Increased IRS-2 content and activation of IGF-I pathway contribute to enhance \( \beta \)-cell mass in fetuses from undernourished pregnant rats

Elisa Fernández,* M. Angeles Martín,* Susana Fajardo, Fernando Escrivá, and Carmen Álvarez

Departamento de Bioquímica y Biología Molecular II, Facultad de Farmacia, Universidad Complutense, Ciudad Universitaria, Madrid, Spain

Submitted 15 June 2006; accepted in final form 10 August 2006

Fernández E, Martín MA, Fajardo S, Escrivá F, Álvarez C. Increased IRS-2 content and activation of IGF-I pathway contribute to enhance \( \beta \)-cell mass in fetuses from undernourished pregnant rats. Am J Physiol Endocrinol Metab 292: E187–E195, 2007. First published August 15, 2006; doi:10.1152/ajpendo.00283.2006.—We have previously shown that fetuses from undernourished (U) pregnant rats exhibited an increased \( \beta \)-cell mass probably related to an enhanced IGF-I replicative response. Because IGF-I signaling pathways have been implicated in regulating \( \beta \)-cell growth, we investigated in this study the IGF-I transduction system in U fetuses. To this end, an in vitro model of primary fetal islets was developed to characterize glucose/IGF-I-mediated signaling that specially influences \( \beta \)-cell proliferation. We found that U fetal islets showed a greater replicative response to glucose and IGF-I than controls. Furthermore, insulin receptor substrate (IRS)-2 protein and its association with p85 were also increased. In the complete absence of IGF-I or stimulatory glucose, U islets presented an increased basal phosphorylation of IRS-2/PI3K/PKB pathway. These molecular changes may be responsible for the greater glucose/IGF-I islet replication and contribute to the increased \( \beta \)-cell mass found in these fetuses.

undernutrition; fetal islet; insulin-like growth factor I pathway; insulin receptor substrate-2

AN ADVERSE FETAL OR NEONATAL ENVIRONMENT can lead to changes in the development of a key endocrine axis with deleterious consequences for function in later life (reviewed in Ref. 16). In this regard, the endocrine pancreas seems to be particularly affected by the metabolic conditions of the mother. Perinatal period is considered to be critical for \( \beta \)-cell growth, and maternally derived changes in the fetal plasma concentration of nutrients clearly influence the development and function of the fetal endocrine pancreas (24). For example, maternal diabetes and experimentally induced hyperglycemia result in increased insulin secretion and hyperplasia of \( \beta \)-cells in the fetuses, while maternal malnutrition is generally associated with low insulin secretion and decreased development of \( \beta \)-cell (reviewed in Ref. 24). We have also shown that a 65% food restriction during the last week of gestation provoked glucose intolerance in pregnant rats, leading to an increase in \( \beta \)-cell mass and hyperinsulinemia in their fetuses at term (3). Consequently, the adaptation of the endocrine pancreas to an adverse intrauterine environment can lead to an inappropriate pancreatic \( \beta \)-cell mass, which is a well-known causative factor for subsequent type 2 diabetes (18). Identifying the factors and the mechanisms that control endocrine pancreas growth in physiological and pathological conditions has thus become a priority in the prevention of diabetes and later diseases.

The number of islet \( \beta \)-cells present at birth is mainly determined by the proliferation and differentiation of pancreatic progenitor cells in a process called neogenesis (28). The growth of the endocrine pancreas is controlled by many factors including nutrients, like glucose and certain amino acids, and a variety of peptide growth factors (22). Among these, insulin-like growth factors (IGFs) have been specially implicated in fetal pancreas development, since they potentiate \( \beta \)-cell growth, maturation, and function and are expressed by \( \beta \)-cells in early life (reviewed in Ref. 46). The potential of IGFs as \( \beta \)-cell mitogenic factors has been demonstrated in vitro on primary islets (23) and in \( \beta \)-cell lines (25) and in vivo using transgenic mice (17). Furthermore, the mitogenic action of IGF-I is glucose dependent, and glucose itself is able to promote \( \beta \)-cell proliferation in a manner that is dependent on glucose metabolism (25).

