8 Programming of Diabetes: Experimental Models

BRIGITTE REUSENS, LUISE KALBE AND CLAUDE REMACLE

Laboratoire de Biologie Cellulaire, Catholoc University of Louvain, Place Croix du Sud 5, B-1348, Louvain-la-Neuve, Belgium

Introduction

The mechanism by which fetal malnutrition increases the risk for glucose intolerance and type 2 diabetes is not well understood. Besides the insulin resistance that has been proposed to develop as a consequence of the lack of nutrient availability during early life, a primary insult in β-cell development has also been observed. Fetal malnutrition would lead to inappropriate β-cell ontogeny, resulting in a population of β-cells that does not cope adequately with metabolic or oxidative stress later in life. Since, obviously, this hypothesis can not be verified in humans, various animal models have been established.

The time course of development is different from one species to another. For instance, humans and guinea-pigs are born more mature than rats or mice. The same is true at the level of the endocrine pancreas. In humans, the initial formation of the endocrine cells in the pancreas occurs at 10 weeks' gestation (Bouwens *et al*., 1997). In the guinea-pig, islet formation and remodelling also begin at an early stage of gestation and continue for a longer time after birth. In rodents, the islets develop relatively late in gestation and undergo a remodelling 2 weeks after birth. This means that the critical window for islet development is narrower in rodents than in other species. This could be an advantage for the identification of mechanisms involved when the consequences of nutritional or other experimental manipulations are analysed. However, results from such studies should be viewed with caution until confirmed in other species, before extrapolating them to humans

Development of the Endocrine Pancreas in the Rat

Endocrine cells of the pancreas are organized in highly vascularized and innervated microorgans distributed throughout the exocrine tissue. These

 Fetal Nutrition and Adult Disease: Programming of Chronic Disease through Fetal Exposure to Undernutrition (ed. S.C. Langley-Evans)

microorgans, called 'islets of Langerhans', contain four endocrine cell types and represent only 1% of the pancreatic tissue at adulthood: α-cells produce glucagon, β-cells produce insulin, δ-cells produce somatostatin and PP-cells produce pancreatic polypeptide. The most abundant cell is the β-cell, which represents 60–80% of the islet cell population. The development of the pancreas is a fascinating event, starting from a pool of common progenitor cells (multipotent endodermal progenitors) which will be committed into the endocrine or exocrine cell lineage or duct cell. Then, within the endocrine compartment, the cells will have to further differentiate into α-, β-, δ- or PP-cells. This is regulated by the expression of distinct genes, under the control of a hierarchy of various and specific networks of transcription factors. It is thus obvious that any disturbance in the environment of the future endocrine cell, for instance by intrauterine malnutrition or other injuries, will alter the relative participation of factors involved in this network, and may drive the β-cell mass into the corner, contributing to β-cell failure and diabetes later in life.

Figure 8.1 illustrates the different steps of pancreas development The pancreas develops from two anlagen of the primitive gut, which fuse to form both the exocrine and endocrine pancreas. In the mouse and rat, the dorsal pancreatic bud appears at embryonic day (E) 9.5 closely followed by the ventral bud. The buds fuse together at E16.5. A branched structure is distinguishable at E14.5 and the endocrine cells can be identified by E15.5. The endocrine tissue is derived from epithelial duct cells by rotation of the polarity of the mitotic axis. These cells will divide to form small clusters budding out the pancreatic ducts. These immature endocrine cell clusters become vascularized and coexpress several pancreatic hormones and neuropeptides, eventually becoming 'islets of Langerhans' (Reusens *et al*., 2000).

One of the important components of the early specification of the pancreatic programme within the region of the gut endoderm involves the exclusion of the expression of the hedgehog gene family (*Shh* and *Ihh*). The *hh* signalling molecules are expressed throughout the primitive gut endoderm except in the regions destined to become the pancreas (endocrine and exocrine). In other words, the *hh* molecules promote the intestinal differentiation but impair pancreatic development. The classical way of specifying a particular cell fate within the field of initially equivalent cells is the lateral specification mediated by the Notch pathway. Notch signalling involves the cell expressing high quantities of ligands (Delta or Serrate) that signal to activate Notch receptors on the neighbouring cells in which the activated intracellular Notch receptors (IC) suppress the primitive cell fate (Edlund, 2001). In the Notch-signalling pathway, IC interacts with the DNA-binding protein RBP-Jx to activate expression of repressor genes such as *hes* genes that, in turn, repress expression of other genes that otherwise will promote the primary fate. Notch signalling controls the choice between differentiated endocrine and progenitor cell fate in the developing pancreas. Blocking the activation of Notch receptor results in high Neurogenin-3 gene (*ngn3*) expression, and promotes the endocrine fate. Neurogenin-3 downstream of the endoderm factors will specify which cells in the pancreatic epithelium will differentiate into endocrine cells and will initiate the differentiation programme. In contrast, cells with active Notch signalling adopt the exocrine fate and/or

