Development of Insulin Release by Fetal Rat Pancreas In Vitro

Effects of Glucose, Amino Acids, and Theophylline

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SUMMARY

The dynamics of insulin release by pieces of fetal rat pancreas from 17.5 to 21.5 days of gestation was measured in an in vitro perifusion system. Interactions between glucose, theophylline, and a mixture of 12 amino acids at physiologic concentration (mix. A.A.) were studied.

On day 17.5, 13.9 mM glucose induced only a small (10 min) early phase of insulin release. The late phase of insulin secretion appeared on day 18.5 and gradually increased as the gestation proceeded. The mix. A.A. (9 mM) or theophylline (5 mM) potentiated the two phases of insulin release induced by 13.9 mM glucose from days 18.5 to 21.5 of gestation. On day 21.5, the combination of theophylline and mix. A.A. at 2.2 mM glucose stimulated insulin release by fetal pancreas.

The dose-dependent curves of the early phase of insulin release, due to glucose or glucose and mix. A.A. (9 mM), showed half maximal responses in the term fetal pancreas at glucose concentrations of 7.2 and 4 mM, respectively. The values were 7.9 and 5.9 mM for the late phase.

The results indicate that the mechanisms controlling biphasic insulin release develop during the late fetal life in the rat. Transition from the fetal to adult type of insulin secretion more likely parallels quantitative, rather than qualitative, changes within the B cells.

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The rat fetus responds in vivo to a one hour hyperglycemia by increasing its plasma insulin concentration.[1–5] This response is present as early as day 18.5 of gestation[2] and exhibits a clear biphasic pattern by days 20.5 and 21.5.[4,5]

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It has been claimed repeatedly that the pancreas of the rat fetus did not respond to glucose in vitro when incubation procedures were used[6–8] or responded only by a small initial peak of insulin release when studied dynamically with a perifusion system.[9–11] An adult-like insulin release was observed only in the presence of phosphodiesterase inhibitors.[7,9,12]

In preliminary reports, we indicated that the biphasic insulin release induced by glucose in vitro was already present in the term rat fetus.[13,14] We also suggested that differences in the magnitude of the response between in vivo and in vitro experiments might be caused by a potentiating effect of the high circulating amino acid levels reported in the term fetus.[15,16] Recently, it was reported that islets isolated from rat fetuses at 2 days before term responded to glucose, leucine, and arginine during incubation in vitro.[17]

We investigated the development of the biphasic insulin response of the fetal rat pancreas to glucose using an in vitro perifusion system. The effects of theophylline or of a mixture of amino acids (mix. A.A.) were also studied.

MATERIALS AND METHODS

Animals. Virgin female Wistar rats, weighing 190 to 220 g, were fed ad libitum with UAR BO3 laboratory chow. Pregnancy was induced by caging females overnight with a male. The beginning of gestation was estimated to occur at 1 a.m., the time of ovulation. The following morning was day 0.5 of gestation. Pregnant rats were screened by palpation 14 days later. Experiments were performed on days 17.5 to 21.5 of gestation and on adult virgin females without previous fasting of the animals.

Perifusion procedure. The perifusion device was similar to that described by Kikuchi et al.[18] Briefly, a Krebs-Ringer bicarbonate buffer (KRB), continuously gassed with 95% O_2 and 5% CO_2 (pH 7.4), containing 0.5 mg/ml of bovine serum albumin (fraction V, Sigma), was driven by means of a peristaltic pump (Ismatec, France) through the perifusion chambers at a flow rate of 0.75–0.80 ml/min. The buffer flasks and the chambers were kept at 37°C in a water bath. The effluent was collected in test tubes with a fraction collector. As a

function of fetal age, 2 (at 21.5 days) to 20 (at 17.5 days) pancreases were rapidly excised, mechanically sectioned (McIlwain tissue chopper, England) in small square fragments of 0.5 mm sides, and washed 2 min in 20 ml KRB. Minced tissue was placed into the perifusion chambers (5 to 10 mg in each of them). During the washing and the prestimulatory periods, the glucose concentration in the KRB was 2.2 mM. The effluent of the last 10 min of the prestimulatory period was taken as the basal rate of insulin release for each preparation. It was followed by a 45 min stimulation period. After the experiment, the pancreatic tissue was recovered and the wet weight was determined.

