Role of IGFBP-3 in the Regulation of β-Cell Mass during Obesity: Adipose Tissue/β-Cell Cross Talk

Nuria Palau,† Sandra A. Rebuffat,† Jordi Altirriba, Sandra Piquer, Felicia A. Hanzu, Ramon Gomis,* and Albert Barbera*

Diabetes and Obesity Laboratory (N.P., S.A.R., J.A., S.P., F.A.H., R.G., A.B.), Endocrinology and Nutrition Unit, Institut d'Investigations Biomediques August Pi i Sunyer (IDIBAPS), Hospital Clinic de Barcelona, and Centro de Investigación Biomédica en Red de Diabetes y Enfermedades Metabólicas Asociadas (CIBERDEM) (S.A.R., J.A., S.P., F.A.H., R.G.), E-08036 Barcelona, Spain

In obesity an increase in β-cell mass occurs to cope with the rise in insulin demand. This β-cell plasticity is essential to avoid the onset of hyperglycemia, although the molecular mechanisms that regulate this process remain unclear. This study analyzed the role of adipose tissue in the control of β-cell replication. Using a diet-induced model of obesity, we obtained conditioned media from three different white adipose tissue depots. Only in the adipose tissue depot surrounding the pancreas did the diet induce changes that led to an increase in INS1E cells and the islet replication rate. To identify the factors responsible for this proliferative effect, adipose tissue gene expression analysis was conducted by microarrays and quantitative RT-PCR. Of all the differentially expressed proteins, only the secreted ones were studied. IGF binding protein 3 (*Igfbp3*) was identified as the candidate for this effect. Furthermore, in the conditioned media, although the blockage of IGFBP3 led to an increase in the proliferation rate, the blockage of IGF-I receptor decreased it. Taken together, these data show that obesity induces specific changes in the expression profile of the adipose tissue depot surrounding the pancreas, leading to a decrease in IGFBP3 secretion. This decrease acts in a paracrine manner, stimulating the β-cell proliferation rate, probably through an IGF-I-dependent mechanism. This cross talk between the visceral-pancreatic adipose tissue and β-cells is a novel mechanism that participates in the control of β-cell plasticity. **(*Endocrinology* 153: 177–187, 2012)**

Under normal conditions β-cells can increase their insulin secretion sufficiently to respond to the organism's demands and, in the case of obesity, to overcome the rise in insulin demands. This ability is partially due to an expansion in pancreatic β-cell mass (1, 2). The capacity of β-cell mass to increase is a key factor in the pathogenesis of type 2 diabetes. When this response is efficient enough, hyperglycemia can be avoided, this being the case for approximately two thirds of obese subjects (3). However, in the remaining one third, type 2 diabetes finally appears because the β-cell mass is insufficient to cope with the development of insulin resistance. Indeed, it has been demonstrated that β-cell mass in type 2 diabetic patients not only fails to expand but is also reduced by about 50% of normal levels (4).

This β-cell mass compensatory response can be achieved through β-cell replication, β-cell hypertrophy, or the formation of new islets through neogenesis (5). The dominant mechanism in humans remains unclear, but in adult rodents it appears to be β-cell replication (6). Nevertheless, the molecular mechanisms that control the obesity-induced β-cell mass increase are not completely understood, although important advances have been made in this field. For example, the insulin receptor substrate (IRS)-2/phosphatidylinositol 3-kinase (PI3K)/AKT signaling pathway has been shown to be critical for β-cell sur-

ISSN Print 0013-7227 ISSN Online 1945-7170
Printed in U.S.A.

doi: 10.1210/en.2011-0181 Received February 16, 2011. Accepted October 14, 2011.
First Published Online November 8, 2011

* R.G. and A.B. contributed equally to this study.
† N.P. and S.A.R. are coauthors of this paper.
Abbreviations: BrdU, Bromodeoxyuridine; CM, conditioned medium; eWAT epididymal depot; IGFBP3, IGF binding protein 3; IGTT, ip glucose tolerance test; IRS, insulin receptor substrate; iSUB, inguinal sc depot; PI3K, phosphatidylinositol 3-kinase; pMES, pancreatic mesenteric depot; Sfrp4, secreted frizzled-related protein 4.

vival and growth. However, the extracellular signals that participate in this compensatory mechanism are less clear. Glucose metabolism seems to play a critical role (7), but a baseline IRS-2/PI3K/AKT activation is also an essential requirement. Although most evidence suggests that insulin is the activator for the IRS-2/PI3K/AKT pathway (8), IGF-I may also exert a compensatory effect when the insulin signal is not functional (9).