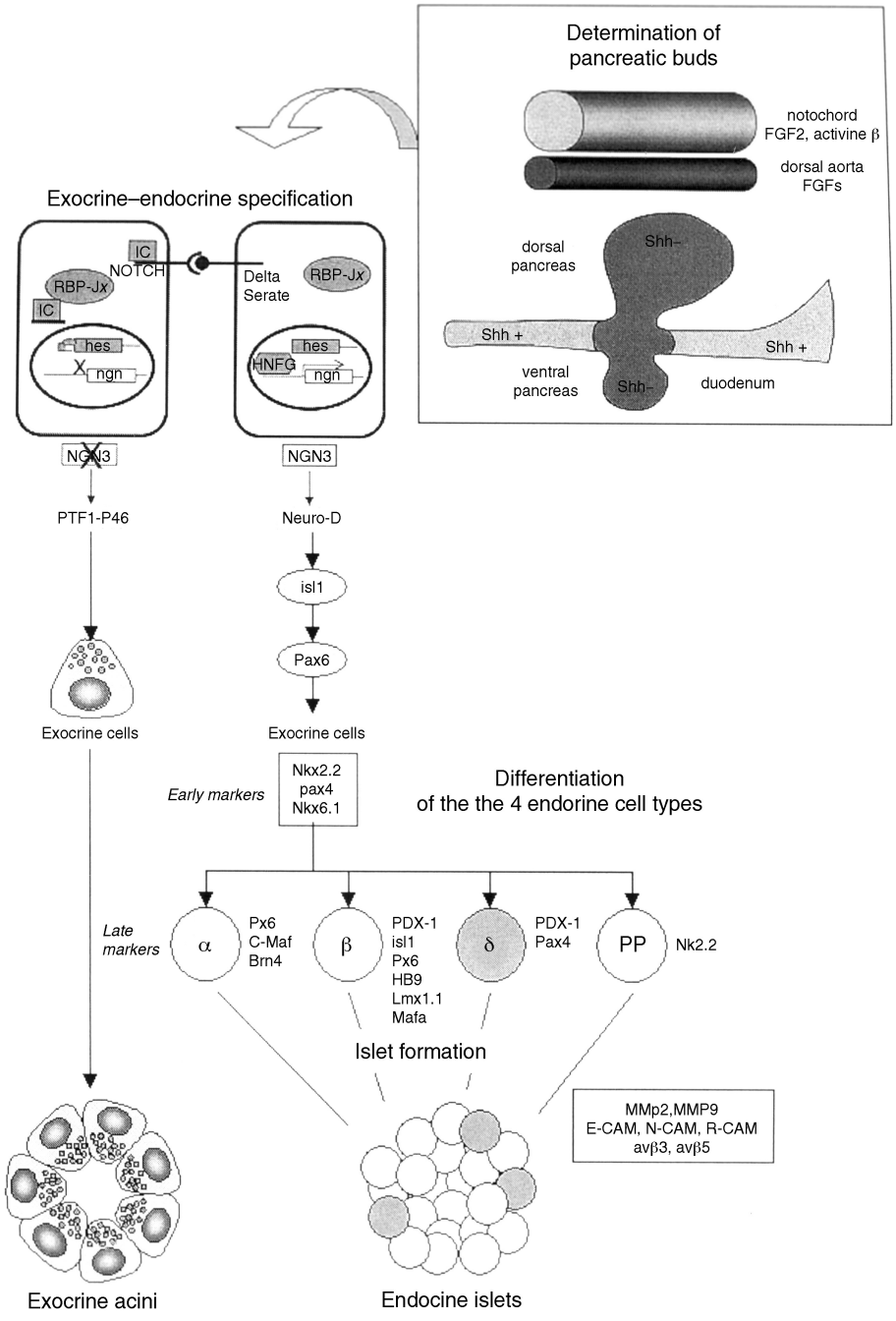

Fig. 8.1. Schematic representation of the different steps of the development of the pancreas (adapted from Grapin-Botton *et al.*, 2001; Wilson *et al.*, 2003). See text for explanation.

remain as undifferentiated progenitor cells (Edlund, 2001). As stated before, the endocrine cells delaminate from the epithelium upon differentiation and migrate into the mesenchyme where they cluster. This migration is likely to result in a decrease in the Notch signal among the progenitor cells and will allow the formation of the islets.

The final fate of the individual endocrine cells is determined by the expression of a series of transcription factors specific for each type of endocrine cell. Some of them are early markers, such as Pax4, Nk2.2 and Nk6.1, co-expressed with neurogenin-3, whereas others are late markers, such as Px6, isl1, HB9 and PDX-1. The transcription factor network is highly complex and several factors involved are expressed more than once during the differentiation process and play more than one role. One of the key players is the homeodomain transcription factor PDX-1, which is not only important in the regulation of insulin gene expression, but is also required for the differentiation of the mature pancreas. At embryonic day 8.5 in the mouse (1 day before the appearance of the dorsal pancreatic bud), cells of the endoderm are already committed to the pancreatic fate (Reusens *et al*., 2000) and will give rise to all pancreatic tissues. These cells already express *Pdx-1*, also known as *Sft-1*, *Idx-1* or *Ipf-1* (Sandler and German, 1997), and *hb9* (Wilson *et al*., 2003) under the control of signals coming from the surrounding tissues. Cells expressing *Pdx-1* between E9.5 and E11.5 give rise to pancreatic duct, endocrine islets and exocrine acinar cell. Cells expressing *Pdx-1* at E8.5, or after E11.5, only give rise to exocrine acini or endocrine islets. Stated otherwise, the duct progenitors express detectable PDX-1 only around E10.5, while the endocrine and exocrine progenitors express PDX-1 throughout embryogenesis. The lineage for pancreatic duct and the rest of the pancreas must thus separate before E12.5 (Gu *et al*., 2003). Expression of *Pdx-1* in undifferentiated ductal epithelium is associated with the glucose transporter GLUT 2. By day 15 in the rat, both remain in the developing β-cell but are lost in the acinar cell.

Early morphogenetic signalling in the formation of the pancreas may depend on the interplay between peptide growth factors and tissue transcription factors, an interaction which is apparent throughout islet formation. Indeed, it was found in chick and mouse embryo, that activin-βB (a member of the transforming growth factor family (TGF)) and fibroblast growth factor-2 are notochord factors that can repress endodermal *Shh*, permitting expression of pancreas genes, including *Pdx-1* and *insulin* (Hebrok *et al*., 1998). Other growth factors, such as platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF)-7, which are expressed within the pancreatic stroma adjacent to the ductal epithelium, may also intervene. The ongoing proliferation and developmental differentiation of β-cells, once formed, is highly dependent on the expression of the insulin-like growth factors (IGFs) within the islets, as we will see below.

β-Cell Function and Insulin Action

In adults, β-cells store insulin and C-peptide within their granules. After stimulation by glucose uptake via the low-affinity glucose transporter GLUT 2, glucose

enters the cell, is phosphorylated by glucokinase, and metabolized, leading to a rise in the ATP/ADP ratio. This increase induces a blockage of the K^+ ATP-dependent channels, which in turn will lead to membrane depolarization and, subsequently, to the activation of the voltage-dependent Ca^{2+} channels. Ca^{2+} will enter into the β-cell and stimulates the release of insulin by exocytosis. In addition to glucose, amino acids have been shown to stimulate insulin release in the absence of glucose, the most potent secretagogues being leucine, arginine and lysine (Fajans and Floyd, 1972). In the fetus, insulin secretion is more sensitive to amino acids than to glucose (de Gasparo *et al*., 1978), its full response to glucose beginning after birth. Fetal pancreas is capable of insulin synthesis and release in response to agents that increase cyclic AMP, activate protein kinase-C or raise calcium levels. In humans, as well as in rats, fetal β-cells show an immature or poor response to nutrients, especially to glucose. In contrast to the adult pancreas, fetal β-cells never display a biphasic pattern (Hughes, 1994).