Recent investigations have focused on the importance of the IGF signaling transduction pathway to endocrine pancreas development. Growth-promoting effects of IGFs on \( \beta \)-cells are mediated by their binding to the IGF-I receptor (IGF-IR) and subsequent tyrosine phosphorylation of the insulin receptor substrate-2 (IRS-2) (47). Different studies have widely shown that IRS-2 plays a critical role in the regulation of \( \beta \)-cell growth. IRS-2 signaling has been implicated in \( \beta \)-cell mitogenesis and survival (33) and has also been associated with \( \beta \)-cell neogenesis in pancreatectomized rats (26). Downstream of IRS-2, the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (PKB)/mammalian target of rapamycin (mTOR) pathway seems to be highly related to cell cycle regulation. Expression of a constitutively active PI3K increases cell size by affecting mTOR and the ribosomal S6 kinase (p70S6K) activity (2). On the other hand, IGF-I can also activate the mitogen-activated protein kinase (MAPK) pathway, leading to activation of the extracellular signal-related kinases-1 and -2 (ERK1/
2), which are required for β-cell proliferation (35). In addition, glucose can activate these pathways in β-cells independently of growth factors (35). Taken together, these studies point out the relevance of these signalings as regulators of β-cell growth and show the importance of the study of the molecular mechanism that controls this process.

The aim of the present study was to investigate the intracellular glucose- and IGF-I-mediated signal transduction pathway(s) in islets of fetuses from undernourished (U) pregnant rats (65% daily reduction of food intake during the last week of gestation). These fetuses exhibited an increased β-cell mass that has been recently related to a higher stimulation of β-cell replication induced by an increase in both local IGF-I expression (36) and glucose oxidation (37).

MATERIAL AND METHODS

Animals and diets. Wistar rats bred in our laboratory under controlled temperature and an artificial dark-light cycle (from 0700 to 1900; 24-h clock) were used throughout the study. 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 (19 g protein, 56 g carbohydrate, 3.5 g lipid, 4.5 g cellulose/100 g, plus salt and vitamin mixtures) and were divided into two groups. Control (C) pregnant dams were fed ad libitum, and the U group received 35% of the food intake of a C pregnant dam during the third part of pregnancy. Water was given ad libitum. Food intake of C and U rats has been previously reported (15).

All studies were conducted in strict conformance with the National Institutes of Health guidelines for animal care, and the studies were submitted to and approved by the Bioethics Committee of the Universidad Complutense, Ciudad Universitaria, Madrid.

Islet isolation and culture. Islets from fetal rats (21.5 days of gestation) were obtained according to the method of Hellerstrom et al. (19). Briefly, five to seven pancreases were minced in sterile Hanks’ solution. The fragments were transferred to a sterile vial containing Hanks’ solution supplemented with 5–7 mg of collagenase (Boehringer Mannheim). The vial was shaken for 10 min at 37°C, and the tissue digest was washed three times with Hanks’ solution. The pellet was resuspended in culture medium and transferred to six plastic dishes containing the same medium. The culture medium consisted of RPMI 1640 (ICN, Nuclear Iberica) supplemented with 2 mmol/l glucose, penicillin, and streptomycin and 10% fetal bovine serum (ICN, Nuclear Iberica). The islets were maintained at 37°C in an atmosphere of 5% CO₂ during 2 days to allow for recovery and eliminate acinar cells. After that, the islets were gently detached from the plates, handpicked under a dissecting microscope, washed with PBS, and used for the diverse experiments. As it has been shown previously (1), the structural integrity of the islets after 2 days of culture was well preserved, and islets were ready to use for different functional studies.

Cell proliferation assay. A colorimetric immunooassay [Cell Proliferation ELISA, 5-bromo-2’-deoxyuridine (BrdU); Roche Diagnostic] was used for the quantification of cell proliferation. This method is based on the measurement of BrdU incorporation into genomic DNA during DNA synthesis of proliferating cells. Islets were cultured for 2 days with 11 mM glucose and maintained in a serum-free medium with 3 mM glucose for 20 h. After that, groups of 20 islets were seeded in 96-well multiwells and grown for 48 h at different glucose concentrations (3, 9, and 17 mM glucose) with or without IGF-I (1–1,000 ng/ml). During the last 24 h, they were labeled by the addition of BrdU. Then the anti-BrdU antibody was added, and the immune complexes were detected by the subsequent substrate (tetramethylbenzidine) reaction and quantified by measuring the absorbance at 620 nm, using a scanning multiwell spectrophotometer (ELISA reader).

