Maternal undernutrition increases pancreatic IGF-2 and partially suppresses the physiological wave of β-cell apoptosis during the neonatal period

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Abstract

Replication, neogenesis, and apoptosis play a main role in neonatal endocrine pancreas remodeling. IGFs are major contributors to β-cell growth and function and are highly sensitive to nutritional status. We previously showed that maternal malnutrition caused an increase in β-cell mass in fetuses related to the stimulation of β-cell proliferation due to increased pancreatic IGF-1. At 4 days of life, the β-cell mass was decreased in undernourished neonates and persisted until adult age. To clarify whether undernutrition disrupts islet remodeling, we quantified β-cell mass, neogenesis, replication, and apoptosis on days 4, 14, and 23. To determine the impact of food restriction on IGF ontogeny and the consequences for β-cell growth, we measured IGF-1/-2 protein content in pancreas and liver and pancreatic IGF-1 receptor (IGF-1R)-signaling pathway at the same days. Our results indicate that undernutrition alters the timing and intensity of neonatal β-cell ontogeny. However, although malnutrition causes β-cell deficiency in neonates, an active process of β-cell neogenesis and a lower incidence of β-cell apoptosis maintain the regenerative capacity of the endocrine pancreas. Interestingly, our data provide evidence that local production of IGFs seems to be instrumental in these processes. In particular, increased pancreatic IGF-2 in undernourished rats may contribute to the partial suppression of the developmental wave of β-cell apoptosis probably through the inhibition of glycogen synthase kinase-3. In addition, decreased pancreatic levels of IGFBP-1/-2/-3 in undernourished neonates could enhance IGF availability for interacting with IGF-1R/IR.

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Introduction

It is generally accepted that the developing endocrine pancreas undergoes substantial remodeling during the neonatal period and that replication, neogenesis, and apoptosis play main roles in this process. In rodents, many of the β-cells proceeding from fetal origin are destroyed during neonatal life in a developmental wave of apoptosis to be later replaced by neogenesis or replication (Bonner-Weir 2000). Consequently, the new islets have insulin release characteristics suited to postnatal life and diet. Along these lines, the balance between β-cell growth and death determines an adequate development of β-cell mass, and any deregulation of these processes due to abnormal availability of hormones and growth factors could be of great importance for the functionality of the pancreas.

There is considerable evidence for the role of insulin and insulin-like growth factors (IGF-1 and -2) in the development, maturation, and function of β-cells. It is known that IGFs and their binding proteins (IGFBPs) are expressed by islet cells throughout life (Hill *et al.* 1999). In rats, IGF-2 levels are relatively high in fetal and early neonatal period and then they decline sharply toward adulthood, while IGF-1 levels increase progressively with age (Hogg *et al.* 1994, Petrik *et al.* 1998). Both IGFs are considered as survival factors by inhibiting β-cell apoptosis. It has been shown that the transient wave of apoptosis that occurs in developing rat islets between the first and second week of postnatal life (Scaglia *et al.* 1997, Petrik *et al.* 1998) is temporally associated with a lack of expression of IGF-2 within pancreatic islets (Petrik *et al.* 1998); in fact, persistent circulating IGF-2 abolishes neonatal islet apoptosis (Hill *et al.* 2000). Similarly, IGF-1 has also been

implicated in β-cell protection from cytokine-induced cell death (Casellas *et al.* 2006). IGFs mediate their biological actions via the IGF-1 receptor (IGF-1R), which involves the interaction of the IGF-1R with the insulin receptor substrates (IRS-1/-2) and the activation of phosphatidylinositol 3-kinase and Akt (Liu *et al.* 2002). Once activated, Akt integrates multiple survival signals inducing the phosphorylation–inactivation of pro-apoptotic proteins such as Bcl-associated death promoter (Bad) or glycogen synthase kinase-3 (GSK-3; Liu *et al.* 2002).

A worldwide series of epidemiological data support the concept that dietary influences during early stages of development present a risk factor for the onset of both perinatal and later life diseases. The extensive changes in islet cells throughout the neonatal life could explain why pancreatic morphology and function are so sensitive to nutritional insult at this time. Indeed, several studies in experimental models with rats submitted to different patterns of malnutrition have reported that maternal food restriction significantly affects the β-cell mass in the fetuses (Alvarez *et al.* 1997, Bertin *et al.* 2002) and in the offspring neonates at day 1 (Garofano *et al.* 1999) and day 4 of postnatal life (Martín *et al.* 1997). This effect persists until adulthood (Martín *et al.* 1997) and can provoke long-lasting consequences related to the plasticity of the endocrine pancreas under situations of increased insulin demand such as aging (Garofano *et al.* 1999) and pregnancy (Blondeau *et al.* 1999). Accordingly, nutritional insufficiency can alter the timing and amplitude of the ontological events, which trigger the remodeling of endocrine pancreas (Petrik *et al.* 1999).

We previously reported that protein-caloric food restriction (65% of *ad libitum* intake) applied during the last third of gestation caused an increase of β-cell mass in fetuses at term (Alvarez *et al.* 1997), associated to the stimulation of β-cell proliferation due to locally increased pancreatic IGF-1 and islet IRS-2 production (Martín *et al.* 2005, Fernández *et al.* 2007). Nevertheless, food restriction continued after birth induced a decrease in β-cell mass at 4 days of life and this effect persisted until adult age (Martín *et al.* 1997). We consider that this chronic severe deficiency represents better condition of undernourished humans in developing countries, and it is a very useful tool to investigate the impact of a permanent undernutrition state on pancreas plasticity. Therefore, in order to clarify whether the programmed turnover of pancreatic β-cells occurs following the same pattern of growth and by the same mechanisms in control and undernourished neonates, in this study, we quantified the β-cell mass, β-cell neogenesis, replication, and apoptosis at 4, 14, and 23 days of age. Since the IGF system is highly sensitive to nutritional status (Thissen *et al.* 1994), the second aim in the present study was to determine the impact of food restriction on the IGF protein expression profile and signaling pathways through the suckling period and the consequences for the growth of β-cells.