Insulin promotes the storage of glucose as glycogen in the liver and muscle, the incorporation of amino acids into the muscle proteins and the accumulation of triglycerides in adipose tissues. In muscle and adipose tissue, the action of insulin on glucose uptake is mediated through the translocation of the glucose transporter GLUT4 from the intracellular site to the plasma membrane. To achieve this action, insulin binds to the α- and β-receptors (type α predominates during development, while type β predominates in adulthood). These receptors are composed of an extracellular ligand-binding domain that controls the activity of an intracellular tyrosine kinase. Insulin fixation on the α-subunit of the receptor activates the β-subunit kinase activity and induces tyrosine phosphorylation of the insulin receptor substrate (IRS) family (IRS1, 2, 3 and/or 4). IRS-1 and IRS-2 function as scaffold proteins to coordinate separate branches of the insulin cascades (and IGFs). IRS proteins couple insulin to the phosphatidylinositol (PI) 3-kinase and extracellular signal-regulated kinase (ERK) cascades. The phosphorylation of IRS proteins recruits the phosphatidylinositol 3-kinase (PI3K) by its association with the regulatory subunit p85a. PI3K is central in the action of insulin, including the stimulation of glucose uptake and inhibition of lypolysis (Shepherd *et al*., 1998). It is a heterodimereric enzyme, comprising a regulatory subunit (p85) and a catalytic subunit (p110). PI3K activation is an absolute requirement for the insulin-induced translocation of GLUT4, from both the endosomal and GLUT4 storage vesicules, to the plasma membrane. However, subsequent steps are vague, as various serine kinases have been involved, including PKB, PKC-λ and PKC-ξ (White and Myers, 2001).

Early Malnutrition Programmes: The Endocrine Pancreas and its β-cells

In the rat fetus, the β-cell mass increases rapidly at the end of gestation, due to both replication and recruitment of undifferentiated β-cell precursors in the pancreatic duct. Following birth, the growth rate of each islet cell population, including β-cells, declines within 3–4 days. A wave of apoptosis occurs in the neonatal rat islets between 2 and 3 weeks of age (Scaglia *et al*., 1997). Since

the total number of β-cells is not substantially modified during this period of time, this suggests that replication and neogenesis compensate for the loss. It is obvious that the development of the β-cell mass contributes to the accumulation of the islet mass in adulthood. Therefore, any deficiency occurring *in utero* or soon after birth is unlikely to be completely compensated for later in life. Alteration of the β-cell mass by maternal malnutrition in humans had already been demonstrated more than 25 years ago (Winick and Noble, 1966; Weinkove *et al*., 1974), but the mechanisms involved in this alteration were only deciphered when animal models were developed.

The cellular area staining immunopositively for insulin increases twofold over the 2 days before birth in normal rats (Kaung, 1994). A maternal low-protein diet (LP, 8% of protein instead of 20% in the control diet) modifies this process of β-cell expansion. The islet area was reduced by 30–50% between 19.5 and 21.5 days of gestation in fetal offspring from protein-restricted dams (Snoeck *et al*., 1990; Petrik *et al*., 1999; Boujendar *et al*., 2002). This is due partially to a relative deficiency in α-, β- and somatostatin-secreting cells, although the β-cell population was the most severely affected (Petrik *et al*., 1999). As a consequence, the total islet and β-cell mass were depleted at birth. When the protein restriction was maintained until weaning, the deficiency was even more pronounced. Cells traversing the S phase of the replication cycle were investigated after tritiated thymidine or bromodeoxyuridine (BrdU) incorporation in these LP fetal and neonatal islet cells. The replication rate of islet cells was reduced by almost 50% in the malnourished offspring, and preferentially in the β-cells, which is consistent with the reduction in the proliferation of the β-cells in the endocrine cell mass observed at these stages. Further analysis has revealed that the cell cycle was lengthened in the LP islets because more β-cells from LP pups contained cyclin D1 (a marker of G_1 phase) but fewer islet cells contained NIMA-related kinase 2 (NEK2; an indicator of cells in G_2 and mitosis) (Petrik J *et al*., 1999).

A modulation of the β-cell area by the maternal diet might also involve β-cell death. After a low rate of apoptosis in fetal islets, a dramatic increase in apoptosis occurs after birth, with a return to a minimal level by weaning. This was also observed in the malnourished pups. Although the timing of the neonatal wave was not affected by the maternal diet, the rate of islet cell apoptosis was increased in LP-exposed offspring at every age analysed. This suggests that the event is quantitatively dependent upon, but qualitatively independent of, the diet. This is further supported by the fact that the presence of inducible nitric oxide synthase (iNOS), which precedes the peak of apoptosis in normal islets by 2 days (Petrik *et al*., 1998), was not changed in LP islets (Petrik *et al*., 1999).

The timing of the neonatal wave of apoptosis coincides not only with an increase of iNOS but also with the disappearance of the expression of insulin-like growth factor-II (IGF-II) in the endocrine pancreas. IGF-II can act functionally as a growth factor, but also as a survival factor to prevent apoptosis in β-cells (Petrik *et al*., 1998) and in other cell types (Geier *et al*., 1992; Jung *et al*., 1996). We have demonstrated reduced expression of IGF-II mRNA, and a lower number of islet cells positive for the protein, in the pancreas of LP pups. This suggests that the increased apoptosis and the reduced β-cell proliferation seen in islets of LP

rats may be linked functionally to this reduction of IGF-II expression (Petrik *et al*., 1999).

The development of fetal islet cells is dependent on the availability of glucose. However, at 17–18 days of gestation in the rat, amino acids appear to be more important than glucose in stimulating β-cell differentiation and proliferation (de Gasparo *et al*., 1978). In protein-restricted animals, plasma glucose levels were normal but the amino acid profile was perturbed in the feto-maternal unit (Reusens *et al*., 1995). Maternal and fetal total amino acid levels were not perturbed by the maternal diet but several amino acids were significantly decreased. Essential amino acids levels were reduced by 10% in the fetal plasma on the last day of gestation. Taurine, which does not participate in protein synthesis, but which is very important for several tissues during development (Sturman, 1993), was the most affected, being reduced by 30% in the fetal and maternal plasma by protein deficiency. It was shown that amino acids, but not glucose, potentiate the release of immunoreactive IGF-II from isolated fetal rat islets (Hogg *et al*., 1993). Interestingly, as mentioned above, less IGF-II was observed in the pancreas of fetuses that grew in an intrauterine milieu where amino acids were limiting. Supplementation of the drinking water of the LP-fed pregnant rat by 25 g/l of taurine prevented the reduction of the β-cell mass by enhancing β-cell proliferation and decreasing islet cell apoptosis. We demonstrated that this could be achieved partially by the normalization of the number of islet cells expressing IGF-II (Boujendar *et al*., 2002) (Figs 8.2–8.4)

The endocrine pancreas is a richly vascularized tissue. In adult islets, irrigation represents 10% of the total blood flow, while the endocrine mass represents only 1% of the total mass of the pancreas. Pancreatic cell–endothelial cell