Islet protein determination. Batches of 300–500 islets cultured for 2 days with 11 mM glucose were maintained in a serum-free medium with 3 mM glucose for 20 h. After that, islets were incubated at 37°C with basal (3 mM) or stimulatory (17 mM) concentrations of glucose with or without 100 ng/ml IGF-I (Recombinant Human IGF-I, R&D Systems) at 5, 10, or 15 min. After the incubations, the islets were sonicated in a homogenization 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 X, pH 7.6). The tissue extracts were centrifuged at 12,000 rpm at 4°C for 15 min, and the supernatant was used for the determination of protein. The concentration of protein was determined by the Bradford method (4) using a protein assay (Bio-Rad Laboratories), with γ-globulin as standard.

Immunoprecipitation. Islet extracts containing 150–300 μg of protein were immunoprecipitated overnight at 4°C with gentle rotation in the presence of 2–5 μg of the corresponding primary antibody, followed by the addition of protein A-agarose (Roche Diagnostic, Indianapolis, IN) or anti-mouse IgG-agarose (Sigma, St. Louis, MO) for the rabbit polyclonal and mouse monoclonal antibodies, respectively. 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. Equal amounts of protein were submitted to SDS-PAGE on 10% polyacrylamide gels at 125 V. Proteins were then electrophoretically transferred to polyvinylidene difluoride (PVDF) filters (PVDF Protein Sequencing Membrane, Bio-Rad Laboratories) at 100 V for 2 h. After the transfer, the filters were blocked with 5% (wt/vol) nonfat dry milk (for general antibodies) or 3% BSA (for anti-phosphotyrosine antibodies) in PBS followed by incubation with primary antibodies overnight. The PVDF filters were then washed four times for 10 min at 37°C with PBS and 0.1% Tween 20, followed by 1-h incubation with appropriate secondary antibody conjugated to horseradish peroxidase (Sigma BioSciences, St. Louis, MO). The PVDF membranes were then washed as indicated above and subsequently exposed to an enhanced chemiluminescence reagent (Amer sham Life Sciences, Little Chalfont, Buckinghamshire, UK). The bands were quantified by laser scanning densitometry (Molecular Dynamics, Sunnyvale, CA). Immunoblots were performed under conditions of linearity according to the amount of protein loaded on the gel. The PVDF filters were finally stained with Coomassie blue to confirm that, in the same Western assay, equal amounts of protein were analyzed as well as to ensure the heterogeneity of the protein composition pattern in the different samples.

Antibodies used. Antibodies included the following: anti-IGF-IR β-subunit sc713 (Santa Cruz Biotechnology, Santa Cruz, CA); anti-IRS-2 (Upstate Biotechnology, Lake Placid, NY); anti-p85, which recognizes all variants of p85α and p85β (Upstate Biotechnology); anti-phosphotyrosine (Upstate Biotechnology); anti-pKB and anti-phospho-pKB, recognizing phosphorylated Ser547 of PKB (Cell Signaling Technology, Beverly, MA); anti-PI3K, sc216 (Santa Cruz Biotechnology) and anti-phospho-PI3K, recognizing phosphorylated Thr410 (Cell Signaling Technology); anti-phospho-OSK3α/β, recognizing phosphorylated Ser47 of cyclic synapse kinase-3α (GSK3α) or Ser49 of GSK3β (Cell Signaling Technology); anti-mTOR (Upstate Biotechnology); anti-phospho-mTOR, recognizing phosphorylated Ser2448 of mTOR (Biosource International); anti-p70S6K and anti-phospho-p70S6K, recognizing phosphorylated Thr389/Ser424 of p70S6K (Cell Signaling Technology); anti-4E-BP1 sc6936 (Santa Cruz Biotechnology) and anti-phospho-4E-BP1, recognizing phosphorylated Thr70 of the initiation factor 4E-binding protein (4E-BP1) (Cell Signaling Technology); anti-ERK1/2 and anti-phospho-ERK1/2, recognizing phosphorylated Thr202/Tyr204 of ERK1/2 (Cell Signaling Technology).

Statistics. Values are given as means ± SE. The two-tailed t-test for independent observations was used for comparisons of the two
groups. For multiple comparisons, significance was evaluated by two-way analysis of variance (ANOVA), followed by the protected least significant difference test.

