A Protein-Restricted Diet during Pregnancy Alters in Vitro Insulin Secretion from Islets of Fetal Wistar Rats 1,2

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ABSTRACT Previous studies indicate that insulin secretion from the fetuses of dams fed a low protein (LP) diet is reduced in response to leucine or arginine. The aim of this study was to locate the defect in the insulin secretion pathway induced by a LP diet during gestation. The effects of various secretagogues acting at different levels of the insulin secretion cascade were investigated in vitro in fetal islets from dams fed either a normal or a LP diet during pregnancy. Insulin content, insulin secretion and the cAMP content were then measured. Although insulin content of LP islets did not differ from that of control islets, insulin secretion from LP fetal islets was reduced when challenged by amino acids or cAMP enhancers. This reduction did not appear to be related solely to an altered islet cAMP content. An impairment of insulin secretion remained after stimulation of fetal LP islets with either metabolic or nonmetabolic secretagogues. The insulin secretion by LP islets was restored to normal, however, with barium or cytochalasin-B. These findings demonstrate that an in utero isocaloric LP diet impairs insulin secretion of the fetus. This alteration is located at the exocytosis step in the insulin secretion cascade and not in the insulin pool of the β cell. J. Nutr. 131: 1555–1559, 2001.

KEY WORDS: protein malnutrition • glucose • amino acids • pregnancy • insulin • cAMP • rats

The appropriate maternal metabolic environment is essential for the development of the fetus, including its endocrine pancreas. Indeed, when the former is disturbed such as in diabetic pregnancy, many changes occur in the structure and function of the fetal endocrine pancreas. Fetuses from mildly diabetic dams feature hypertrophy and hyperplasia of the islets (1), leading to a higher insulin content (2). The in vivo and in vitro proliferative capacity of these islets is increased (3,4). Such islets are more sensitive to glucose stimulation than normal fetal islets (2,5). In situations of severe maternal diabetes, fetal β cells are degranulated (2) and their pancreatic insulin content is lower (4). In response to glucose perfusion, these islets secrete less insulin (5).

Major perturbation of the metabolic environment also occurs when the dams are fed a low protein (LP) diet during gestation. When an isocaloric LP diet containing 8% instead of 20% protein is given to dams from d10 of pregnancy and maintained throughout gestation, the glycemia of dams and fetuses remains normal, whereas the amino acid profile in their serum is altered (6). LP fetuses and newborns are smaller, and the development of their endocrine pancreas is impaired. Islet cell multiplication in vivo, islet size and pancreatic insulin content are decreased compared with pancreata of control fetuses. Moreover, these LP islets are less vascularized (7). These islet alterations observed in vivo are associated with a decreased insulin release in response to leucine and arginine in vitro. In adulthood, insulin secretion remains lower in these rats even when weaned onto a normal diet (8).

The importance of insulin for fetal growth throughout late gestation is well established (9). In humans, specific fetal hypoinsulinemia is associated with intrauterine growth retardation (10). For normal insulin-dependent fetal growth, insulin must then be produced by the fetal pancreatic β cells in an appropriate quality and quantity, which is not the case in protein-restricted fetuses (10).

Previous experiments could not attribute the deficiency in insulin release to a lower pool of insulin, a reduced secretory capacity or a lower sensitivity to secretagogues. This paper attempts to address this question by triggering different steps leading to insulin release. Therefore, metabolic (glucose, leucine, glutamine or α-ketoisocaproate) or nonmetabolic (high extracellular potassium concentration, acetylcholine, tolbutamide, barium chloride, cytochalasin-B) secretagogues were used to analyze specific changes in intracellular pathways leading to insulin secretion. Cyclic AMP, which is a major second messenger in the amplification of the stimulus secretion coupling in the β cell (11), was also investigated.

The mechanistic basis of the secretory defect in LP islets is not known. The aim of the present study was to investigate the


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4 Abbreviations used: C, control; Ig, immunoglobulin; KIC, α-ketoisocaproate; LI, labeling index; LP, low protein diet; TPA, 5-tetradecanoyl-13-phorbol acetate.
site of this defect. This was achieved using a range of metabolic and nonmetabolic secretagogues, which act at different steps of the insulin secretory pathway.