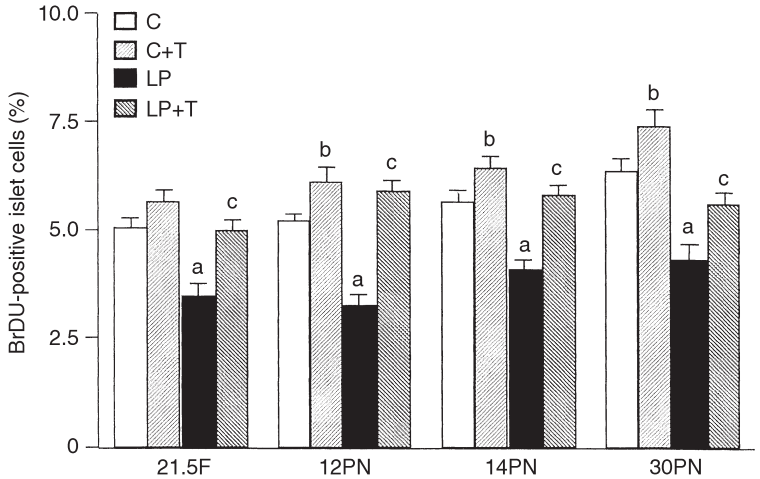

Fig. 8.2. Effect of a maternal low-protein (LP) diet during gestation and lactation on islet cell proliferation of the progeny, and its prevention by taurine (T). F, Fetus; PN, postnatal; BrDU, bromodeoxyuridine. a, $P < 0.05$, LP versus control (C); b, $P < 0.05$, C + T versus C; c, $P < 0.05$, LP + T versus LP. (Reproduced from Boujendar *et al*., Figure 5 in *Diabetologia* (2002) 45, p. 861, Springer-Verlag.)

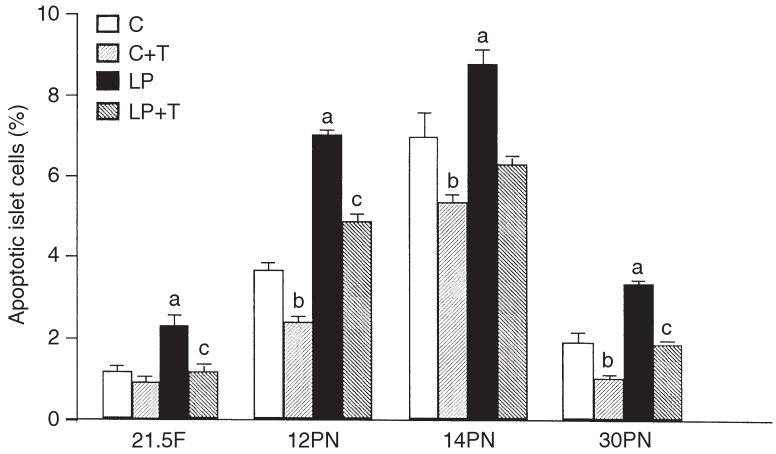

Fig. 8.3. Effect of a maternal low protein (LP) diet during gestation and lactation on the islet cell apoptotic rate of the progeny, and its prevention by taurine (T). F, Fetus; PN, postnatal. a, $P < 0.05$, LP versus control (C); b, $P < 0.05$, C + T versus C; c, $P < 0.05$, LP + T versus LP. (Reproduced from Boujendar *et al.*, Figure 7 in *Diabetologia* (2002) 45, p. 863, Springer-Verlag.)

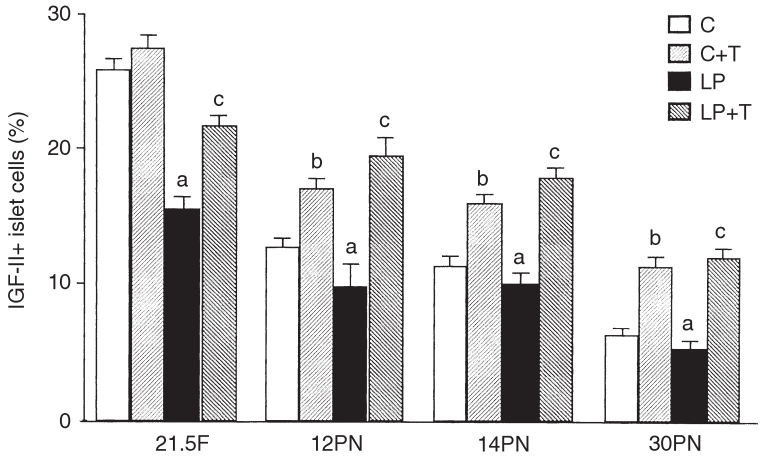

Fig. 8.4. Effect of a maternal low protein (LP) diet during gestation and lactation on the percentage of islet cells positive for insulin-like growth factor-II (IGF-II) in the progeny, and its prevention by taurine (T). F, Fetus; PN, postnatal. a, $P < 0.05$, LP versus control (C); b, $P < 0.05$, C + T versus C; c, $P < 0.05$, LP + T versus LP. (Reproduced from Boujendar *et al.*, Figure 10 in *Diabetologia* (2002) 45, p. 863, Springer-Verlag.)

interactions begin long before islets are functionally mature, and are maintained throughout islet formation. Recently, blood vessels have been shown not only to provide metabolic sustenance, but also inductive signals for the development of endocrine pancreas. Vascular endothelial growth factor (VEGF) is a major factor in these endothelium–endocrine cell interactions (Lammert *et al.*, 2001). At

E8.5–E9.5 of mouse development, pancreatic buds develop precisely where endoderm previously contacted the endothelium of the dorsal aorta and two vitelline veins (Lammert *et al*., 2001, 2003). Thus pancreatic and vascular development cooperate during embryogenesis.

Islet blood vessel development has been shown to be very sensitive to the lack of protein availability *in utero,* as the volume occupied by blood vessels was lower in islets from LP-exposed fetuses, and this was associated with a reduction in the blood vessel number (Snoeck *et al*., 1990; Boujendar *et al*., 2003). The proportion of VEGF-positive cells was reduced in fetal islets from LP rats compared to controls, suggesting that the impaired vascularization could be a consequence of the reduction of VEGF production by the surrounding β-cells, since VEGF stimulates the growth of the endothelial cells. VEGF could play a role in β-cell differentiation from duct precursor cells because its receptor, Flk-1, is found on these cells and VEGF is able to stimulate their proliferation (Oberg *et al*., 1994; Oberg-Welsh *et al*., 1997). In parallel, we investigated the VEGF receptor, Flk-1, in the pancreas of the LP offspring. We found that the percentage of cells immunopositive for Flk-1 was decreased in the islets, while it was increased in the duct cells. Maternal protein deprivation thus induces an up-regulation of the Flk-1 expression in duct cells as a reaction to the decreased endocrine cell mass. However, such an up-regulation seemed inefficient to maintain a normal β-cell mass, probably because the level of VEGF was too low (Boujendar *et al*., 2003).