Materials and methods

Animals and diets

Neonatal Wistar rats bred in our laboratory under a controlled temperature and artificial dark–light cycle (from 0700 to 1900) were used throughout the study. Virgin females were caged with males, and mating was confirmed by the presence of spermatozoa in a vaginal smear. Each dam was housed individually from the 14th day, and maternal food restriction was established. All animals were fed a standard laboratory diet (Panlab No. A04: 19·3% protein, 2·9% lipids, 57% carbohydrates (starch and sucrose), 4·5% cellulose, a vitamin and mineral mix, and 12% water; Barcelona, Spain) and were divided into two groups: control pregnant dams were fed *ad libitum*; and the undernourished group received 35% of the food intake of a control pregnant along the third week of gestation and suckling period. The animals had free access to water. At the time of birth, litters were uniformed to eight pups per nursing dam to minimize the effects of litter size on postnatal growth, and pups were killed on days 4, 14, and 23 of postnatal life.

Before killing by decapitation, the neonates were weighed, blood samples were collected, and serum and plasma were separated and stored frozen at $-20\,°C$ until analyzed. Pancreases and livers were removed and weighed. For western blot analyses, pancreases and livers were immersed in liquid nitrogen and stored at $-70\,°C$ until analyses. For immunohisto-chemistry and morphometric studies, pancreases were fixed in aqueous Bouin's solution during 24 h and embedded in paraffin.

All studies were conducted according to the principles and procedures outlined in the National Institute of Health Guidelines for Care and Use of Experimental Animals and approved by the Animal Ethics Committee of the Complutense University of Madrid (Spain).

Analytical determinations

Aliquots of 10 μl obtained from 30 μl $Ba(OH)_2$–$ZnSO_4$ deproteinized blood were used to determine glucose by a glucose oxidase method (Boehringer–Mannheim). Serum insulin was determined with a rat insulin RIA (Linco Research Inc., St Louis, MO, USA) with rat insulin used for standard curve. Sensitivity of 0·1 ng/ml was achieved with overnight equilibration using a 100 μl serum sample. Serum glucagon was determined with a glucagon RIA purchased from Linco; sensitivity of

20 pg/ml was achieved using 100 μl serum sample in a 2-day disequilibrium assay. Serum growth hormone (GH) was determined with a rat GH ^{125}I assay system (Amersham, GE Healthcare). The sensitivity of the assay was 1·6 ng/ml. Serum IGF-1 was measured by a mouse/rat IGF-1 RIA (Diagnostic System Laboratories, Websters, TX, USA). The method incorporates a sample pretreatment to avoid interference from IGFBPs. Serum IGF-2 was measured by radioreceptor assay as previously described (Rivero *et al.* 1995).

Immunohistochemistry and morphometry

Immunohistochemistry was performed as previously described (Fernández *et al.* 2006). Pancreas sections (5 μm) at fixed intervals throughout the paraffin block were immunostained for insulin using guinea pig anti-porcine insulin (ICN, Biolink, Barcelona, Spain) and a peroxidase-conjugated rabbit anti-guinea pig IgG (Dako Diagnostics, Barcelona, Spain). Immunoreactivity was localized using a peroxidase substrate kit (DAB; Vector, Grupo Taper, Madrid, Spain). Quantitative evaluation was performed using computer-assisted image analysis performed by means of an Olympus BX40 microscope connected via a video camera to a PC computer and using the Histolab 5.2 software (Microvision Instruments, Evry, France). The area of insulin-positive cells and that of total pancreatic sections was evaluated in each stained section. β-Cell relative volume was determined by calculating the ratio between the area occupied by insulin immunoreactive cells and that occupied by total pancreatic cells. Total β-cell mass per pancreas was derived by multiplying the total pancreatic weight by the β-cell relative volume. The total islet number per section (cm^2 of pancreatic tissue) was counted, and islets were arbitrarily classified as very small (≤ 30 μm in diameter), small (31–50 μm in diameter), medium (51–150 μm in diameter), or large (≥ 151 μm in diameter) to evaluate the distribution of islet sizes. To obtain an estimation of β-cell neogenesis activation, we quantified on every section immunostained for insulin the number of single β-cells and β-cell clusters (2–4) incorporated into the ductal epithelium. The values obtained were related to the total area of the pancreatic section and calculated per micrometer squared of the pancreatic area. Results represent the average of four to five tissue sections per animal from four to six animals from each experimental group and age.

β-Cell replication

Other pancreas sections were used to measure β-cell replication rate as previously described (Fernández *et al.* 2006). In each group, neonates were injected with 5′-bromo-2′deoxyuridine (BrdU), 50 mg/kg body weight i.p. (Sigma–Aldrich) 1 h before killing. Sections were double stained. First, for detection of BrdU-positive cells, it was used with a cell proliferation kit (Cell Proliferation kit; Amersham, GE Healthcare) with a mouse monoclonal anti-BrdU antibody and a peroxidase anti-mouse IgG. Immunoreactivity was localized using the peroxidase substrate kit DAB (Vector). Then insulin staining was performed using a guinea pig polyclonal anti-insulin antibody (ICN). This antibody was recognized with a rabbit anti-guinea pig alkaline phosphatase-conjugated antibody (ICN), which was revealed with alkaline phosphatase substrate kit I (Vector). The β-cell replication rate was expressed as the percentage of BrdU-positive β-cells. At least, 1500 β-cells were counted per pancreas.

β-Cell apoptosis

In situ detection and quantification of β-cell apoptosis were done within histological sections of pancreas by using a TUNEL cell death detection kit (ApopTag Peroxidase *In Situ* Apoptosis detection kit, Chemicon International, Millipore, Madrid, Spain) according to the manufacturer's protocol and coupled to insulin staining in similar conditions to those described for BrdU/insulin labeling. The tissue was counterstained with Harri's hematoxylin for 30 s. The β-cell apoptosis rate was expressed as the percentage of apoptotic β-cells. At least 1500 β-cells were counted per pancreas.

Immunoprecipitation

Tissue extracts containing 250 μg protein were immuno-precipitated overnight at 4 °C with gentle rotation in the presence of 2–4 μg anti-phosphotyrosine mouse monoclonal antibody (Upstate Biotechnology, Millipore, Madrid, Spain), followed by the addition of anti-mouse IgG agarose (Sigma–Aldrich). After mixing for 2 h, the pellets were collected by centrifugation, and the supernatants were discarded. Then, the pellets were washed and saved for western blot analyses.