RESULTS

Characteristics of the U animals. The present animal model matches all physiological characteristics of those reported in previously published studies (3, 36, 37, 38). At the end of gestation, body weight of U mothers was significantly lower than that of C mothers (394.1 ± 12.3 vs. 312.2 ± 11.5 g, P < 0.05). Accordingly, maternal food restriction provoked a clear decrease in body weight of fetuses at term compared with C (5.1 ± 0.2 g, P < 0.05). Nevertheless, no significant differences were found in the size of C and U fetal islets.

Proliferation response induced by glucose and IGF-I in pancreatic fetal islets. Because glucose and IGF-I are able to stimulate β-cell growth in the fetal period in a synergistic way, we addressed the effect of increasing IGF-I concentrations on islet proliferation in C and U fetal islets at different glucose concentrations. After 2 days of culture, isolated fetal islets were cultured with 3 mM glucose without serum for 20 h. Then groups of 20 islets were treated for 2 more days with 3, 9, or 17 mM glucose with or without IGF-I at different concentrations (1–1,000 ng/ml). Four different wells (samples) for each condition and 4 different experiments (n = 16) were used for statistical purposes. Results are expressed as the relative increase over the value of C group at 3 mM glucose without IGF-I and are means ± SE. *P < 0.05 when U group is compared with the respective C group within the same treatment. †P < 0.05 when a group (C or U) is compared with the value of the identical group (C or U) at 3 mM glucose without IGF-I. *P < 0.05 when a group (C or U) is compared with the value of the identical group (C or U) without IGF-I within the same glucose concentration.

To assay the protein content of IRS-2, C and U islets were cultured with 3 mM glucose and without serum for 20 h. Islets were lysed, and their total protein content was evaluated. Because no difference was found between C and U islets in total protein content (0.72 ± 0.2 ng/islets, respectively), this parameter was used for normalization of samples in the immunoblot analysis.

IRS-2 protein levels and its association with p85 in pancreatic fetal islets. The autophosphorylation of the IGF-IR and activation of its tyrosine kinase activity lead to tyrosine phosphorylation of various intracellular substrates, including IRS-2. To assay the protein content of IRS-2, C and U islets were
cultured for 20 h in a serum-free medium containing 3 mM glucose. After the culture period, islets were lysed, and equal protein content (100 µg) was analyzed by Western blotting with anti-IRS-2 antibody. As shown in Fig. 3A, the protein levels of IRS-2 were twofold increased in islets from U fetuses compared with those in C islets. However, we could not detect the tyrosine phosphorylation of IRS-2 because it was below the sensitivity of the immunoblotting techniques employed.

Once phosphorylated, IRS-2 binds proteins that contain Src homology 2 domains, such as the p85 regulatory subunit of PI3K. To evaluate the association of IRS-2 with the p85 subunit of PI3K, fetal islets were cultured for 20 h in a serum-free medium containing 3 mM glucose. Then islets were stimulated for 5 min with 3 and 17 mM glucose with or without IGF-I. Next, equal amounts of protein (300 µg) were immunoprecipitated with the 85-kDa regulatory subunit of PI3K antibody and immunoblotted with anti-IRS-2 and anti-p85 antibodies. As shown in Fig. 3B, total p85 levels were equivalent between C and U fetal islets. IRS-2-p85 complexes were ninefold higher in U islets than in C at the basal glucose concentration. When glucose was augmented in the culture medium to 17 mM, the association of IRS-2 with p85 increased in C islets but did not change in U. Similarly, the presence of IGF-I in C islets led to marked increases of this association, both at 3 and 17 mM, but in U islets the IRS-2-p85 complexes were further increased only in presence of 17 mM glucose and IGF-I. Levels of IRS-2 binding the p85-kDa regulatory subunit of PI3K were significantly higher in the U group throughout the study.

Protein expression and phosphorylation of PKB, PKCζ, and GSK3α/β. To investigate the participation of downstream proteins of the PI3K pathway, C and U fetal islets were cultured for 20 h in a serum-free medium containing 3 mM glucose and treated for different time periods with basal (3 mM) or stimulating (17 mM) concentrations of glucose, with or without IGF-I. Then islets were lysed and separated by SDS-PAGE, and equal amounts of protein (70 µg) were blotted with anti-PKB, anti-phospho-PKB, anti-GSK3α/β, anti-PKCζ, and anti-phospho-PKCζ antibodies. The maximum activation time was established at 10 min for PKB and 15 min for PKCζ and GSK3α/β (data not shown).