The appropriate final concentrations of the mix. A.A. in the perifusion medium (3-6-9 or 18 mM) were obtained by dilution of a 600 mM stock solution of 12 amino acids (Multène, Laboratoires Robert et Carrière, Paris; supplemented with alanine). The composition in amino acids of the stock solution was as follows (in millimolar): alanine, 107.8; arginine, 28.6; glycine, 79.9; histidine, 10.5; isoleucine, 40.8; leucine, 47.6; lysine, 44; methionine, 67; phenylalanine, 58.1; threonine, 42; tryptophan, 12.2; and valine, 61.4. In all experiments the stimulation medium was corrected in order to maintain a constant molarity.

Analysis and expression of results. The pancreases of four to five fetuses per litter were weighed individually and extracted in 2 ml of acid-ethanol (ethanol 75 ml, distilled water 23.5 ml, and concentrated hydrochloric acid 1.5 ml) by ultrasonic disintegration at 4°C (M.S.E. MK_2 Crowley, England). The concentrations of insulin in the pancreatic extracts and the perifusion effluents were determined by radioimmunoassay using rat insulin as standard (21.4 IU/mg; batch R 170, Novo Industry, Denmark). The lower limit of detection of the assay system was 1.25 μU/ml, with a coefficient of variation of 10%.[19]

The insulin secretion rate from the pancreatic fragments is expressed as microunits of insulin released per minute and per milligram of pancreas (wet weight). The values for the dose-response curves were expressed in terms of their increase over the mean basal rate of insulin release obtained during the last 10 min of the prestimulatory period.

The results are given as $\bar{x} \pm$ SEM. The significance of changes between the prestimulatory periods and the early or late phases of insulin release was evaluated by paired Student's *t* test.

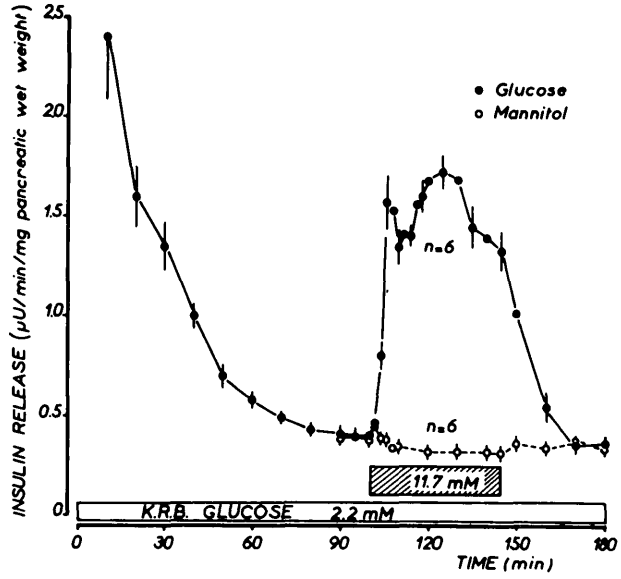

FIGURE 1. Insulin release by pieces of pancreas from term rat fetus during the prestimulatory period with KRB 2.2 mM glucose and the stimulation period with (●) 11.7 mM glucose or (○) 11.7 mM mannitol. Results are $\bar{x} \pm$ SEM of six perifusions.

RESULTS

Characteristics of the perifusion system. The pattern of insulin secretion by perifused 21.5-day-old fetal rat pancreas during the prestimulatory and stimulatory periods is depicted in Figure 1. After a high initial insulin release, which is largely artifactual,[18] the secretion rate fell to a stable baseline 90 to 100 min later. In the following experiments, a prestimulatory period of 100 min preceded the addition of any test substance. The specificity of the insulin response was tested by using a nonsecretagogue sugar (mannitol, 11.7 mM); no change of the basal secretion rate was observed. In contrast, 11.7 mM glucose induced a biphasic insulin release. After cessation of the stimulus, insulin secretion returned to basal values within 20 min.