Western blot analyses

Frozen pancreases and livers were homogenized in ice-cold lysis buffer (10 μM leupeptin, 2 mM *O*-vanadate, 2 mM benzamidine, 10 μg/ml aprotinin, and 2 mM phenylmethylsulfonyl fluoride in 12·5 mM EGTA, 1·25 mM EDTA, and 0·25% Triton $\times$, pH: 7·6) using the IKA T10 basic Ultra-Turrax. Then, tissue extracts were cleared by microcentrifugation at 15 700 *g* at 4 °C for 20 min, and the supernatant was used for the determination of total protein content.

The concentration of protein was determined by the Bradford method (Bradford 1976) using a protein assay (Bio-Rad Laboratories, Inc.), with γ-globulin as standard.

Equals amount of protein were separated by SDS-PAGE, transferred to Immobilon membranes, and incubated overnight with the corresponding primary antibodies (anti-IGF-I, clone Sm1.2, anti-IGF-II, clone S1F2, and anti-IR β-subunit purchased from Upstate Biotechnology, Millipore; anti-IGF-1Rβ, anti-GSK-3α/β, anti-Bad, and anti-Bcl-x$_{S/L}$ purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA); anti-phospho-Akt (Ser473), anti-Akt, anti-phospho-GSK-3α/β (Ser21/9), and anti-phospho-Bad (Ser136) purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA)). Immunoreactive bands were visualized using the ECL western blot protocol (Amersham). Normalization of western blot was ensured by β-actin (Sigma–Aldrich), and the intensity of bands was quantified by laser-scanning densitometry (Molecular Dynamics, Sunnyvale, CA, USA).

Statistical analyses

Values are given as means ± S.E.M. for the number of rats studied. For all data comparisons, the ANOVA, followed by the protected least significant difference test, was used. Statistical analyses were performed using SPSS version 15.0 software (SPSS Inc., Chicago, IL, USA).

Results

Biological characteristics of neonates

The biological characteristics of neonates are shown in Table 1. Postnatal development is associated with a high growth rate. Here, we show that the body, pancreas, and liver weight of neonatal rats increased linearly, and this increase was significantly higher at

each age studied as compared with the previous time point. In undernourished pups, the weight values were significantly lower at each time point as compared with control neonates. However, because body weights are different between control and undernourished rats when pancreas and liver weights were normalized to the body weight, no dissimilarities were found in these parameters (data not shown).

Circulatory glucose levels were similar between control and undernourished offspring through the suckling period. On the other hand, serum insulin levels were similar in both groups on day 4, but food-restricted neonates developed hypoinsulinemia from 14 days of age onwards. Although undernourished pups showed lower glucagonemia than controls on days 4 and 14, serum glucagon concentration was normalized on day 23.

Serum GH levels decreased progressively from day 4 to day 23 of age, and no differences were found between control and undernourished values at any time point considered. On the contrary, serum IGF-1 levels increased along the period of time considered in both populations. However, from 14 days of age until the end of suckling period, serum IGF-1 levels were significantly higher in undernourished neonates than in controls. Regarding circulatory IGF-2 levels, we could not find any difference between the ages considered or the type of diet.

Postnatal evolution of β-cell mass, β-cell relative volume, and islet size

β-Cell mass increased in control neonates from day 4 to day 14. However, it remained stable from 14 to 23 days of age. With regard to β-cell growth in the undernourished population, we did not observe any difference between 4 and 14 days of age, but there was a significant increase from postnatal day 14 to 23. Nevertheless, the β-cell mass was markedly lower at each

Table 1 Biological characteristics of neonates at 4, 14, and 23 days of age from control and undernourished pregnant rats

	4 days of age		14 days of age		23 days of age	
	C	U	C	U	C	U
Body weight (g)	12·28 ± 0·07	9·45 ± 0·10*	30·69 ± 0·31†	15·56 ± 0·19*,†	58·40 ± 1·09†,‡	16·62 ± 0·29*,†
Pancreas weight (mg)	23·8 ± 0·5	19·0 ± 1·0	60·21 ± 4·25†	29·7 ± 1·6*,†	220·0 ± 7·0†,‡	51·0 ± 2·10*,†,‡
Liver weight (mg)	277·0 ± 4·0	189·0 ± 2·0*	783·3 ± 22·0†	378·1 ± 12·2*,†	2160 ± 22·0†,‡	468 ± 17·1*,†,‡
Glycemia (mg/dl)	101·7 ± 1·5	94·4 ± 2·3	104·9 ± 2·6	95·7 ± 3·5	110·9 ± 3·9	84·8 ± 3·6
Serum insulin (ng/ml)	0·31 ± 0·1	0·29 ± 0·1	0·58 ± 0·06†	0·125 ± 0·01*	0·41 ± 0·03	0·092 ± 0·01*,†
Serum glucagon (pg/ml)	465 ± 6·7	128·9 ± 1·42*	543·3 ± 28	256·9 ± 11·6*,†	114·9 ± 44†,‡	193 ± 25·8
Serum GH (ng/ml)	30 ± 5·5	29 ± 4·2	15·2 ± 4·4	14·6 ± 1·9	7·84 ± 0·4†	11 ± 2·2†
Serum IGF-1 (ng/ml)	71·2 ± 6·8	72·9 ± 14·2	83 ± 3·3†	261·6 ± 11·5*,†	264 ± 10·6†,‡	363·6 ± 20·1*,†,‡
Serum IGF-2 (ng/ml)	26·2 ± 1·2	28·3 ± 0·81	27·47 ± 1·1	27·1 ± 1·02	22·3 ± 2·7	27·0 ± 0·96