As shown in Fig. 4A, the amount of total PKB was not altered in the U group. The presence of 17 mM glucose significantly increased PKB phosphorylation in C islets (1.8-fold). However, PKB was found significantly increased at 3 mM glucose (8-fold) and remained constitutively activated in the presence of 17 mM glucose in U rats compared with C. In both groups, the magnitude of the phosphorylation was further increased by the addition of IGF-I. In all cases, the amount of phosphorylated PKB in the U group was markedly higher than in C.

Once phosphorylated, PKB can evoke PKCζ activation. Total PKCζ levels were equivalent between samples in both C and U fetal islets (Fig. 4B). PKCζ phosphorylation was increased in C islets with 17 mM glucose (2.1-fold) and further by the addition of IGF-I. Compared with C, U islets showed a significantly higher basal phosphorylation of PKCζ (5.2-fold) that was only slightly increased in the presence of both glucose (17 mM) and IGF-I.

Downstream of PKB, phosphorylation of GSK3α/β takes place. Compared with C, in U islets GSK3α/β phosphorylation was significantly increased at basal (3 mM) glucose (1.7-fold). In the C group, increasing glucose concentration and the additional presence of IGF-I at 3 mM enhanced the amount of GSK3α/β phosphorylation. Unlike PKB and PKCζ, there were no differences in GSK3α/β phosphorylation between C and U islets under these conditions (Fig. 4C).

Protein expression and phosphorylation of mTOR, p70S6K, and 4E-BP1. PKB phosphorylation of mTOR leads to mTOR activation, which in turn phosphorylates at least two proteins involved in translational control of protein synthesis, 4E-BP1 and p70S6K. Besides, glucose itself can activate mTOR independently of PKB. To evaluate the effect of glucose and IGF-I in these proteins, C and U fetal islets were cultured for 20 h in a serum-free medium containing 3 mM glucose and stimulated for different time periods with basal (3 mM) and stimulating (17 mM) concentrations of glucose, with or without IGF-I. Then islets were lysed and separated by SDS-PAGE, and equal amounts of protein (100 µg) were blotted with anti-mTOR, anti-phospho-mTOR, anti-p70S6K, anti-phospho-p70S6K, anti-4E-BP1, and anti-phospho-4E-BP1 antibodies. The maximum activation time was established at 15 min (data not shown).

Figure 5A shows that there were no differences in mTOR total content between U and C fetal islets. The presence of IGF-I at 3 mM glucose and the increase in glucose concentration were able to induce mTOR phosphorylation in C islets. Compared with C, in U islets, the mTOR phosphorylation was
significantly increased at basal (3 mM) glucose (2.4-fold). In U islets, increasing glucose concentration enhanced the mTOR phosphorylation, which slightly increased further in the presence of IGF-I. Thus mTOR phosphorylation was ever higher in the U group compared with C.

As shown in Fig. 5B, glucose and IGF-I stimulation evoked an increase in the phosphorylation of p70S6K in C islets. In the U group, p70S6K phosphorylation was found elevated at basal (3 mM) glucose (6.5-fold over C value), and it underwent a slight increase in the presence of 17 mM with IGF-I. Therefore, p70S6K phosphorylation was significantly higher in the U group than in the C group, even though total p70S6K levels were similar in both populations.

No differences were found in 4E-BP1 protein content between C and U islets (Fig. 5C). In C and U islets, the phosphorylation of 4E-BP1 was increased in the presence of 17 mM glucose and further enhanced by the addition of IGF-I. However, levels of phosphorylated 4E-BP1 were significantly higher in the U group than in the C group, even though total p70S6K levels were similar in both populations.

Protein expression and phosphorylation of ERK1/2. Activation of ERK1/2 has been shown to be an essential requirement for glucose- and IGF-I-induced β-cell mitogenic responses. Consequently, to evaluate potential effects of undernutrition on the ERK1/2 pathway, we determined the glucose- and IGF-I-induced ERK phosphorylation in C and U fetal islets. Therefore, fetal islets were cultured for 20 h in a serum-free medium containing 3 mM glucose and treated for different time periods with basal (3 mM) and stimulating (17 mM) concentrations of glucose with or without IGF-I. Then, islets were lysed and separated by SDS-PAGE, and equal amounts of protein (40 μg) were blotted with anti-ERK1/2 and anti-phospho-ERK1/2 antibodies. The maximum activation time was established at 15 min (data not shown).