Development of glucose-induced insulin release. Pancreatic insulin concentration increased progressively during the late fetal period, from 0.20 ± 0.03 mU/mg (n = 10) on day 18.5 up to 1.5 ± 0.08 mU/mg (n = 35) on day 21.5. It was 1.7 ± 0.2 mU/mg (n = 7) in the adult rat.

The dynamics of insulin release in response to 13.9 mM

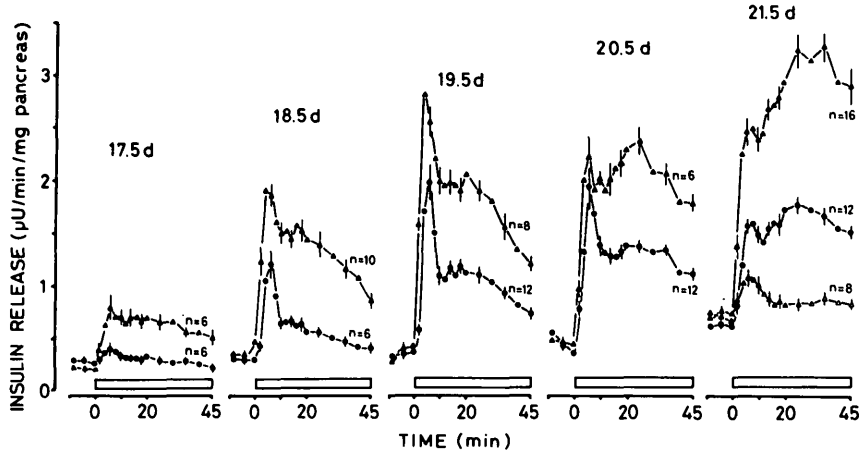

FIGURE 2. Perifusion of fetal rat pancreas from 17.5 to 21.5 days of gestation. Effects of 13.9 mM glucose alone (●) or with 9 mM mix. A.A. (▲) on insulin release. Insulin secretion elicited by 9 mM mix. A.A. at 2.2 mM glucose is reported for age 21.5 days (△). Results are $\bar{x} \pm$ SEM of the number (n) of perifusions. The white bars represent the time of stimulation.

TABLE 1
Insulin release by perifused fetal or adult pancreas in response to 13.9 mM glucose.

Age	n	Insulin secretion rate (μU/min/mg wet pancreas) Prestimulatory phase (t-10–t0)	Early phase (t0–t10)	Late phase (t10–t45)	Total release (μU/45min/mg)
Fetus 17.5 days	6	0.28 ± 0.03	0.35 ± 0.04*	0.28 ± 0.03	13.4 ± 1.2
18.5 "	6	0.32 ± 0.04	0.85 ± 0.08*	0.53 ± 0.03*	27.0 ± 1.8
19.5 "	12	0.35 ± 0.02	1.33 ± 0.10*	0.95 ± 0.06*	46.4 ± 2.2
20.5 "	12	0.45 ± 0.03	1.32 ± 0.09*	1.19 ± 0.05*	55.0 ± 2.3
21.5 "	12	0.62 ± 0.05	1.31 ± 0.05*	1.60 ± 0.07*	69.3 ± 3.0
Adult	6	0.60 ± 0.09	1.33 ± 0.18*	4.06 ± 0.19*	156.0 ± 7.0

Significant difference between the prestimulatory phase and the early or late phases is indicated by *P < 0.001
n = number of perifusions.

glucose by fetal pancreas is summarized in Figure 2. The average rates of insulin secretion during the first 10 min (early phase) and the following 35 min of stimulation (late phase) are shown in Table 1. The insulin secretion in basal conditions (KRB glucose 2.2 mM) increased from day 17.5 to day 21.5, when it was identical to that of the adult. On day 17.5 of gestation, the passage from 2.2 mM to 13.9 mM glucose induced only a small transient rise of insulin secretion during the first 10 min of stimulation. From day 19.5 onwards, the insulin secretion rate during the early phase was similar in fetuses and adults. The late phase increased gradually between 18.5 and 21.5 days of gestation. At that stage the insulin secretion rate was almost 40% that obtained in the adult (Table 1).

The insulin release during 45 min of stimulation (expressed in percent of the pancreatic insulin content) decreased from day 18.5 (13.5) to day 21.5 of gestation (4.6); it was at this stage half that of the adult (9.2).