Data are means ± S.E.M. of 10–15 animals per group and age. *$P<0.05$ relative to control rats at the same age; †$P<0.05$ relative to 4 days of age within the same condition; ‡$P<0.05$ relative to 14 days of age within the same condition.
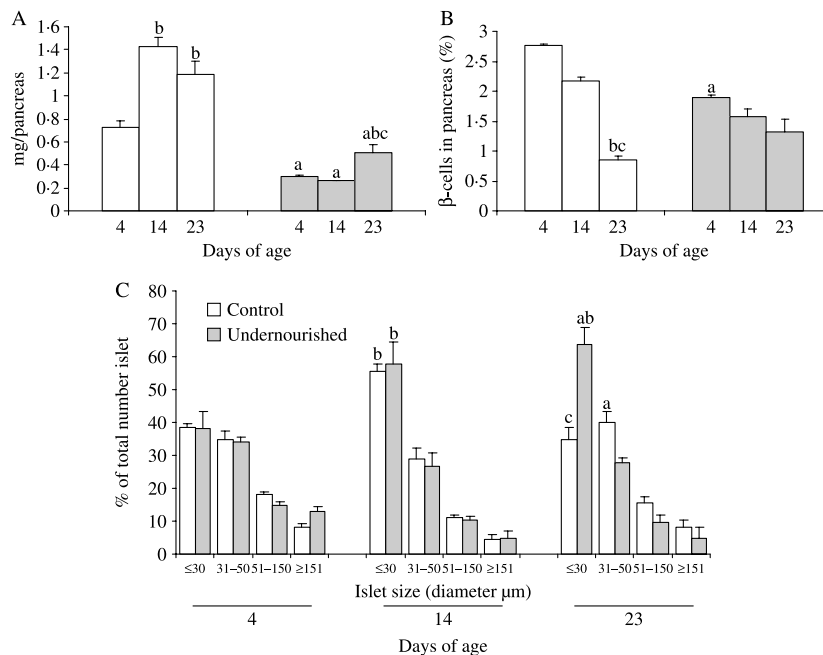
Figure 1 β-Cell mass (A) and β-cell relative volume (B) in control and undernourished neonatal rats. Values are mean ± S.E.M. for four to six observations in each experimental group and age. [a]$P < 0.05$ relative to control rats at the same age; [b]$P < 0.05$ relative to 4 days of age within the same condition; [c]$P < 0.05$ relative to 14 days of age within the same condition. (C) Relative distribution of arbitrarily defined very small (≤ 30 μm), small (31–50 μm), medium (51–150 μm), or large (≥ 151 μm) pancreatic islets in control and undernourished neonates at 4, 14, and 23 days of life. The comparative distribution of islet size from control and undernourished neonates at different ages is expressed in each group as a percentage of total islet number. Values are mean ± S.E.M. for four to six observations in each experimental group and age. [a]$P < 0.05$ relative to control neonates for the same age and islet size category, [b]$P < 0.05$ relative to 4 days of age within the same condition and islet size category, [c]$P < 0.05$ relative to 14 days of age within the same condition and islet size category.

time point considered in undernourished rats as compared with the control group (Fig. 1A). On the other hand, since both populations depict different pancreas weights, we have expressed the β-cell mass data as β-cell relative volume (Fig. 1B). In control pups, the relative β-cell volume decreased progressively with time becoming three times lower on day 23 as compared with day 4. The relative volume of β-cells remained stable over the neonatal period studied in food-restricted animals, although, at 4 days of age, this parameter was decreased by 30% as compared with the corresponding control neonates at the same time point. During all the neonatal period, most of the islets from control as well as from undernourished rats were included in the small size range (≤ 30–50 μm in diameter); however, the relative size distribution of arbitrarily defined very small, small, medium, or large islets significantly differed between control and undernourished neonates at 23 days of life, with the food-restricted animals showing a greater percentage of very small islets as compared with control rats (Fig. 1C).

Representative images are shown in Fig. 2(A and B).

β-Cell neogenesis, replication, and apoptosis

β-Cell neoformation is shown in Fig. 3(A). In control neonates, the β-cell neogenesis decreased progressively throughout the neonatal period, while, in undernourished animals, the neoformation of β-cells remained constant between 4 and 14 days of age and then declined on day 23. The β-cell neogenesis rate was significantly higher in undernourished rats at 14 and 23 days of age as compared with age-matched control animals.

As shown in Fig. 3(B), β-cell replication peaked on day 14 in control animals, but decreased on day 23 to values lower than those observed at 4 days of life. In undernourished rats, β-cell replication remained stable from 4 to 14 days of age and then decreased significantly on day 23. The frequency of β-cell replication in undernourished neonates was significantly lower than in controls at 14 days of age.

The occurrence of apoptotic β-cell death in control neonates was <1% on postnatal day 4, but, at 14 days of age, the β-cells experienced a peak of apoptosis
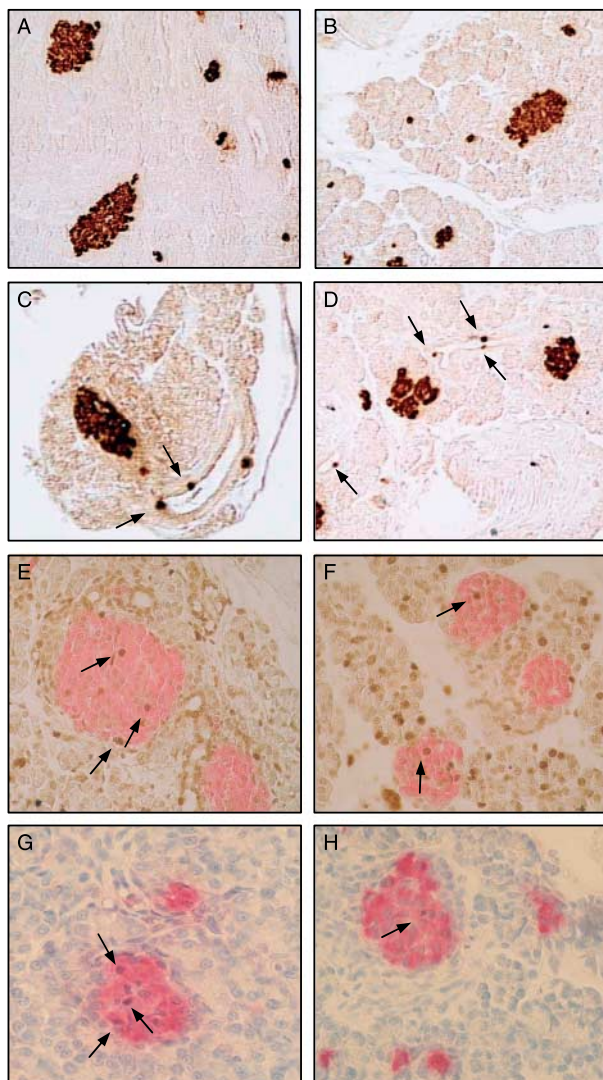
Figure 2 Immunostaining for pancreatic insulin in control and undernourished neonates (A and B) was used to measure β-cell mass. β-Cell neoformation from ducts in control and undernourished neonates (C and D) was illustrated by single β-cells and β-cell clusters (2–4), incorporated into the ductal epithelium. Double immunostaining for BrdU and insulin was used to measure β-cell replication in control and undernourished neonatal pancreases (E and F). Double immunostaining for apoptosis and insulin was used to measure β-cell apoptosis in control and undernourished neonatal pancreases (G and H). All images correspond to postnatal day 14. Magnification: 20× for A–D and 40× for E–H.