Islet vascularization is also sensitive to the depletion of taurine that occurs in the LP fetus, since addition of this amino acid to the LP diet of the dam was sufficient to restore a well-vascularized endocrine pancreas in the progeny. Taurine supplementation of the LP dams led to a normalization of the number of the blood vessels, and the proportion of cells positive for VEGF and its receptor in islets. However, it is again unclear whether the prevention of the alteration of the vascular system by taurine in the LP islets is the cause, or the consequence, of the restoration of the β-cell mass by this specific amino acid (Boujendar *et al*., 2003).

As mentioned previously, the transcription factor, pancreatic and duodenal homeobox 1 (PDX-1), is important for the development and the maintenance of β-cells. The expression of this factor was also modulated in the offspring when the dam was a fed another low-protein diet (Arantes *et al*., 2002) containing 6% of protein during pregnancy and lactation. At birth and after 28 days of life, islet volume and PDX-1 protein expression were reduced in pups fed this diet during gestation and lactation. In contrast, *Pdx-1* mRNA levels in the islets from 28-day-old low-protein rats were no different from those of controls. If the low-protein diet was present only during fetal life, PDX-1 protein expression in pancreatic islets, the volume of islets and insulin secretion were restored in recovered rats, whereas PDX-1 mRNA levels were higher than in normal rats. These results suggest links between diminished PDX-1 protein expression, reduction in islet volume and impaired insulin secretion in pups exposed to a low-protein diet.

The deleterious effect of fetal malnutrition on the development of the β-cell mass is also apparent in other animal models of fetal malnutrition. Fetuses whose mothers were caloric restricted by 50% during the last week of gestation

were growth retarded. Such animals were also born with important alterations in the development of their endocrine pancreas, including a lower β-cell mass, brought about by different mechanisms than those observed in the case of protein deprivation (Garofano *et al*., 1997). In this model, the reduction in β-cell mass appears to be due to reduced neogenesis expressed through a smaller number of islets rather than a lower rate of islet cell proliferation and increased apoptosis, as occurred with protein deprivation. Interestingly, the islet blood vascularization was not affected (Kalbe *et al*., unpublished data). In a study comparing the effect of maternal low-protein but isocaloric, low calorie and low-protein–calorie restricted diets, Bertin *et al*. (2002) demonstrated that, although the three approaches to inducing fetal malnutrition all induced 50% reduction of the fetal β-cell mass, plasma taurine was only depleted in the fetuses that were protein deprived. Taurine can thus not be involved in the reduction of β-cell mass observed in caloric-restricted fetuses, and other factors have been suggested. During development, a negative role of glucocorticoids on the fetal β-cell has been demonstrated (Blondeau *et al*., 2001). Maternal food restriction increased maternal and fetal corticosterone levels (Lesage *et al*., 2001). A normalization of the glucocorticoid levels in the intrauterine growth retardation (IUGR) calorie-restricted fetus restored a normal β-cell mass that was associated with the correction of the decreased β-cell neogenesis (Blondeau *et al*., 2001).

To better address the key issue of the fetal programming of the endocrine pancreas, two approaches were used. In a first step, we highlighted the short-term programming by culturing fetal β-cells, which were thus withdrawn from the influence of a disturbed metabolic maternal environment. In a second step, by feeding the offspring with a normal diet after birth or after weaning and analysing the adult progeny, long-term consequences were revealed. This will be addressed in the next part of this chapter.

Pancreatic cells cultured for 7 days proliferate and differentiate into pseudo-islets rich in β-cells (Hellerström, 1979). Although they were cultured in the same medium as the control islets and were free of the poor maternal milieu, low-protein islet cells retained the lower proliferation rate (Cherif *et al*., 2001) and the higher apoptotic rate (Merezak *et al*., 2001) that were observed *in vivo*. In addition, insulin secretion in response to glucose and various amino acids was dramatically altered in islets of the LP fetus, depending on the secretagogues used. The lowering of insulin release was more pronounced when the islets were challenged with amino acids than with glucose (Cherif *et al*., 1998, 2001). Although a reduction of cAMP concentration was detected in these islets, and might explain, in part, the reduced insulin release, we demonstrated that the main defect in insulin secretion was at the level of exocytosis (Cherif *et al*., 2001). The persisting reduction of the insulin secretion and replication, as well as the increased apoptotic rate of fetal β-cells in LP offspring, even when they have been withdrawn from the maternal environment and cultured for 1 week, demonstrates that protein restriction during pregnancy induced lasting impairments. The secretory and proliferative actions of the fetal β-cells are stimulated by amino acids (Fajans and Floyd, 1972; Swenne *et al*., 1980). Since a decrease in essential, branched amino acids and taurine was reported in the LP feto-maternal unit (Reusens *et al*., 1995), lower sensitivity to several amino acids

might have been due to their low availability during development. However, a low level of taurine was also involved, since taurine supplementation to the LP diet of the pregnant rat restored a completely normal insulin secretion from these fetal islets.

Early Malnutrition Programmes, Glucose Metabolism and Insulin Action: Type 2 Diabetes

Type 2 diabetes is characterized by hyperglycaemia, resulting from insulin resistance, β-cell dysfunction or both. β-Cell dysfunction occurs because of an inability to synthesize and secrete active insulin in sufficient amount to meet the increased demand for insulin (Kahn, 1998). In addition, type 2 diabetic patients often show a reduced β-cell number compared to weight-matched non-diabetic patients. This suggests that these patients had fewer cells prior to the onset of diabetes, or that they failed to enhance their β-cell mass in response to the demand (Clark *et al*., 1988). Individuals with type 2 diabetes are not at increased risk for autoimmune diseases, but do have higher prevalence of metabolic abnormalities, including obesity, hypertension and dislipidaemia. It should be mentioned, however, that a recent study showed that one-third of patients aged 15–34 with clinical type 2 diabetes had objectively type 1 diabetes on the basis of the evolution of their anti-islets antibodies; namely, islet cell antibody (ICA), glutamic acid decarboxylase (GAD) and insulin antibody (IA) (Borg *et al*., 2003).