Effects of a mixture of amino acids on the insulin response to glucose. We investigated whether physiologic concentrations of amino acids enhanced the insulin response to glucose by the fetal pancreas. At the end of the prestimulatory period with 2.2 mM glucose, a mixture of 12 amino acids was added to the medium containing either low (2.2 mM) or high (13.9 mM) glucose concentrations.

On day 21.5 of gestation the mix. A.A. (9 and 18 mM) stimulated noticeably the insulin release at 2.2 mM glucose (Table 2). At 13.9 mM glucose, the mix. A.A. enhanced both phases of insulin secretion. A near maximal stimulation of insulin release was attained at 13.9 mM glucose, with amino acid concentrations within the physiologic range (6–9 mM).

The stimulation by the mix. A.A. (9 mM) on glucose-induced insulin release was evaluated at earlier stages (Figure 2). On day 17.5, the mix. A.A. induced the appearance of the late phase, which was inconspicuous with glucose alone. At later stages, the mix. A.A. enhanced both phases of insulin release. The total amount of insulin secreted during the 45 min of stimulation was increased an average of 90% over the five stages studied.

Glucose-insulin dose-response curves. The insulin response of the pancreas from term fetus to glucose alone or with 9 mM mix. A.A. is shown in Figure 3. The dose-response curve for the early and late phases of insulin secretion suggested a sigmoid shape.

TABLE 2
Insulin release by perifused 21.5-day-old fetal rat pancreas in response to a mixture of 12 amino acids.

Amino acid mixture (mM)	Glucose (mM)	n	Insulin secretion rate (μU/min/mg wet pancreas) Prestimulatory phase (t-10–t0)	Early phase (t0–t10)	Late phase (t10–t45)	Total release (μU/45min/mg)
0	2.2	4	0.65 ± 0.02	0.57 ± 0.03	0.62 ± 0.02	27.4 ± 1.1
	13.9	12	0.62 ± 0.05	1.31 ± 0.05*	1.60 ± 0.07*	69.3 ± 3.0
3	2.2	4	0.65 ± 0.06	0.70 ± 0.05	0.60 ± 0.05	27.9 ± 2.2
	13.9	8	0.66 ± 0.03	2.07 ± 0.17*	2.59 ± 0.26*	111.4 ± 10.5
6	2.2	4	0.64 ± 0.02	0.66 ± 0.06	0.46 ± 0.06	26.1 ± 2.4
	13.9	6	0.57 ± 0.06	2.29 ± 0.12*	2.96 ± 0.24*	126.5 ± 9.5
9	2.2	8	0.71 ± 0.04	1.02 ± 0.06*	0.86 ± 0.04*	40.3 ± 2.0†
	13.9	16	0.71 ± 0.05	2.17 ± 0.12*	2.98 ± 0.17*	126.1 ± 6.7
18	2.2	6	0.68 ± 0.03	0.86 ± 0.03*	0.90 ± 0.02*	40.2 ± 0.9†
	13.9	6	0.65 ± 0.02	2.45 ± 0.12*	3.56 ± 0.19*	149.0 ± 7.8

Significant difference between prestimulatory phase and early or late phases is indicated by *P < 0.001.
† Significantly different from the total release obtained with 2.2 mM glucose alone (unpaired *t* test, P < 0.001)
n = number of perifusions.