doubling the 4-day-old values (Fig. 3C). The incidence of β-cell apoptosis in undernourished pups also showed a rise on day 14, but at all the neonatal ages studied the percentage of apoptotic β-cells was significantly lower in undernourished rats as compared with the age-matched controls.

Representative images are shown in Fig. 2(C–H).

Pancreatic and hepatic IGF-1/-2 protein levels

Because the IGF system is highly responsive to nutritional status and there is enough evidence of the relationship between IGFs and β-cell growth, we then studied the IGF-1/-2 ontogeny in pancreas and liver along the neonatal period.

Both groups of animals showed the same IGF-1 protein expression profile in the pancreas and liver (Fig. 4A and B). Although pancreatic IGF-1 levels were barely detectable at the beginning of the neonatal period, we did observe significantly lower values in undernourished neonates on day 4 than age-matched controls, followed by a peak in concentration at 14 days of age in both groups without differences between control and undernourished animals. Pancreatic IGF-1 levels declined in both groups at 23 days of age, but continued to be higher than at 4 days of age (Fig. 4A). As shown in Fig. 4(B), the protein levels of hepatic IGF-1 increased linearly with time in both groups, but, on day 23, the values were significantly higher (twofold) in undernourished rats as compared with controls at the same age.

Both pancreatic and hepatic IGF-2 levels decreased progressively with time in control neonates, becoming hardly detectable by postnatal day 23 (Fig. 4A and B). In undernourished animals, the timing but not the intensity of the signal for IGF-2 content was similar to control data. We found that pancreatic IGF-2 was highly expressed in undernourished rats during all the suckling period (Fig. 4A), whereas liver IGF-2 protein content was only higher in food-restricted animals at 4 and 23 days of age (Fig. 4B).

Pancreatic IGFBP-1, IGFBP-2, and IGFBP-3 protein levels

Because IGF biological actions are closely related to their binding proteins, we analyzed the local production of IGFBP-1, -2, and -3 in pancreas (Fig. 5). Both IGFBP-1 and -2 proteins showed a substantial increase with time in control neonates, whereas pancreatic IGFBP-3 protein production did not change during the neonatal period. In undernourished rats, IGFBP-1 and -2 were significantly decreased as compared with control values at 14 and 23 days of age, whereas IGFBP-3 values were lower than controls at each time point considered.

Pancreatic IGF/insulin-signaling pathway

To establish the mechanism underlying the neonatal spur of β-cell apoptosis and its partial suppression in malnourished animals, we analyzed the pancreatic IGF/insulin-signaling pathway during the neonatal life under basal conditions. IGF-1 and -2 survival and
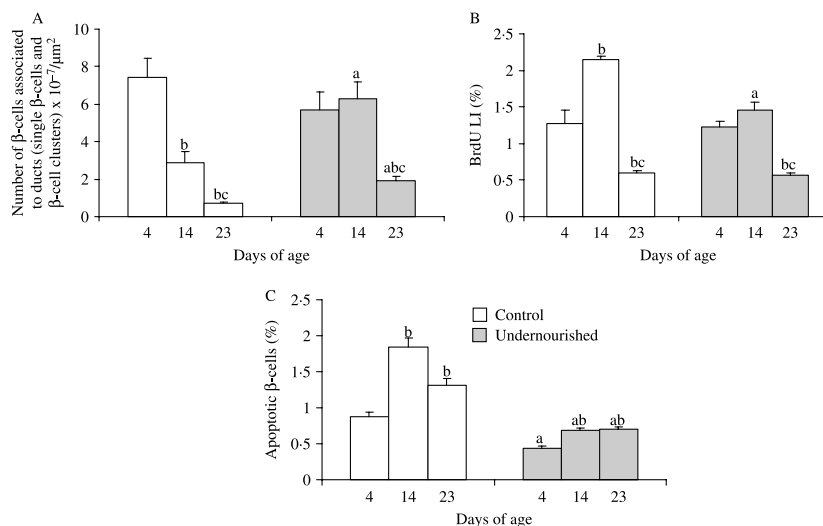
Figure 3 (A) β-Cell neoformation from ducts in control and undernourished neonatal pancreases was evaluated through quantification of single β-cells and β-cell clusters (2–4), incorporated into the ductal epithelium per unit of total tissue area. (B) Bromodeoxyuridine (BrdU) labeling index of β-cells represents β-cell replication rate in control and undernourished neonates. (C) β-Cell apoptosis rate in control and undernourished rats along the neonatal period. Apoptotic β-cells were quantified on sections double-immunostained for apoptosis and insulin. Values are mean ± S.E.M. for four to six observations in each experimental group and age. [a]$P<0.05$ relative to control rats at the same age; [b]$P<0.05$ relative to 4 days of age within the same condition; [c]$P<0.05$ relative to 14 days of age within the same condition.