**MATERIALS AND METHODS**

**Animals and diets.** Control fetuses (C) were from Wistar rats fed 20% protein diet during gestation; the LP fetuses were from dams fed an isocaloric, low protein diet (8%). Virgin female rats, 110 d old and weighing 200 g, belonging to a local stock bred at the Animal Center of the Université Catholique de Louvain, Belgium, were caged overnight with males. The upper limit of the pregnancy was determined in the morning by detection of a vaginal plug. Midnight was considered as the time of mating on d 0 of gestation. Pregnant females were then housed singly under controlled conditions of light (14 h light, 10 h darkness), temperature (24°C), and humidity (60%). The rats were then given free access to their respective diets and to water. They were divided randomly into two groups starting from d 1 of gestation. After 21.5 d of gestation, dams and their fetuses were killed by decapitation.

**Islet isolation.** Rat islets were prepared from 200 g rats (300 g; Charles River, L’Arbresle, France) of either sex at 21.5 d of gestation. The islets were isolated as described elsewhere (2). The rats were fasted for 18 h before sacrifice. Immediately after decapitation, the pancreata were removed aseptically and placed in cold Hanks medium. They were minced and transferred into a sterile tube containing 2 mL of medium with 5 mg collagenase (Boehringer, Mannheim, Germany). The tubes were shaken by hand at 37°C for 7–8 min and the digestion was stopped by adding cold medium. The tissue digest was then washed twice with RPMI medium. Thereafter, the resulting cell pellets were suspended in 20 mL of medium and gently stirred at room temperature for 60 min. After centrifugation (180 × g for 10 min), the pellets were resuspended in medium (1 pancreas/2 mL RPMI 1640 medium). This suspension was distributed into 35-mm Petri dishes (2 mL/dish; Falcon Plastics, Los Angeles, CA). The culture dishes were incubated for up to 7 d at 37°C in a humidified atmosphere of 5% CO2/95% air. By d 7, the islets were detached from the surrounding connective tissue network and the purity of the islet preparation was confirmed by microscopic observation of the digestion of the pancreatic tissue.

**Incubation of cultured islets.** RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum, 11 mmol/L glucose and antibiotics (penicillin 0.2 × 10^7 U/L, streptomycin 0.2 g/L) was used during the entire culture process, including the digestion of the pancreatic tissue. The culture medium was replaced every 24 h and stored at 20°C for insulin secretion.

**Fetal islet cell proliferation.** On d 7 of culture, tritiated thymidine (Amersham, Arlington Heights, IL) was added to the Petri dishes (1 mCi/L). After 24 h, the dishes were washed with RPMI 1640 medium containing excess of nonradioactive thymidine. The islets were fixed in Bouin Allen solution, embedded in paraffin and serially sectioned at a thickness of 7 μm. The slides were covered with L4-Ilford nuclear track emulsion, exposed for 18 d at 4°C and revealed with Kodak D 19 developer. The labeling index (LI) was calculated by dividing the number of labeled islet cell nuclei by the total number of islet cell nuclei. The LI was expressed as a percentage.

**Insulin secretion.** Batches of 10 neofomed fetal islets were incubated in Krebs-Ringer medium with different secre-
tages. C and LP islets had insulin contents that did not differ [4.9 ± 0.2 pmol/islet (n = 164) and 4.7 ± 0.2 pmol/islet (n = 139), respectively]. In response to either 5.6 or 16.7 mmol/L glucose, the fractional insulin release was significantly lower in the LP group than in controls (P < 0.05) (Fig. 1). In the presence of 5.6 mmol/L glucose with 10 mmol/L leucine or arginine, fetal islets from both groups exhibited increased insulin secretion (P < 0.01), but it remained significantly lower in the LP islets than in the C group (P < 0.01) (Fig. 1). In both groups, the inclusion of theophylline, a methylxanthine inhibitor of phosphodiesterase, with glucose and leucine in the incubation medium induced a significant potentiation of the insulin release (P < 0.05). However, the secretion was lower in the LP group compared with the C group (P < 0.01) (Fig. 1). Arginine added to glucose also induced a lower insulin secretion compared with controls. LP islets secreted less insulin in response to all of these nonmetabolic secretagogues tested except for barium, for which the insulin secretion was not significantly different. When cytochalasin-B (Fig. 5) was added to the incubation medium containing either glucose (16.7 mmol/L) or leucine and glutamine, insulin was secreted by the fetal LP islets and reached values not different from those of controls.