As shown in Fig. 6, undernutrition did not alter the total amount of ERK1/2. At basal (3 mM) glucose, IGF-I promoted the activation of ERK1/2 in C and U islets. Increasing the glucose concentration to 17 mM further enhanced phosphorylation in both groups of islets. There was no difference in phosphorylated ERK1/2 between C and U islets.

DISCUSSION

In this work, we have investigated the IGF-I signal transduction system in islets from fetuses with increased β-cell mass proceeding from U pregnant rats (36). To this end, we have developed an in vitro model of primary fetal islets in which elements of the above-mentioned signal transduction pathway are present and can be stimulated by glucose and IGF-I. Through this in vitro system, we have established that, compared with C, U fetal islets show a higher protein content of IRS-2 and an increased basal and glucose/IGF-I-induced phosphorylation of several proteins implicated in the mitogenic and survival pathway.
In the present study, islet mitogenesis was evaluated in freshly isolated islets that were cultured with 11 mM glucose for 48 h. After that, islets were maintained for 20 h in a serum-free medium with 3 mM glucose. This experimental procedure allowed us to obtain better physiological conditions and alleviate the effects on the IGF-I signaling of exposing the islets to glucose and serum for a prolonged period of time (5). By using this in vitro system, we found that glucose in the physiological range (3–17 mM) was able to stimulate islet cell replication in fetal islets, especially in the U group. Thus, the additional presence of IGF-I (10–1,000 ng/ml) induced a greater islet replication in both groups. As previously shown in pancreatic β-cells (25), the effect of IGF-I on increased DNA synthesis in C islets was apparent only at 9 and 17 mM glucose; however, in the U group, this increase was also evident at 3 mM glucose. These results suggest that U fetal islets show increased sensitivity to glucose and to IGF-I compared with C.

To identify the mechanism underlying the islet replication response observed in the U population, we evaluated in fetal islets the levels of different proteins implicated in the IGF-I mitogenic pathway and their phosphorylation on stimulation by IGF-I and glucose. It is widely known that the growth effect of IGF-I is mediated by its interaction with IGF-IR and the subsequent activation of IRS-2 within the β-cells (47). Here we show that IGF-IR content and its phosphorylation on IGF-I stimulation were increased in U fetal islets compared with C. Although IGF-IR is not crucial for β-cell development (29), it seems to be instrumental in activating IRS-2-mediated signaling, strongly implicated in β-cell growth. Therefore, the increased IGF-IR content and phosphorylation shown by U islets may be favoring the mitogenic action of IGF-I, which is also enhanced in U fetal pancreas (36).

Unlike IGF-IR, the importance of IRS-2 to pancreatic β-cell growth has become apparent from several in vivo and in vitro studies. Increased IRS-2 in vitro can promote β-cell replication, neogenesis, and survival, whereas decreased IRS-2 causes β-cell apoptosis (reviewed in Ref. 12). In addition, transgenic expression of IRS-2 in vivo promotes β-cell survival (20), while IRS-2 knockout mice show a marked decrease in β-cell mass (48). Interestingly, we found that IRS-2 protein levels were markedly increased in U islets. As previously shown in INS-1 cells (34) and pancreatic islets (41), the endogenous expression levels of IRS-2 can be specifically increased by glucose. This effect seems to be dependent on glucose metabolism and requires a downstream increase in cytosolic Ca$^{2+}$ concentration (32). In addition, enhanced glucose oxidation is also able to increase IRS-2 levels via cAMP response element-binding protein (CREB) activation (27). Accordingly, we have previously demonstrated that U islets show a greater glucose oxidation (37). Therefore, we suggest that the enhanced content of IRS-2 found in these islets can be related to the higher glucose oxidative metabolism shown by the U group.