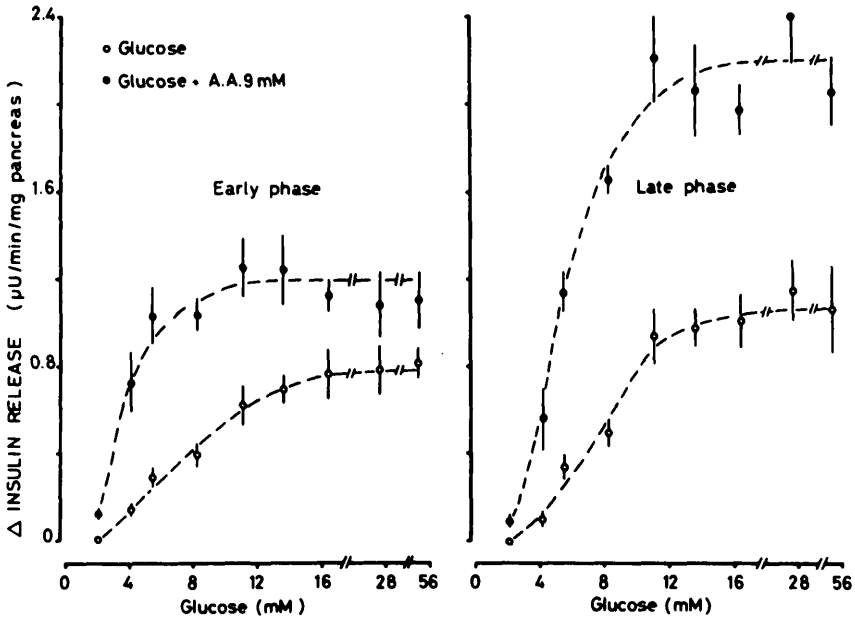

FIGURE 3. Dose-response curves for the early (0–10 min) and late (10–45 min) phases of insulin release induced by glucose (○) or glucose and 9 mM mix. A.A. (●). The values represent $\bar{x} \pm$ SEM of the secretion rates of insulin after subtraction of the basal secretion. Number of values ranged from 4 to 12, and all results were statistically different from the values at 2.2 mM glucose ($P < 0.001$).

The threshold for glucose-induced insulin release was near 4 mM and maximum stimulation occurred at about 12–16 mM glucose for both early and late phases. The addition of the mix. A.A. increased the insulin secretion rate obtained with glucose alone.

As proposed by Kobayashi et al.,[20] Hill's equation was applied to calculate the affinity or activation constant (K_A) of the fetal pancreas to glucose alone or with the mix. A.A. The K_A for the early and late phases of insulin release were, respectively, 7.2 mM and 7.9 mM with glucose; they were 4.0 mM and 5.9 mM when mix. A.A. was added to glucose.

Effects of theophylline on the insulin response to glucose or mix. A.A. The insulin release induced by 13.9 mM glucose was enhanced by the addition of 5 mM theophylline to the stimulation medium at all fetal ages studied (Figure 4). On day 17.5, theophylline increased only the early phase of insulin secretion using 13.9 mM glucose; later, it enhanced the total amount of insulin released an average of 65%.

On day 21.5, 5 mM theophylline with 2.2 mM glucose increased slightly the two phases of insulin release when compared with the secretion rate during the prestimulatory phase for each perifusion (paired t test, $P < 0.05$) (Table 3). However, the overall secretion during the 45 min of stimulation was not statistically different from that obtained with 2.2 mM glucose alone: 33.8 ± 2.8 μU/45 min/mg (n = 4) v.s. 27.4 ± 1.1 μU/45 min/mg (n = 4); unpaired t test $p < 0.05$.

At 2.2 mM glucose, theophylline potentiated markedly the mix. A.A.–induced insulin release (Table 3). At 13.9 mM glucose, theophylline did not enhance further the effect of mix. A.A. on insulin release.

DISCUSSION

The present results indicate that glucose is able to stimulate insulin release by the fetal pancreas in vitro during the last 5 days of gestation in the rat. The response of the pancreas of the term fetus to glucose is qualitatively similar to that reported with adult pancreas using the same perifusion procedure,[18] with a typical biphasic pattern. The main differ-