growth-promoting actions mainly implicate the binding to IGF-1R, although, at high IGF concentrations, the IR can also be stimulated. For this reason, we determined the total pancreatic protein content of IGF-1R/IR and its tyrosine phosphorylation in control and food-restricted rats. IGF-1R protein content increased significantly in rats fed *ad libitum* on postnatal day 23, whereas the pancreatic production of this receptor did not change with age in undernourished neonates. However, the ratio P-IGF-1R/IGF-1R was significantly higher at 14 and 23 days of age in undernourished neonates as compared with control group (Fig. 6A). On the other hand, the IR levels increased with time in both populations without differences between control and undernourished animals. Again, when we normalized the IR rate of phosphorylation to total IR protein content, we observed a higher ratio in food-restricted neonates at 14 days of age (Fig. 6B) and no change along the suckling period in controls. The autophosphorylation of the IGF-1R/IR and activation of its tyrosine kinase activity trigger several signals including serine phosphorylation of Akt. Once activated, Akt regulates the expression and activity of many proteins involved in cell survival and apoptosis. We found no differences in total pancreatic Akt levels due to maternal malnutrition, although the activated form of Akt was markedly higher in the pancreas

from undernourished neonates as compared with controls from 14 days of age onwards (Fig. 6C). Because Akt phosphorylation of Bad on Ser136 causes its inactivation, preventing its association with the anti-apoptotic protein Bcl-x$_{S/L}$ and, therefore, resulting in increased cell survival, we analyzed both proteins by western blot (Fig. 6C). Bcl-x$_{S/L}$ protein expression gradually increased with age in both populations, although we did not find any difference between groups because of diet. On the contrary, and despite pancreatic Bad content also increased linearly with age in both groups of animals, total Bad protein was lower in food-restricted neonates than in controls at each time point considered. However, pancreatic phospho-Bad levels were similar in both groups. Another mechanism of Akt for promoting cell survival is the phosphorylation–inactivation of GSK-3α/β. We observed by western blot significantly lower activation of GSK-3α/β in the pancreas of food-restricted animals at all the neonatal ages studied. Since total pancreatic GSK-3α/β protein content peaked on day 14 similarly in control and undernourished neonates when we expressed the data as the ratio of phospho-GSK-3α/β/total GSK-3α/β, this ratio described a marked decrease at this age, which correlates with the increase on β-cell apoptosis observed in both groups (Fig. 6D).
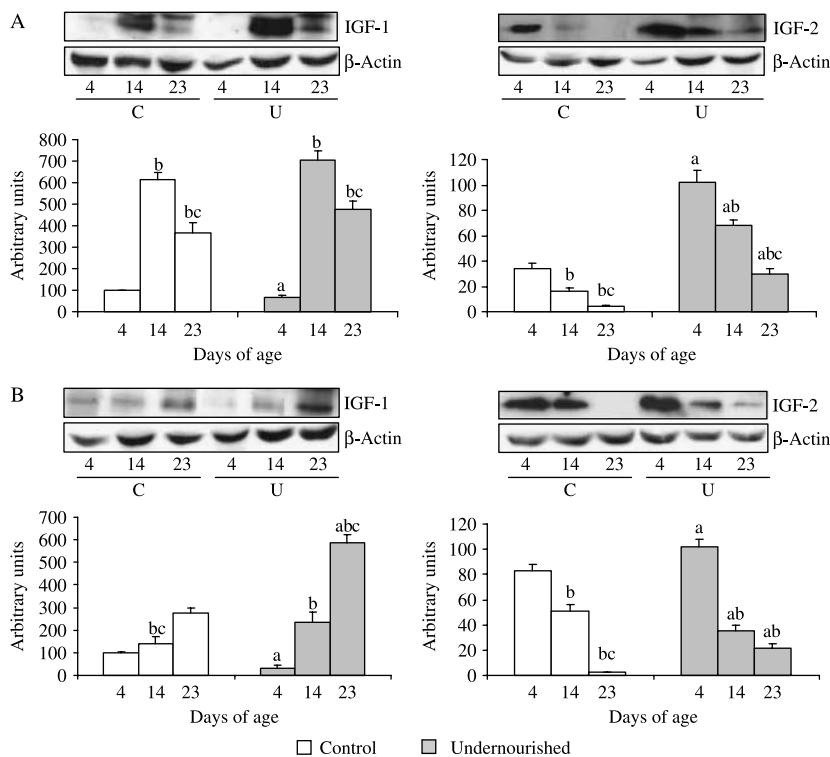
Figure 4 Pancreatic (A) and hepatic (B) IGF-2 and IGF-1 protein production in control and undernourished neonates at 4, 14, and 23 days of age. Representative western immunoblots are shown. Densitometric measurements of western immunoblots' bands are expressed as arbitrary units. Values are means ±s.e.m. for 10–12 observations in each experimental group. Neonates were obtained from six to eight different litters. [a]$P<0.05$ relative to control rats at the same age; [b]$P<0.05$ relative to 4 days of age within the same condition; [c]$P<0.05$ relative to 14 days of age within the same condition.

Discussion

After birth, the β-cell population is exposed to critical challenges such as lactation with a lipid-rich milk diet followed by weaning and the change to a solid diet high in carbohydrates. These events mark a change from a fetal phenotype of β-cells to adult phenotype with altered glucose thresholds and the ability to rapidly release insulin. In this context, any alteration of dietary milieu could affect endocrine pancreas remodeling and functionality. Herein, our results indicate that the timing and intensity of neonatal β-cell ontogeny are altered by undernutrition. However, although undernutrition evokes β-cell deficiency in neonates, the endocrine pancreas preserves the capacity to regenerate as a result of an active process of β-cell neogenesis and a lower incidence of β-cell apoptosis. Interestingly, the decrease in β-cell apoptosis coincides with pancreatic IGF-2 overproduction and increased survival of insulin/IGF signaling. Therefore, our data point to that locally produced IGFs may be instrumental for the endocrine pancreas capacity of adaptation to nutritional insult.

In this developmental stage, β-cell mass from undernourished neonates was markedly lower than control values along the study. Decreased β-cell mass correlates with hypoinsulinemia but normal glycemia during all the neonatal period. This phenomenon suggests increased insulin sensitivity, as we have previously demonstrated in our animal model at 10 days of age (Gavete *et al.* 2005) and 3-month-old rats (Escrivá *et al.* 1992). Similarly, others have reported that dietary insult such as diets with high-carbohydrate (Petrik *et al.* 2001) or low-protein content (Petrik *et al.* 1999) during lactation induces alterations in islet size, number, and composition. However, specific adaptations of the endocrine pancreas to the nutritional environment differ between animal models.