Increased IRS-2 in U islets was accompanied by a rise in both basal and glucose/IGF-I-induced association of IRS-2 with p85, the regulatory subunit of PI3K. Surprisingly, the increased IRS-2 in fetal pancreas (36).
magnitude of the increase found in the basal association of p85 with IRS-2 in U islets was greater than that expected just from the increase of IRS-2 content. Thus it seems fair to assume that tyrosine phosphorylation of IRS-2 could be augmented in U islets under basal conditions too. In this regard, it has been shown recently (32) that glucose not only regulates IRS-2 expression levels but also IRS-2 intracellular localization. At stimulatory glucose concentrations (32) or in the presence of IGF-I (33), IRS-2 is translocated to the β-cell plasma membrane where it can be tyrosine phosphorylated, leading to an increase in IRS-2/p85 association. Accordingly, it is possible that, in U islets at 3 mM glucose, IRS-2 was mostly localized in the plasma membrane, favoring the tyrosine phosphorylation of IRS-2. Although there was no activation of the IGF-IR at 3 mM glucose in fetal islets, IRS-2 could then be tyrosine phosphorylated by basal tyrosine kinase activities localized in the basal membrane (40, 43).

The increase in IRS-2/p85 association in both populations leads to a consequential increase in PKB phosphorylation. PKB has been involved in the regulation of β-cell mass and function (14). The phosphorylation activation of this protein plays a pivotal role mediating β-cell mitogenesis (12) and β-cell survival (45, 49). Besides, it has been shown that glucose itself can promote pancreatic β-cell survival through activation of PI3K/PKB signaling pathway (44). It can be assumed then that the greater PKB phosphorylation activation

Fig. 5. Protein content and phosphorylation of mammalian target of rapamycin (mTOR), p70S6K, and the initiation factor 4E-binding protein1 (4E-BP1) on glucose/IGF-I stimulation. Two-day-cultured U and C fetal islets were cultured at 3 mM glucose without serum for 20 h. After this period, islets were cultured for 15 min with 3 or 17 mM glucose with or without 100 ng/ml IGF-I. C and U islets were lysed, and total protein (40 µg) was submitted to SDS-PAGE and analyzed by Western blotting with the anti-phospho-mTOR and anti-mTOR antibodies (A), the anti-phospho-p70S6K and phospho-p70S6K antibodies (B), and anti-phospho-4E-BP1 and anti–4E-BP1 antibodies (C). Blots representative of 6 independent determinations are shown. Bars represent the relative increase over C value at 3 mM glucose without IGF-I and are means ± SE. a P < 0.05 when a group (C or U) is compared with the value of the identical group (C or U) at 3 mM glucose without IGF-I. b P < 0.05 when a group (C or U) is compared with the value of the identical group (C or U) without IGF-I within the same glucose concentration.

Fig. 6. Protein content and phosphorylation of ERK1/2 on glucose/IGF-I stimulation. Two-day-cultured U and C fetal islets were cultured at 3 mM glucose and without serum for 20 h. After this period, islets were cultured for 15 min with 3 or 17 mM glucose with or without 100 ng/ml IGF-I. C and U islets were lysed, and total protein (40 µg) was submitted to SDS-PAGE and analyzed by Western blotting with the anti-phospho-ERK1/2 and anti-ERK1/2 antibodies. Blots representative of 6 –7 independent determinations are shown. Bars represent the relative increase over C value at 3 mM glucose without IGF-I and are means ± SE. a P < 0.05 when a group (C or U) is compared with the value of the identical group (C or U) at 3 mM glucose without IGF-I. b P < 0.05 when a group (C or U) is compared with the value of the identical group (C or U) within the same glucose concentration.
found in U islets, even at basal conditions, could lead to increased β-cell replication and promote survival under restricted conditions.

To determine the role of PKB in mediating mitogenic response in fetal islets, we examined downstream PKB targets that could be involved in promoting this process. PKB can activate an atypical isoform of PKC (PKCζ) that has been implicated in increasing β-cell mitogenesis in response to glucagon-like peptide-1 (GLP-1) (8) and to IGF-I (21). In this regard, basal and glucose/IGF-I-induced PKCζ phosphorylation was increased in U islets compared with C, suggesting that PKCζ could be implicated in the increased replication response found in U islets. Unlike PKCζ, the increased PKB phosphorylation in U islets did not evoke higher levels of GSK3α/β in response to IGF-I and/or glucose, and it was increased only under basal conditions. It can be assumed then that GSK3α/β is not implicated in the increased replication response found in U islets. Nevertheless, inactivation by phosphorylation of GSK3α/β decreases phosphorylation of β-catenin, which has been previously associated with increased β-cell survival (9). Therefore, the increased phosphorylation found in the basal situation in U islets could be playing an important role in promoting β-cell survival at low levels of glucose.

