the maturation of the stimulus-secretion coupling mechanism in the B cell.

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Dr. A. Ktorza
Laboratoire de Physiologie du Développement
associé au CNRS No.307
Tour 23-33, 1er étage
Université Paris VII
2, Place Jussieu
F-75251 Paris Cédex 05
France