cAMP content. Islet cAMP content estimated per pmol of islet insulin was not different in low and high concentrations of glucose, in either the LP or the C group (Table 1). However, cAMP content was significantly lower in the LP group at both concentrations of glucose (5.6 or 16.7 mmol/L) (P < 0.01). This was also true in response to leucine, but not to arginine added to glucose. Moreover, in both groups, cAMP content was enhanced in the presence of pharmacologic agents such as theophylline, forskolin or TPA, and reached values that did not differ between the two groups, notwithstanding alterations in insulin secretion (Fig. 2). The greatest effect on cAMP content was observed in the presence of forskolin.

Proliferation of fetal islets. As analyzed by the labeling index, the cell proliferation in 19.34 islets from 6 cultures was...
DISCUSSION

This study was designed to investigate the cellular basis of the secretory defect present in islets from fetuses deprived of maternal protein.

The insulin content per islet was similar in the C and LP groups, suggesting that the reduced insulin release was not attributable to a decreased available pool of insulin but to alterations in the insulin secretion pathways. To test the metabolic disturbance in insulin secretion of fetal LP β cells, secretory effects of glucose, glutamine, leucine and KIC were investigated. Glucose has to be metabolized in the cytosol as well as the mitochondria, whereas leucine and KIC require only mitochondrial metabolism to stimulate insulin release. In response to glucose, albeit a poor stimulus in the normal fetus (14,15), fetal LP islets exhibited a lower insulin secretion than control islets. A similar reduction was observed when fetal LP islets were stimulated by leucine or KIC. In another study (16), we found that insulin release stimulated by nutrient secretagogues was altered in the islets of adult rat offspring of dams fed the low protein diet during pregnancy and lactation and maintained on the LP diet throughout life. In parallel, the activity of the mitochondrial FAD-dependent glycerophosphate dehydrogenase was diminished in these islets, and coincided with an alteration in the oxidative metabolism of glucose. Moreover, L-leucine transamination to KIC was impaired in these rats (17). These alterations, which are evident in adults, may also be present during fetal life and contribute to the deficient insulin secretion by the LP fetuses.

Because metabolic secretagogues were unable to restore to normal the lower insulin secretion from LP fetal islets, the secretory effect of arginine was then tested on C and LP islets. Arginine is weakly metabolized in islet cells differently from glucose and leucine; this amino acid is thought to depolarize the β cell membrane because of its transport into the cell in a positively charged form (19). It activates voltage-dependent Ca2+ channels, and Ca2+ influx ensues. Insulin secretion by fetal LP islets remained reduced in response to arginine. This indicates that there is a defect in the insulin secretory pathway, which is independent of metabolic processes.

Ca2+ influx into β cells is a key step in the insulin secretory pathway. To investigate the involvement of Ca2+ in the altered insulin secretion of LP islets, KCl, tolbutamide, acetylcholine and Ba were used. KCl and tolbutamide induced insulin release by increasing the rate of entry of Ca2+ into the β cell. Acetylcholine was added to increase cytosolic Ca2+ by releasing Ca2+ from intracellular stores in addition to the increase of Ca2+ influx through voltage-dependent Ca2+ channels (20). In response to the two kinds of stimulation, insulin was secreted by the C as well as by the LP fetal islets, but the response of LP islets remained reduced compared with controls. In substituting Ca2+ with Ba2+, which permeates membranes through Ca2+ channels (21) and triggers directly exocytosis of insulin without any contribution of Ca2+ (22), insulin was secreted by C and LP fetal islets and was not different in the two groups. This suggests that in fetal LP β cells, defects in Ca2+ movements and/or activity of exocytotic enzymes including kinase proteins, which are Ca2+ dependent, are implicated in their reduced insulin secretion.