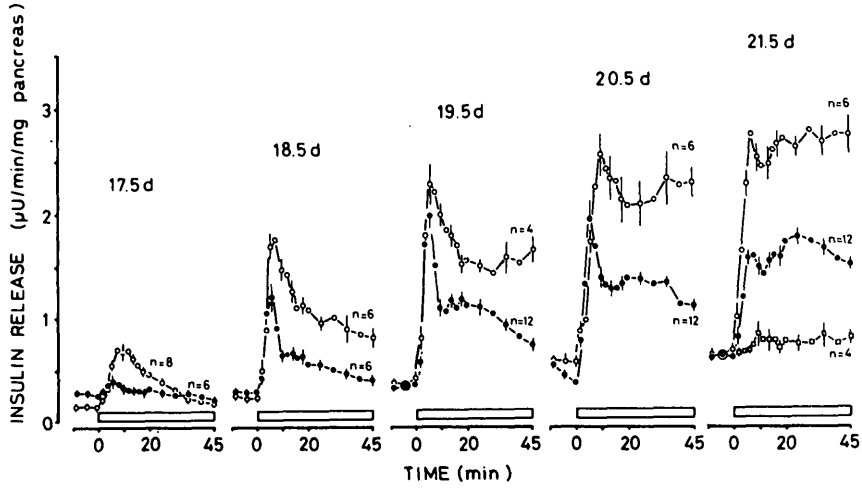

FIGURE 4. Perifusion of fetal rat pancreas from 17.5 to 21.5 days of gestation. Effect of 5 mM theophylline with 13.9 mM glucose (○) on insulin release. The effect of 5 mM theophylline with 2.2 mM glucose is reported on day 21.5 only (□). The insulin response with 13.9 mM glucose alone is presented for comparison (●). Results are $\bar{x} \pm$ SEM of the number (n) of perifusions. The white bars represent the time of stimulation.

TABLE 3
Insulin release by perifused 21.5-day-old fetal rat pancreas. Effects of mix. A.A. and (or) theophylline at 2.2 and 13.9 mM glucose during the 45 min stimulation period.

Glucose (mM)	Mix. A.A. (mM)	Theophylline (mM)	n	Insulin secretion rate (μU/min/mg wet pancreas) Prestimulatory phase (t-10–t0)	Early phase (t0–t10)	Late phase (t10–t45)	Total release (μU/45min/mg)
2.2	—	5	4	0.62 ± 0.05	0.70 ± 0.05†	0.77 ± 0.07†	33.8 ± 2.8
2.2	9	—	8	0.71 ± 0.04	1.02 ± 0.06*	0.86 ± 0.04*	40.3 ± 2.0
2.2	9	5	10	0.67 ± 0.03	1.51 ± 0.07*	2.37 ± 0.13*	98.0 ± 5.0
13.9	—	5	6	0.65 ± 0.14	2.01 ± 0.10*	2.66 ± 0.12*	113.0 ± 5.2‡
13.9	9	—	16	0.71 ± 0.05	2.17 ± 0.12*	2.98 ± 0.17*	126.1 ± 6.7§
13.9	9	5	12	0.66 ± 0.03	2.23 ± 0.26*	3.11 ± 0.41*	131.3 ± 17.0‡

Significant differences between prestimulatory and early or late phases are indicated by *$P < 0.001$, †$P < 0.05$.
‡ $P > 0.05$ } Statistical differences from the total release with mix. A.A., theophylline, and 2.2 mM glucose.
§ $P < 0.01$ }
n = number of perifusions.

ence appears in the magnitude of the late phase of insulin secretion which is, in the term fetal pancreas, only 40% that of the adult.

The results confirm and extend those reported from our laboratory both in vivo[1,2,4,5] and in vitro[13,14] and recently by others in vitro.[17,21] The results disagree with earlier studies, in which no response to glucose or only a small early peak of insulin release was observed up to the second postnatal day.[22] We are unable to explain the reasons for such a discrepancy.

Development of the fetal B cell response to glucose. In our study, the first manifestation of the appearance of the glucose level–sensing mechanism is, on day 17.5 and more obviously on day 18.5, an early phase of insulin release in response to 13.9 mM glucose. The magnitude of this first phase remains rather constant from day 19.5 onwards. The late phase increases progressively during the last 4 days of gestation.

It has been reported that, for cultured pancreatic rudiments, the secretory competence was correlated with the accumulation of insulin in B granules, suggesting that packaging and release of the hormone was regulated as a single coordinated mechanism.[21] In our experiments (table 1) as in others,[17,23] the proportion of insulin released in response to glucose, expressed in percent of the pancreatic content, decreases with fetal age. Thus the increasing secretory competence does not merely reflect differences in content.