Adipose tissue has always been considered to be an energy store with few interesting attributes. However, it has now been clearly shown that adipocytes integrate a wide array of physiological processes (10), all of which are coordinated mainly through the synthesis and release of adipokines, which can act either locally or through the blood stream (11). It is worth noting that several of the adipokines secreted by the adipocyte have proliferative properties. This capacity is greater in obese patients and significantly correlates with the size of the adipocyte (12).

Taking into account the key role of adipose tissue as an endocrine organ, as well as the proliferative capacity of some of its secreted adipokines, it is reasonable to hypothesize that they might participate in obesity-induced β-cell mass expansion. The aim of this study was to evaluate the feasibility of this role and to identify the signals involved in this β-cell mass adaptation. Using a diet-induced obese model, we show that the adipose tissue surrounding the pancreas is able to secrete proliferative signals, which are partially responsible for the adaptation of the β-cell mass observed in obesity.

Materials and Methods

Animals

Adult male Wistar rats (Charles River Laboratories, Wilmington, MA), 7 wk old (weighing 225–250 g), were caged individually in a 12-h light, 12-h dark cycle in a temperature- and humidity-controlled environment. Animals were divided into two dietary sets for 30 or 60 d. One group was fed with standard chow diet (supplying 8% of calories as fat; type AO4 from Panlab, Barcelona, Spain). The second group was fed with a cafeteria diet (66% of calories as fat), as previously described (13). The animals were allowed to eat and drink *ad libitum*. During the experimental period, body weight was measured every 5 d. At the end of the experiment, rats from each group were anesthetized and killed by decapitation. For morphological studies, pancreases were removed, weighed, and fixed in 10% buffered formalin. Adipose tissue from different depots, *i.e.* the inguinal sc (iSUB), the epididymal white adipose tissue (eWAT), and the mesenteric surrounding the pancreas (pMES), was excised, weighed, cut, and either used for the preparation of conditioned medium or rapidly frozen in liquid nitrogen for RNA isolation. All animal procedures were approved by the Animal Research Committee of the University of Barcelona.

Metabolic parameters and ip glucose tolerance test (IGTT)

Animals were fasted 6 h before metabolic measurements or IGTT. Blood glucose was measured using an automatic glucose monitoring device. Plasma insulin levels were measured by ELISA kits from Mercodia (Uppsala, Sweden). The IGTT was performed by the administration of an ip injection of D-glucose (2 g/kg body weight), and blood samples were collected from the tail vein at time 0 (before injection) and 15, 30, 60, and 120 min after glucose or insulin injection.

Islet morphology studies

The pancreases fixed in formalin were embedded in paraffin and serial sections (4 μm) were cut from three different levels for each pancreas block. For β-cell mass and replication studies, paraffin sections were stained for insulin or Ki67 (Dako, Glostrup, Denmark), respectively, using the indirect peroxidase technique. In these sections, toluidine blue dye was used for islet counterstaining. The individual β-cell area was measured in sections immunofluorescently stained for insulin (Dako) and costained with propidium iodide to detect the nuclei. The cross-sectional areas were measured with Leica DRM software (Leica Microsystems GmbH, Wetzlar, Germany).

Conditioned medium (CM)

The CM was prepared as previously described (12), with some modifications. Adipose tissue from three different adipose depots (iSUB, eWAT, and pMES) was removed, weighed, and finely minced in PBS. Under sterile conditions the tissue aliquots were filtered through nylon gauze (70 μm mesh, Cell Strainer; BD Biosciences, Bedford, MA), washed in PBS, and cultured for 4 h in RPMI 1640 (Biosera, Ringmer, UK) containing 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mmol/liter L-glutamine, 11.1 mmol/liter D-glucose, and 0.2% BSA. The tissue to medium ratio was set at 1 g of tissue to 5 ml of medium. The CM was subsequently collected, carefully avoiding the lipid floating on the top, and then filtered with a 0.22 μm sterile filter (Millipore, Bedford, MA) and kept frozen at −80 C until use.

Proliferation assays

INS-1E cells (kindly provided by Dr P. Maechler, University of Geneva, Geneva, Switzerland) were maintained as described elsewhere (14). Two days after seeding, the cells were cultured for 24 h in RPMI 1640 containing 5.5 mmol/liter glucose, 1 mmol/liter sodium pyruvate, 50 μmol/liter 2-mercaptoethanol, 2 mmol/liter glutamine, 10 mmol/liter HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.1% BSA. The cells were then washed with PBS and cultured in medium containing an aliquot of CM for 20 h (CM was diluted 1:3 in RPMI 1640 and supplemented up to 1 mmol/liter sodium pyruvate, 50 μmol/liter 2-mercaptoethanol, 2 mol/liter glutamine, 10 mmol/liter HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.1% BSA, and either 5.5 or 15 mmol/liter glucose). Subsequently [methyl-^{3}H]thymidine (370 kBq/ml) was added to the medium for 4 h. The medium was then removed and cells were washed twice with cold PBS and kept frozen until cell radiation was measured to assess cell proliferation (15).