In agreement with our present results, it has been previously shown that pancreatic β-cells undergo dynamic proliferative activity during the first month of life (Kaung 1994, Scaglia *et al.* 1997). In addition to proliferation, β-cell neogenesis contributes to β-cell mass expansion not only during fetal development but also in postnatal life (Finegood *et al.* 1999, Paris *et al.* 2004, Bonner-Weir & Weir 2005). On this basis, in the
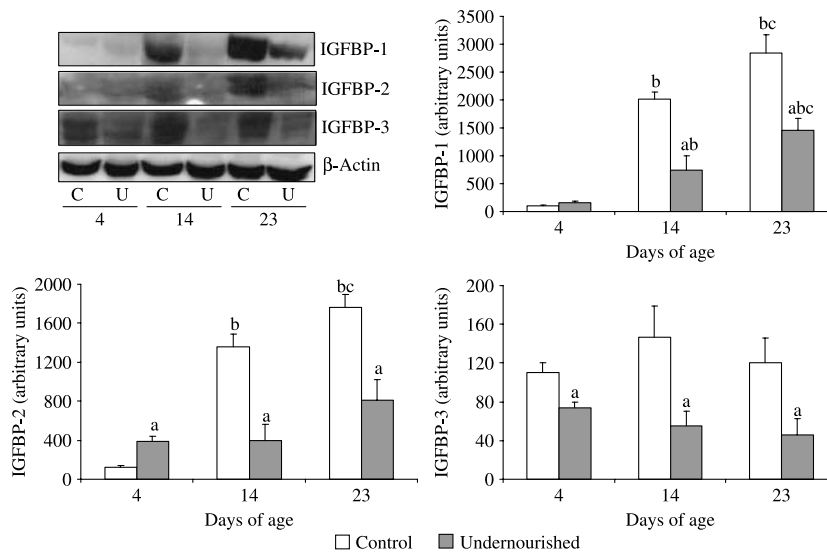
Figure 5 Pancreatic IGFBP-1, IGFBP-2, and IGFBP-3 protein levels in control and undernourished neonates at 4, 14, and 23 days of age. Blots representative of six independent determinations are shown. Results are the means ± s.e.m. of the densitometric measurements of western immunoblots' bands expressed as arbitrary units. Neonates were obtained from six to eight different litters. [a]$P < 0.05$ relative to control rats at the same age; [b]$P < 0.05$ relative to 4 days of age within the same condition; [c]$P < 0.05$ relative to 14 days of age within the same condition.

present study, we observed that β-cell neogenesis stayed markedly active for a longer time in undernourished neonates than in control animals, whereas β-cell proliferation was damaged on day 14. This indicates that there is a predominance of neogenesis versus replication in undernourished neonates, while, in the control group, spontaneous β-cell regeneration occurs mainly by replication. It is possible that undernutrition damages β-cells in such a manner that their ability to replicate is limited. So, although new islets can form by neogenesis in the undernourished animals it appears that they cannot get larger as reflected by the high number of very small islets in restricted pups at the end of the suckling period. This agrees with previous results obtained by our group after partial pancreatectomy of undernourished adult rats (Fernández *et al.* 2006) and with those observed in diabetic models induced by selective perfusion of alloxan (Waguri *et al.* 1997) or by the administration of low dose of streptozotocin (Bernard *et al.* 1999). In contrast, studies performed in rats submitted to a low-protein diet reported impaired islet cell proliferation and increased islet cell apoptosis (Petrik *et al.* 1999, Dumortier *et al.* 2007) but β-cell neogenesis when submitted to a low-energy diet (Garofano *et al.* 2000, Dumortier *et al.* 2007). Despite these discrepancies, there are several ways in which our data could be reconciled with the previously mentioned reports. First, the composition or the daily energy intake of the diet applied in other

undernutrition models differs from ours. Secondly, in our model of maternal undernutrition, pregnant rats exhibit impaired insulin secretion *in vivo* and glucose intolerance at the end of the gestation (Alvarez *et al.* 1997). This disturbance of maternal glucose homeostasis, similar to that reported in mild diabetic mothers (Holemans *et al.* 2003), may induce a specific program in the offspring β-cell growth capacity with later detrimental consequences in β-cell proliferative potential (Fernández *et al.* 2006). In any case, further investigations on all these animal models are required to fully and accurately explain the contrasts.

Since β-cell mass is the result of the balance between cell growth and death, we studied the incidence of β-cell apoptosis. Our results show, in accordance with the literature (Petrik *et al.* 1998), that there is a transient neonatal wave of β-cell apoptosis in the endocrine pancreas of control rats. Surprisingly, the incidence of β-cell apoptosis in food-restricted animals was significantly lower than in controls at all ages examined. Other authors have also reported that intrauterine malnutrition reduces the incidence of type 1 diabetes in non-obese diabetic mice altering β-cell apoptosis levels (Oge *et al.* 2007). Apparently, in view of our results, even increased β-cell neogenesis and decreased β-cell apoptosis are insufficient to restore normal β-cell mass when proliferation and β-cell mass are already affected. Recently, increased islet neogenesis in diabetes-prone rats without increased islet mass has also been reported (Kauri *et al.* 2007).
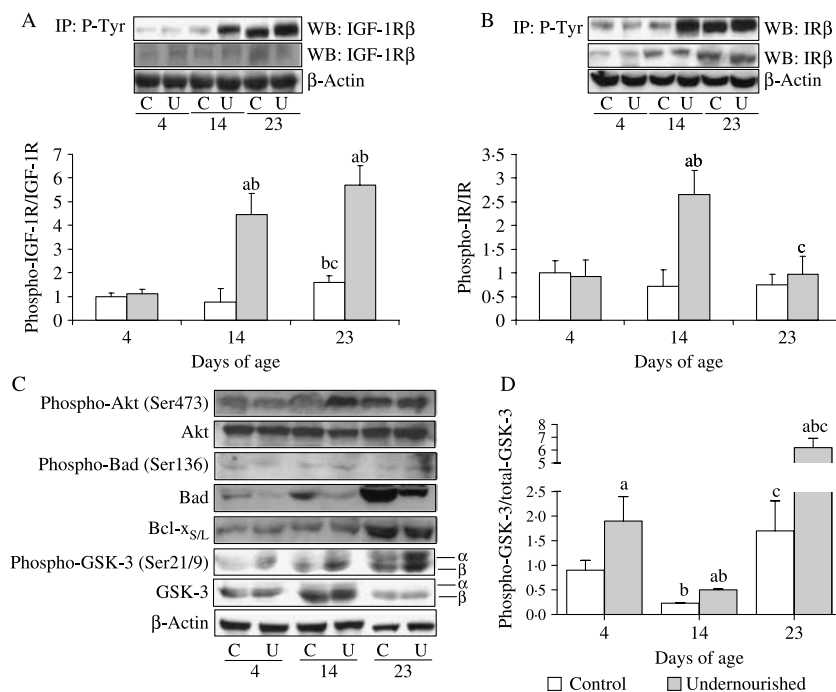
Figure 6 (A and B) Pancreatic content and phosphorylation of IGF-1/insulin receptor (IGF-1R/IR) in control and undernourished neonates at 4, 14, and 23 days of age. About 250 μg of pancreas protein extracts were subjected to immunoprecipitation (IP) with the anti-phospho-tyrosine (P-Tyr) antibody. The resulting immune complexes were analyzed by western blot (WB) with the anti-IGF-1R or IR antibody. Pancreases were homogenized, and total protein was submitted to western blot and immunodetected with antibodies against total IGF-1R (A), IR (B), total Akt, GSK-3α/β, Bad and Bcl-$x_{S/L}$ and the phosphorylated forms of Akt, GSK-3α/β, and Bad (C). Representative blots of at least four independent experiments are shown. The bars represent the ratio of phosphorylated to total form of IGF-1R, IR, and GSK-3α/β (A, B, and D respectively) from densitometric measurements expressed as means$\pm$s.e.m. [a]$P<0.05$ relative to control rats at the same age; [b]$P<0.05$ relative to 4 days of age within the same condition; [c]$P<0.05$ relative to 14 days of age within the same condition.