The underdevelopment of the endocrine pancreas as a response to malnutrition may be a survival advantage in early life, but may be a risk factor for the appearance of diabetes later on. The long-term effect of the early malnutrition depends on the critical time windows at which the poor diet was applied. If protein restriction in rat pregnancy was reversed at birth, the imbalance between β- and α-cell populations observed at birth was restored by 3 months of age (Dahri *et al*., 1991), but females had a lower insulin secretion in response to an oral glucose tolerance test. If the low-protein diet was maintained until weaning, the adult offspring retained a reduced number of pancreatic β-cells and an increased α-cell number (Berney *et al*., 1997) and both males and females exhibited a blunted insulin secretion in response to the oral glucose challenge (Reusens and Remacle, 2001a). This suggests that the LP diet causes fundamental changes in the programming of the β-cell phenotype, with critical periods both in fetal and neonatal life. Changes in such programming of the β-cells were also observed following fetal and postnatal calorie restriction. The β-cell mass, which was depressed by 66% at weaning, remained reduced by 35% at 3 months despite adequate calorie intake after weaning. However, an increased proliferation rate was observed in the tail of the pancreas, which was not sufficient to fully restore a β-cell mass since the latter remained significantly reduced (Garofano *et al*., 1998).

Despite lower insulin secretion in the young offspring protein-malnourished early in life, such animals present better glucose tolerance (Shepherd *et al*., 1997). In contrast, young offspring from mothers that received only 50% of calories during the last week of gestation, featured the same pattern for insulin,

but with a normal glucose tolerance (Garofano *et al*., 1999). To understand this apparent discrepancy between lower insulin secretion and better glucose tolerance, several authors have further investigated the insulin-sensitive tissues in the case of maternal protein restriction. Lower plasma insulin concentration and the increased glucose infusion rates needed to maintain euglycaemia in hyperinsulinaemic clamps suggest an increased sensitivity of peripheral tissues to insulin in the young low-protein-exposed offspring (Holness, 1996). This focuses attention on the three key organs involved in glucose homeostasis: the muscle, the liver and the adipose tissue.

At the level of the muscle and the adipocytes, protein-restricted offspring have an enhanced basal glucose uptake. The number of insulin receptors expressed in these tissues was very considerably increased, as was the number of glucose transporters (GLUT 4) in the plasma membrane (Ozanne *et al*., 1996). Such alterations are likely to have made a significant contribution to the improvement in glucose tolerance observed in the animals' young adult life.

Despite having increased expression of insulin receptors, adipocytes from protein-restricted offspring have revealed a selective insulin resistance in studies of lipolysis (Ozanne *et al*., 1999, 2000). The mechanism of this resistance is unknown, but an alteration in the expression and activities of various components of the insulin-signalling pathway seems to be involved (Ozanne *et al*., 1997; Shepherd *et al*., 1997). Indeed, increased basal and stimulated levels of insulin-receptor-substrate-1-associated phosphatidylinositol 3-kinase (PI3K) activities and increased Akt/protein kinase B activities have been reported. In addition, adipocytes from low-protein-exposed offspring have a relatively low level of the p110-β catalytic subunit of the PI3K.

Resistance of the liver to the effect of glucagon on stimulation of glucose output has also been described in early protein-restricted offspring, and has been associated with a decreased hepatic glucagon receptor number (Ozanne *et al*., 1996). These animals also exhibited an increased insulin receptor number. In addition, the livers of the low-protein-exposed offspring had larger, but fewer hepatic lobules than those of the controls (Burns *et al*., 1997). This was associated with functional differences such as changes in enzyme activity. Increased phosphoenol-pyruvate carboxykinase (PEPCK) and decreased hepatic glucokinase (GK) activity were observed at weaning and at 3 months. Interestingly, it was demonstrated that the gluconeogenic enzyme PEPCK is an important target gene under potent glucocorticoid regulation (Friedman *et al*., 1993) and its expression is inhibited by insulin (Girard *et al*., 1991).

So, early protein restriction induces a number of different effects on glucose metabolism and insulin action, including alteration in the regulation of the hepatic and muscle glucose and disturbance in the suppression of the adipose-tissue lipolysis. The limited capacity of the β-cells to regenerate following poor development of the endocrine pancreas after early malnutrition leaves the offspring with a suboptimal complement of functional units in the pancreas. This will not be a real handicap as long as the individual is young, thin and does not face ageing, overfeeding and pregnancy. Should such events occur, the offspring would develop insulin resistance and would evolve to glucose intolerance and diabetes.

Indeed, whereas glucose tolerance in young adults appeared unaffected, or even enhanced, by the protein deprivation, it deteriorated more rapidly with age than that of controls. Sugden and Holness (2002) showed that exposure to protein restriction during early life alone led to a relative insulin resistance and hyperinsulinaemia associated with a normal glucose tolerance at 5 months of age, but only in males. The animals became glucose intolerant at 15 months (Hales *et al*., 1996) and suffered from frank diabetes at 17 months (Petry *et al*., 2001). The response seemed also to be sex-dependent. The hyperglycaemia was associated with relative hyperinsulinaemia in male low-protein-exposed offspring and relative hypoinsulinaemia in female offspring.

The fetal adaptation to malnutrition takes place to favour survival with poor or cyclic availability of food. The 'thrifty phenotype' hypothesis proposed that following such an adaptation, excessive food availability later in life may be detrimental for health (Hales and Barker, 1992). This part of the hypothesis has been tested in models of malnourished rats receiving several enriched diets in later life. The offspring of dams fed 5% protein during gestation and lactation and fed a high-sucrose or a high-fat diet at adulthood had an impaired glucose tolerance. Feeding a standard lab chow in adult life resulted in normal glucose tolerance in animals subject to protein restriction earlier in life. Glucose-stimulated insulin release was reduced in islets of previously malnourished rats fed sucrose or fat (Wilson and Hughes, 1997). Here again, poor fetal and neonatal nutrition led to impaired pancreatic β-cell function persisting into adulthood. In another study, 8 weeks of feeding a high saturated-fat diet to low-protein-exposed offspring was necessary to provoke an impaired glucose tolerance associated with reduced insulin-stimulated glucose uptake (Holness and Sugden, 1999). Anti-lipolytic effects of insulin were impaired in these animals.