In this study, we detected a low cAMP content in the LP islets when stimulated with glucose and leucine. This might

![FIGURE 4](https://example.com/fig4.png) Fractional insulin release by cultured fetal islets from dams fed 20% (C) or 8% protein (LP) diets throughout gestation. Islets were incubated in Krebs-Ringer medium containing cytochalasin-B (Cyt; 10 μmol/L) added to glucose at 16.7 mmol/L (G16.7) or to leucine and glutamine (Leu + Gln; 10 mmol/L). Values are means ± SEM, n = 9 observations. **P < 0.01, *P < 0.05 vs. C.

![FIGURE 5](https://example.com/fig5.png) Fractional insulin release by cultured fetal islets from dams fed 20% (C) or 8% protein (LP) diets throughout gestation. Islets were incubated in Krebs-Ringer medium containing cytochalasin-B (Cyt; 10 μmol/L) added to glucose at 16.7 mmol/L (G16.7) or to leucine and glutamine (Leu + Gln; 10 μmol/L). Values are means ± SEM, n = 9 observations. **P < 0.01, *P < 0.05 vs. C.

### Table 1

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<th>cAMP content of cultured fetal islets from dams fed 20% (C) or 8% protein (LP) diets throughout gestation1,2</th>
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1 Values are means ± SEM, n = 6. ** P < 0.01 vs. C.
2 The islets were incubated in Krebs-Ringer medium containing various secretagogues, glucose (5.6 mmol/L, G 5.6 or 16.7 mmol/L, G 16.7), leucine (Leu; 10 mmol/L), arginine (Arg; 10 mmol/L), theophylline (The; 2 mmol/L), forskolin (For; 1 μmol/L) and 5-tetradecanoyl-13-phorbol acetate (TPA; 25 mmol/L).
also explain in part their reduced insulin release. However, the level of cAMP in the LP islets in response to arginine, theophylline, forskolin and TPA was normal and was not associated with a recuperation of a normal insulin secretion. This implies that defects in the insulin secretion in the LP group were due to alterations in insulin secretory pathway at steps downstream from cAMP production.

Because none of the secretagogues tested were able to restore insulin secretion from LP fetal islets to normal, alterations in the exocytotic process were analyzed. Fetal islets were then stimulated by cytochalasin-B, which acts by reversibly changing the β cell microfilamentous cell web involved in the last step of the insulin secretion cascade (23). This fungal metabolite similarly increased insulin secretion in the two groups. These findings indicate that the readily releasable pool of insulin is not reduced in size and that a main alteration site of insulin secretion of the LP islets is also situated at the level of exocytotic process.

The persistent reduction of both the insulin secretion and replication of fetal β cells in the LP group in vitro, even when they have been removed from the maternal environment and cultured for 1 wk in RPMI medium, shows that the protein-restricted diet of dams during pregnancy induces a lasting impairment of the sensitivity of the fetal β cells to secretagogues as well as to mitogenic stimuli. The secretory and proliferative activities of the fetal β cell are stimulated by amino acids whose effects are known at 14.5 d of gestation. Indeed, enrichment of the culture medium with essential amino acids results in a higher fetal β cell replication, an increased insulin β cell content as well as a higher basal and glucose-stimulated insulin release (24,25). Because of decreased concentrations of essential and branched amino acids in the serum of LP fetuses and dams (6), we suggest that the lower sensitivity to amino acids observed in vitro might be due to the low availability of amino acids in the fetomaternal unit. Low levels of taurine could also be involved because, when added into the culture medium, this amino acid enhances the insulin secretion by fetal islets in response to secretagogues (26) and restores the reduced insulin secretion by fetal LP islets when added during gestation to the drinking water of LP dams (27).

In conclusion, our findings highlight the crucial role of a normal protein diet during pregnancy to ensure a normal insulin secretory responsiveness in the progeny. The alteration site of the insulin secretion by fetal islets from dams fed a low protein diet might consist of alterations in Ca2+ fluxes into β cells or further events of the exocytotic process of insulin secretion.

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LITERATURE CITED