The loss of regenerative capacity may be caused by the lack of one or more rate-limiting trophic stimuli. In this context, the lack of expression of IGF-2 has been associated with developmental apoptosis in the pancreatic islet cells of neonatal rat (Petrik *et al.* 1998), whereas IGF-2 overexpression abolishes islet cell death (Hill *et al.* 2000). Interestingly, we have found in the present study that undernutrition enhances pancreatic IGF-2 expression in neonates, in agreement with previous works that have reported nutritional upregulation of IGFs in pancreas under conditions of global food restriction (Martín *et al.* 2005, Fernández *et al.* 2007, Gatford *et al.* 2008). However, it contrasts with the effect of low-protein diet on the IGF axis, which induces a reduction in pancreatic IGF-2 expression (Petrik *et al.* 1999). These differences may be due to the nature and severity of the nutrient deficit.

On the other hand, it has been described that IGF-1 is expressed in areas of regeneration after partial pan-createctomy (Smith *et al.* 1991, Fernández *et al.* 2006).

Therefore, it is likely that the increase in pancreatic IGF-1 observed on day 14 responds to the need for growth factors in focal areas, which are undergoing an active process of spontaneous remodeling. To our knowledge, it is the first time that a peak of pancreatic IGF-1 content is reported at this age. Thus, it is noteworthy that this peak coincides temporally with an important rise in the mitotic index of β-cells in control neonates, whereas it does with high levels of β-cell neogenesis in undernourished rats. Likewise, the effect of IGF-2 on β-cell expansion could be due in part to its function as islet neogenic factor; however, we hypothesize that the increased pancreatic IGF-2 production in undernourished neonates is mainly related to the partial suppression of the β-cell apoptosis observed. Nonetheless, because maternal food restriction enhances pancreatic IGF-2 content and delays its physiological drop in this tissue, high levels of IGF-1 and -2 are present in pancreas at the same time, which makes difficult to distinguish the specific effects of each growth factor. In any case, it is known that insulin and IGF-1/-2

mediate their effects via the IR and IGF-1R respectively. Considering the high degree of homology between the IR and the IGF-1R, and that IGF-2 binds with almost comparable affinity to both IR and IGF-1R (Nakae *et al.* 2001), it is likely that under our conditions of local high IGF-2 production, this ligand can also act via IR. Here, we show a higher phosphorylation of IGF-1R/IR in the pancreas from undernourished animals. IGF-1R/IR activation is critical for Akt-mediated survival signaling (Ueki *et al.* 2006). Concerning that, we have observed a higher Ser phosphorylation of Akt in food-restricted animals and, subsequently, a lower activation of GSK-3α/β during the suckling period. Previous studies have demonstrated that inhibition of GSK-3 protected β-cells from non-esterified fatty acids (NEFA)- or endoplasmic reticulum stress-induced apoptosis (Srinivasan *et al.* 2005, Mussmann *et al.* 2007). Certainly, GSK-3 may not be the only mediator of the partial suppression of β-cell apoptosis observed in undernourished neonates, as other distal signals have also been implicated in β-cell apoptosis.

The relative contribution of locally produced IGFs to β-cell expansion could be regulated within islets by changes in the pancreatic set of IGFBPs in response to age or nutritional insult. Since the affinity of IGFBPs for IGFs is greater than the affinity of IGF-1R towards IGFs (Mohan & Baylink 2002), the increase of pancreatic IGFBP production in control neonates at 14 and 23 days of age might prevent the interaction of IGFs with IGF-1R and, consequently, limit the survival actions of IGFs. On the contrary, the lower pancreatic levels of IGFBP-1, -2, and -3 in undernourished neonates could favor an increase in free IGF to interact with IGF-1R/IR. Furthermore, several *in vivo* and *in vitro* studies show IGF-independent actions of IGFBPs, including effects on cell apoptosis (Perks *et al.* 1999, Chen & Ferry 2006, Frommer *et al.* 2006). However, in view of the markedly high serum IGF-1 levels found in undernourished rats, we cannot exclude a systemic effect on β-cell growth.

In summary, our present data show that food restriction induces endocrine pancreas immaturity delaying the physiological drop of pancreatic IGF-2 and the β-cell neogenesis rate. Likewise, the partial suppression of the developmental wave of β-cell apoptosis in undernourished neonates could challenge the correct turnover of β-cells from fetal to adult phenotype, resulting in a loss of islet functionality as we have observed in our animal model at adult age (Martín *et al.* 2004). We hypothesized that this period of natural developmental plasticity of β-cells would allow the identification of mechanisms for β-cell replacement after depletion due to nutritional insult. Therefore, understanding differences in the relationship between the IGF system and processes of β-cell regeneration may lead to therapeutic and preventive interventions in diabetic patients with previous episodes of undernutrition.

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