Long-term consequences of the early malnutrition were also apparent in offspring of mothers receiving 50% of the calories needed during the last week of gestation and during lactation (Garofano *et al*., 1998, 1999). At 3 months, male offspring had fewer β-cells, the reduction being greater when the caloric restriction was maintained until weaning. In fact, the β-cell mass, which normally increases between 3 and 12 months, failed to do so, partially because the apoptotic rate was higher and increased further with ageing (Garofano *et al*., 1999). Insulin secretion in response to an oral glucose injection was depressed at 3 months but, as in low-protein-exposed offspring, no glucose intolerance was observed. At 12 months, the offspring featured increased fasting glycaemia and a dramatically impaired glucose tolerance associated with a profound insulinopenia (Garofano *et al*., 1999). When the calorie restriction was more severe (30% of the *ad libitum* available food) and was applied from the first day of gestation until birth, 4-month-old male offspring developed obesity, hyperinsulinaemia and hyperlipidaemia, which was amplified by hypercaloric nutrition postnatally (Vickers *et al*., 2000). However, such offspring did not exhibit an abnormal fasting plasma glucose level, but the hyperglycaemia induced by the hypercaloric diet after weaning was aggravated by the early malnutrition (Vickers *et al*., 2001). The authors proposed that fetal programming may lead to β-cell dysfunction which, in this case, is induced by hyperlipidaemia. The fact

that leptin suppresses insulin production from β-cells (Poitout *et al*., 1998) and that leptin receptors are present on pancreatic β- and δ-cells (Leclercq-Meyer *et al*., 1996; Emilsson *et al*., 1997) suggests a functional relationship between circulating leptin and insulin secretion. Leptin receptors are evenly distributed throughout the pancreatic islets. In adult offspring from calorie-deprived mothers, however, a higher number of leptin-positive cells were identified as δ-cells and this was amplified by a post-weaning high calorie diet (Vickers *et al*., 2001).

Fetal malnutrition as a consequence of utero-placental insufficiency also programmes the development of the endocrine pancreas and glucose metabolism later in life. Bilateral uterine artery ligation performed on day 19 of gestation in rats leads to intrauterine growth retardation. In this model, IUGR offspring remained growth retarded until 7 weeks after birth, after which they began to recover a normal body weight. They developed marked fasting hyperglycaemia and hyperinsulinaemia at 10 weeks, which deteriorated into glucose intolerance and insulin resistance at 15 weeks. By 26 weeks of age, the IUGR rats were obese (Boloker *et al*., 2002) Interestingly, the IUGR rats presented a lower insulin secretion in response to an intraperitoneal glucose challenge at 1 week, and a blunted insulin secretion at 26 weeks, whereas at 15 weeks they were clearly insulin resistant and glucose intolerant. In this specific experimental condition, the nutrient deprivation was present during a short time window at the end of the development of the β-cell, which corresponded to an expansion phase of the β-cell mass. No measurement of the β-cell mass was performed at birth but, although no difference was detected at 1 and 7 weeks postnatally, a reduction by 50% at 15 weeks and by 70% at 26 weeks was reported (Simmons *et al*., 2001). At 14 days postnatally, intrauterine growth-retardation pups exhibited a reduced β-cell proliferation, but a normal apoptotic rate. The expression of *Pdx-1*, the critical regulator of the pancreatic development and β-cell development, was dramatically reduced at 14 days and remained affected at 3 months (Stoffers *et al*., 2003). Injections of Exendin-4, a long-acting glucagon-like peptide 1 (GLP-1) analogue which promotes expansion of pancreatic β-cell mass, just after birth prevented the defects in the development of the β-cell mass observed postnatally. As a consequence, the glucose intolerance and diabetes observed in the adult were also prevented (Stoffers *et al*., 2003). Here again, long-lasting consequences of early fetal malnutrition at the level of the endocrine pancreas may be prevented by specific intervention during development.

In conclusion, fetal malnutrition due to maternal protein restriction, caloric restriction or limited availability of nutrients to the fetus jeopardizes the development of the β-cell mass, with consequences for insulin secretion and glucose metabolism later in life.

Transgenerational Programming of the Endocrine Pancreas

The lasting consequences of fetal malnutrition are not limited to the first generation, and seem to persist in a second generation. The vulnerability of the β-cell mass acquired early in life may only be frankly revealed in a situation of increased insulin demand, such as obesity, pregnancy and ageing. The

exhaustion of the β-cell mass and the deterioration of the glucose tolerance with obesity and ageing have already been discussed above. Pregnancy is a particular situation during which the endocrine pancreas has to adapt in response to the high demand of insulin coming from the fetus for its anabolism. This specific adaptation can be achieved by several mechanisms A lowering of the threshold at which glucose stimulates insulin secretion occurs in early pregnancy and, as a consequence, increased insulin synthesis and secretion take place. This is achieved by an up-regulation of β-cell proliferation (Parsons *et al*., 1992; Sorenson and Brelje, 1997). β-Cell mass doubles by the end of gestation in rats (Bone and Taylor, 1976; Aerts *et al*., 1997) and this increase is controlled by placental lactogens I and II (Brelje *et al*., 1993). Altered β-cell mass resulting from protein and calorie deprivation in early life, or placental insufficiency, is a limiting factor for this adaptation.

When 3-month-old offspring from dams fed a low-protein diet during gestation became pregnant, they exhibited a very low insulin secretion after an oral glucose challenge performed at 18.5 days of gestation, and became glucose intolerant (Dahri *et al*., 1995). Adaptation of the endocrine pancreas was not fully achieved, as evidenced by the fact that the pancreatic insulin content in mothers fed a low-protein diet during fetal life was significantly lower than in the control mothers (Reusens and Remacle, 2001b). This abnormal intrauterine milieu impacted upon the next generation. Just before birth, the pups whose grandmothers were protein restricted during pregnancy also clearly exhibited lower plasma insulin levels, probably as a consequence of the reduced development of their own endocrine pancreas. These rats therefore had a tendency to be hyperglycaemic (Bone and Taylor, 1976). Indeed, fetal pancreatic insulin content and volume density of the β-cell mass were significantly reduced. An intergenerational effect of the abnormal metabolic intrauterine milieu was thus apparent.

Adaptation to pregnancy was also compromised in the calorie-restricted rat model. Although a normal adaptation was seen at 4 months in mothers that were previously malnourished during development, at 8 months they were no longer able to increase their β-cell mass (Blondeau *et al*., 1999). A study was undertaken to characterize the cellular mechanism responsible for the absence of adaptation. In normally fed pregnant rats, Avril *et al*. (2002) reported a preferential increase in β-cell proliferation in the head of the pancreas at day 12 of gestation. Such an enhanced proliferation rate leads to an important increase in the β-cell fraction on the last day of gestation. Perinatal malnutrition impaired this subsequent adaptation to pregnancy by decreasing the β-cell proliferation in the head of the pancreas at the critical time, i.e day 12 of pregnancy. In addition, the lack of adaptation in the mother was associated with abnormal α- and β-cell development in the second generation at fetal stage (Blondeau *et al*., 2002). The β-cell mass and pancreatic insulin content were reduced. A decreased number of cells expressing PDX-1, which are likely to be precursors for endocrine cells, has been reported in these pancreases at 17 day of gestation. This may partially explain the lower β- and α-cell fraction.