Downstream ofPKB, mTOR regulates cell growth through at least two proteins, 4E-BP1 and p70S6K, whose activation results in a general increase in protein synthesis in β-cells (39). Our results showed that the phosphorylation levels of these proteins were again significantly enhanced in U fetal islets. These findings suggest that the greater activation of these proteins is related to the higher mitogenic response observed in U islets. In addition, it has been shown that glucose can induce mTOR (11) and ERK1/2 (7) phosphorylation in β-cells independently of IRS signaling. It is reasonable to suppose then that the altered glucose oxidation reported in U islets could also be contributing to increased DNA synthesis in these islets by activation of mTOR independently of IRS-2 stimulation. As recently suggested (30), the appropriate regulation of mTOR by nutrients may be an effective strategy to enhance growth and proliferating capacity of β-cells. However, glucose activation in U islets did not evoke an increased stimulation of ERKs compared with C, supporting the idea that glucose-induced ERK activation is mediated by a distinct signaling pathway from mTOR (7).

The increased glucose/IGF-I activation of the PI3K pathway found in U islets correlated with an enhanced glucose- and IGF-I-induced mitogenesis except at basal (3 mM) glucose. Interestingly, in this basal condition, U islets showed increased phosphorylation activation of several proteins implicated in DNA synthesis such as PKB, PKCζ, mTOR, 4E-BP1, and p70S6K but not ERK1/2. Thus these results suggest that a minimum ERK activation needs to be reached to elicit the greater mitogenic response in U islets. As previously shown in the pancreatic β-cell line INS-1 (13) and in the rat clonal β-cell line RIN 1046–38 (21), there is a requirement for the activation of both ERK1/2 and PI3K signal transduction pathways for a full commitment to induce pancreatic β-cell mitogenesis.

Finally, it is worth remarking that the effects of undernutrition on β-cell in our model are different from those described in other models of maternal low-protein diets (42) and intrauterine growth restriction (31), in which β-cell mass and β-cell replication were decreased. However, it has been shown recently that several processes leading to β-cell failure and death seem to be preceded by periods of β-cell hyperfunction (10). It is possible that our U islets are in such a previous hyperfunction stage and that this overstimulation leads to a decrease in β-cell mass later, as it has previously been shown at 4 days of life and at adult age (38). In support of this idea, a recent report (6) has demonstrated that chronic activation of mTOR, as in our U islets, could lead to a decreased IRS-2 expression and an increased β-cell apoptosis, a mechanism that may be contributing to reduce β-cell mass.

In conclusion, we have demonstrated that U islets are characterized by multiple alterations in the IGF-I signal transduction system, leading to an improved activation of the IRS-2/PI3K/PKB/mTOR pathway and to a better mitogenic response to glucose and IGF-I. Thus, all these molecular changes may be contributing to the increased β-cell mass found in U fetuses. Since an increase in IRS-2 has a role in promoting β-cell survival (34) and neogenesis (26), the finding of a raised content of this factor suggests that both processes might also participate in the improved β-cell mass exhibited by U fetuses. Interestingly, the in vitro system shown herein represents a valuable tool for testing the effect that different factors may have on important aspects of β-cell life such as proliferation, survival, or neogenesis. Understanding the intracellular signaling molecules implicated in β-cell growth under physiological and pathological conditions will provide future strategies aimed at the prevention and the treatment of diabetes and other related metabolic disorders.

ACKNOWLEDGMENTS

We thank Dr. Luis Goya for critical review of this manuscript.

Parts of this work were presented at the 41st European Association for the Study of Diabetes (EASD), Athens, Greece (Diabetologia 48, Suppl 1: 160, 2005).

Present addresses: M. A. Martín, Departamento de Metabolismo y Nutrición, Instituto del Frío, Consejo Superior de Investigaciones Científicas, Madrid, Spain, and S. Fajardo, Instituto de Recursos Materiales, Centro de Ciencias Medioambientales, Consejo Superior de Investigaciones Científicas, Madrid, Spain.

GRANTS

This work was supported by Grant Ref. No. BFU2005-02849 from the Ministerio de Ciencia y Tecnología of Spain.

REFERENCES


6. Briaud I, Dickson LM, Linghur MK, McCuaig JF, Lawrence JC, Rhodes CJ. Insulin receptor substrate-2 proteosomal degradation mediated by a mammaliam target of rapamycin (m-TOR)-induced negative