The adenyl cyclase–cyclic AMP system influences the course of glucose-stimulated insulin release in adult rat islets.[24] It has been suggested that the low effectiveness of glucose in the fetus compared with that in the adult might be caused by an inadequate intracellular concentration of cyclic AMP.[9,12,23] However, no difference was observed in cyclic AMP concentration and in phosphodiesterase activity of pancreatic islets from 21-day-old rat fetuses and their mothers.[25] We found that theophylline potentiates the total insulin release elicited by glucose from days 18.5 to 21.5 of gestation by an average of 65%; this is in agreement with the observations of others in the adult rat.[24,26,27] Thus, maturation of the insulin response through qualitative changes in the adenyl cyclase–cyclic AMP system is unlikely.

Glucose metabolism in the adult islets correlates with the amount of insulin released during the late phase.[28] It has been suggested that transition from an immature to an adult insulin response to glucose is parallel to the appearance of a hexokinase with a high K_m value for glucose.[29,30] However, no change in the ability of the B cells to phosphorylate and oxidize the extracellular glucose has been reported between islets from 1-day-old rats and older animals.[31] In the present study, the glucose-insulin dose-response curves are similar to those reported in the newborn[32] or in the adult rat[20,33] with a characteristic sigmoid shape, the same threshold values, and the same levels of glucose that elicited a half maximal response and maximal secretion. In the fetus, the K_As for the early and late phases of insulin release are similar. A same K_A for the two phases was found in the adult of different species and suggests that the action of glucose on insulin secretion is uniform with time.[34]

Our results indicate that the main features of the glucose level–sensing mechanisms are not different in the term fetus and in the adult rats. The transition from the fetal to adult type of insulin release in response to glucose more likely parallels quantitative rather than qualitative changes within the B cells.

Modulation of B cell response to glucose by amino acids. The working hypothesis of the study was that the high level of plasma amino acids in the fetal rat[15,16] might be a potentiator of the glucose-stimulated plasma insulin increase in vivo. Our results show that, at physiologic concentrations, a mixture of 12 amino acids enhanced both phases of glucose-induced insulin secretion in vitro at all ages studied. On day 21.5 of gestation, a near maximal potentiation of the mix. A.A. was observed at 6 mM. This result agrees with those reported in the adult rat with a mixture of 20 amino acids.[35] Arginine in the adult rat[35,36] enhanced glucose-stimulated insulin secretion over a wider range of concentration. The difference may be due to positive cooperativity among the amino acids in the mixture, as already reported.[32,37]

The action of the mix. A.A. on the insulin release by the pancreas of the term fetus depends on the glucose concentration of the perifusion medium. At high glucose (13.9 mM), it increases the maximal response (Vm) for both phases of insulin release and apparently shifts the dose-response curves to the left, with a decrease of the half maximal response (K_A). In isolated islets of adult rat incubated in vitro,

the phosphodiesterase inhibitor IBMX similarly increased Vm and decreased K_A of the dose-response curves.[24] On day 21.5 of gestation, the 9 mM mix. A.A. or 5 mM theophylline potentiated the insulin response to 13.9 mM glucose by the same order of magnitude (Table 3). Furthermore, theophylline does not enhance the release of insulin induced by the combination of 13.9 mM glucose and 9 mM mix. A.A. Taken together, the data suggest that amino acids may interact with the adenylate cyclase–cAMP system to potentiate the effect of high glucose on insulin release.

At low glucose concentration (2.2 mM), the 9 mM mix. A.A. or 5 mM theophylline produces little if any sustained insulin release. In this respect, they may be considered as potentiators of glucose on insulin secretion. However, when associated with theophylline, the mix. A.A. induces a biphasic insulin release by the 21.5-day-old fetal pancreas (Table 3). We may speculate that, under these conditions, the mix. A.A. increases insulin secretion by stimulating some metabolic pathway within the B cell. In this way, it was observed that, at a glucose concentration below 8 mM, the utilization of glucose by pancreatic islets from an adult rat was increased by a mixture of 16 amino acids at a physiologic concentration.[38] Alternatively, the metabolism of amino acids in the B cell[39] may be quantitatively large enough to trigger insulin release. In any case the nature of this mix. A.A.–stimulated pathway is as yet unknown.

From a physiologic point of view the increased sensitivity of the B cells to glucose, induced by amino acids, may provide the basis of the growth-promoting action of insulin during fetal life. The high circulating levels of amino acids in the fetus can allow the modulation of insulin release by small changes of the blood glucose.

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