In the model of placental insufficiency, the female progeny are also less able to cope with pregnancy. In fact, pregnancy prematurely precipitated the

development of diabetes in IUGR that was only clearly apparent by month 6 in non-pregnant rats (Boloker *et al*., 2002). Pregnant rats exhibited a clear insulin resistance and glucose intolerance. The consequences of the disturbed metabolic intrauterine milieu were apparent when the second generation was analysed. Pups were born slightly heavier and remained heavier throughout life. They were insulin resistant very early in life and they featured progressive glucose intolerance. At 1 week of age, β-cell mass was slightly increased, but because β-cell proliferation declined progressively compared to control offspring, the β-cell mass was dramatically reduced at 26 weeks (Boloker *et al*., 2002).

Early Malnutrition and Susceptibility to Type 1 Diabetes

Clinical and animal studies have shown that type 1 diabetes is an autoimmune disease in which both genetic and environmental factors, such as nutrition, participate in the pathogenesis and development of the disease. In type 1 diabetes, β-cells are rapidly and selectively destroyed by apoptosis because of invasion of the islets by immune cells releasing free radicals and cytokines, and also because they have poor defence mechanisms. Type 1 diabetes is not, as is generally believed, a disorder limited to young people. Recent studies support a model in which the disease can occur at any age and that some patients diagnosed with type 2 diabetes actually have type 1 diabetes (Turner *et al*., 1997). The traditional view of type 1 diabetes postulates that an environmental agent triggers the onset of disease in genetically susceptible individuals. However, more recent observations from humans and in animals support a more complex model, wherein the penetration and expression of heritable aberrations, in combination with inherent target organ defects, are part of the life-long influence of multiple environmental factors (Atkinson and Eisenbarth, 2001).

In contrast to type 2 diabetes, there is little epidemiological evidence to suggest a relation between poor nutrition in early life and the occurrence of type 1 diabetes later in life. Only viral infections, such as coxsackie B4, might support the idea of early programming of type 1 diabetes in humans (Hyöty and Taylor, 2002). In spontaneously diabetic animals, the hypothesis of early programming of type 1 diabetes was also proposed, with an exaggerated wave of postnatal apoptosis suggested to be at the origin of insulitis later in life (Trudeau *et al*., 2000).

In view of the defects in the endocrine pancreas, particularly the increased apoptotic rate, that we have highlighted in offspring from mother fed a low-protein diet, we asked the question about the possible increased susceptibility of the β-cell to the effects of agents known to be involved in type 1 diabetes. When β-cells from the fetus of a protein-deprived mother were challenged in culture with a nitric oxide (NO) donor or interleukin-1β (IL-1β), they exhibited a higher apototic rate than the control fetal β-cells (Merezak *et al*., 2001). Moreover, the increased susceptibility was maintained throughout life, despite adequate feeding after weaning, since adult islets were also more vulnerable in the presence of cytokines (Merezak *et al*., 2002). The lack of protein during development has thus generated a β-cell population that is more sensitive to cytokines, perhaps

because of a low level of IGF-II (Petrik *et al*., 1999) and a low level of taurine present during development. Indeed, taurine which is a sulphur amino acid, has antioxidant properties (Huxtable, 1992). When added to the culture medium, it prevented the destruction of β-cells by NO and IL-1β in fetal and in adult islets. The protective role of taurine against NO- and cytokine-induced apoptosis was attributed to the sulphydryl group. Indeed, methionine, which also possesses this sulphydryl group, was also able to prevent apoptosis; however, the effect was less marked than that of taurine. β-Alanine, an analogue of taurine lacking of the sulphydryl moiety, was unable to provide any protection (Merezak *et al*., 2001). Moreover, supplementation of the low-protein diet with taurine, which restored a normal β-cell mass with adequate numbers of cells positive for IGF-II, a normal vascularization and a normal insulin secretion at birth, prevented the hypersensitivity of the fetal and adult islets to cytokines (Merezak *et al*., 2001, 2002). An adequate level of taurine during fetal and early life thus seems thus a critical parameter to achieve normal development and function of the endocrine pancreas. Should that not be present, vulnerability, at the level of proliferation, defence and secretion, would ensue.

Conclusions

Epidemiological studies have revealed strong relationships between poor fetal growth and subsequent development of metabolic syndrome, glucose intolerance and type 2 diabetes (Hales *et al*., 1996; Merezak *et al*., 2002). Persisting effects of early malnutrition become translated into pathology and thereby determine chronic disease risk (Barker *et al*., 1993). These epidemiological observations identify the phenomena of fetal programming without explaining the underlying mechanisms that establish their causal link. Animal models have been established and studies have demonstrated that reduction in the availability of nutrients during fetal development programmes the endocrine pancreas and the insulin-sensitive tissues. Independently of the type of fetal malnutrition

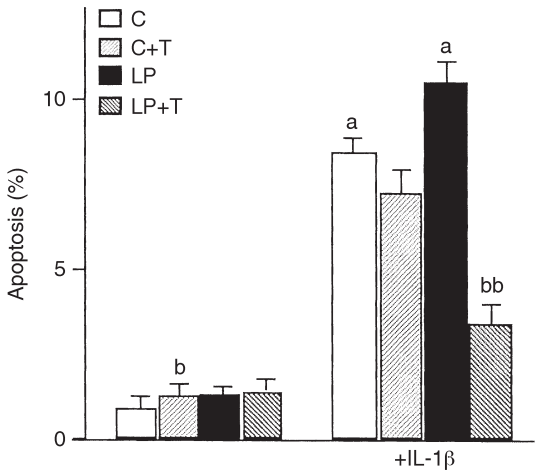

Fig. 8.5. Effect of taurine (T) supplementation on the sensitivity to cytokines of fetal islet cells from control (C) and low-protein-exposed (LP) offspring. a, $P < 0.001$ versus without cytokines; b, $P < 0.05$ versus without taurine; bb, $P < 0.05$ versus without taurine.

(whether there is not enough calories or protein in food or after placental deficiency), malnourished pups are born with a defect in their β-cell mass that will never completely recover, and insulin-sensitive tissues will be definitively altered. Despite the similar defects arising from different approaches to induction of fetal malnutrition, different mechanisms seem to operate and be responsible for the observed endpoints of glucose intolerance and insulin insensitivity. A lack of taurine in the case of protein restriction and high levels of glucocorticoids in case of the calorie-restriction model have been the focus of much interest. Interestingly, addition of taurine to the maternal low-protein diet, or the normalization of the maternal plasma glucocorticoid level in the calorie-restricted mother, prevents the alterations of the endocrine pancreas observed without these interventions.

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