Rat islets were obtained by collagenase digestion from 8-wk-old control rats as described previously (16), and islet proliferation was measured (17). Cell growth of islets was assessed after 4 d of culture using the cell bromodeoxyuridine (BrdU) proliferation kit (Roche,

Basel, Switzerland). Briefly, islets were cultured in groups of 20 on microplates in medium containing an aliquot of CM for 24 h [CM was diluted 1:3 in RPMI 1640 and supplemented up to 2 mmol/liter glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 2% fetal bovine serum and 5.5 mmol/liter glucose]. Hydroxyurea (12.5 mmol/liter) was then added to the medium for another 24 h. After washing with Hanks' balanced salt solution, islets were cultured in the same test medium for another 24 h. BrdU was then added (1:100 dilution) and the microplates were incubated for an additional 24 h. The colorimetric assay was performed according to manufacturer's instructions.

For inactivation experiments, CM was heated at 65 C for 10 min. Igfb3 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and isotype-matched irrelevant antibody were added to the media at 0.1 μg/ml. IGF binding protein 3 (IGFBP-3) protein (R&D Systems, Minneapolis, MN) was added to the media at 10 μmol/liter. Anti-IGF-I receptor-α antibody (Santa Cruz Biotechnology) was added to the media at 10 μg/ml.

IGFBP-3 protein level determination

A commercially available ELISA kit (Uscn Life Science, Wuhan, China) was used to quantify the IGFBP-3 protein level in the CM. An ELISA was performed according to the manufacturer's instructions.

RNA isolation and microarray performance and analysis

Frozen adipose tissue was quickly weighed and total RNA extracted using the RNeasy minikit (QIAGEN, Hilden, Germany). Ten micrograms of total RNA from pMES adipose tissue were converted into cRNA, biotinylated, fragmented, and hybridized to GeneChip Rat Genome 230 2.0 (Affymetrix, Santa Clara, CA). Five microarrays were hybridized, three with independent samples coming from rats fed with standard chow (lean group) and two with independent samples coming from rats fed with the cafeteria diet (obese group). All our arrays passed the quality controls established by the manufacturer (Supplemental Methods, published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org). After several powerful statistical applications to assess their quality, it was concluded that none of them could be considered an outlier (Supplemental Methods).

Background adjustment, normalization, and data summarization of raw data were performed by robust multiarray analysis using the Affymetrix package. Statistical analysis was conducted with those genes that changed at least 1.5-fold between the cafeteria and standard groups, were labeled as present (calculated with microarray suite 5 software from Affymetrix) in at least two arrays of one group and were not Affymetrix internal control genes. Differential expression analysis was carried out with significance analysis of microarrays, setting the false discovery rate at 5%. Heat diagrams were generated using dChip software (Harvard School of Public Health, Cambridge, MA).

Real-time PCR

Total RNA was retrotranscripted with Superscript III (Invitrogen, Carlsbad, CA). Real-time PCR was carried out in a 7900 HT real-time system (Applied Biosystems, Foster City, CA) using a SYBR Green fluorophore. The primers used are described in Supplemental Table 1. A standard curve of each primer set was generated from the serial dilutions of cDNA. The expression levels obtained were normalized with a housekeeping gene (TATA box binding protein).

Separation of stromal and adipocyte fractions

First, 1.5 g of adipose tissue from pMES, eWAT, and iSUB depots was finely minced and digested in 5 ml of DMEM F12 (Gibco BRL, Gaithersburg, MD) containing 2 mg/ml collagenase (Sigma-Aldrich, St. Louis, MO) and 2% BSA for 20 min at 37 C and under constant shaking. After adding 5 ml of DMEM F12 containing 10% newborn calf serum, the samples were filtered through a 25-μm nylon mesh to remove the undigested adipose tissue and then centrifuged at 1600 rpm for 10 min. Afterward, both the floating mature adipocytes from the top and the pellet containing the stromal fraction were kept frozen at −80 C until usage.

Protein extraction

Frozen adipose tissue was homogenized in a buffer containing 50 mmol/liter Tris (pH 7.5), 150 mmol/liter NaCl, 1% Triton X-100, 10 mmol/liter sodium phosphate, 10 mmol/liter sodium fluoride, 1 mmol/liter

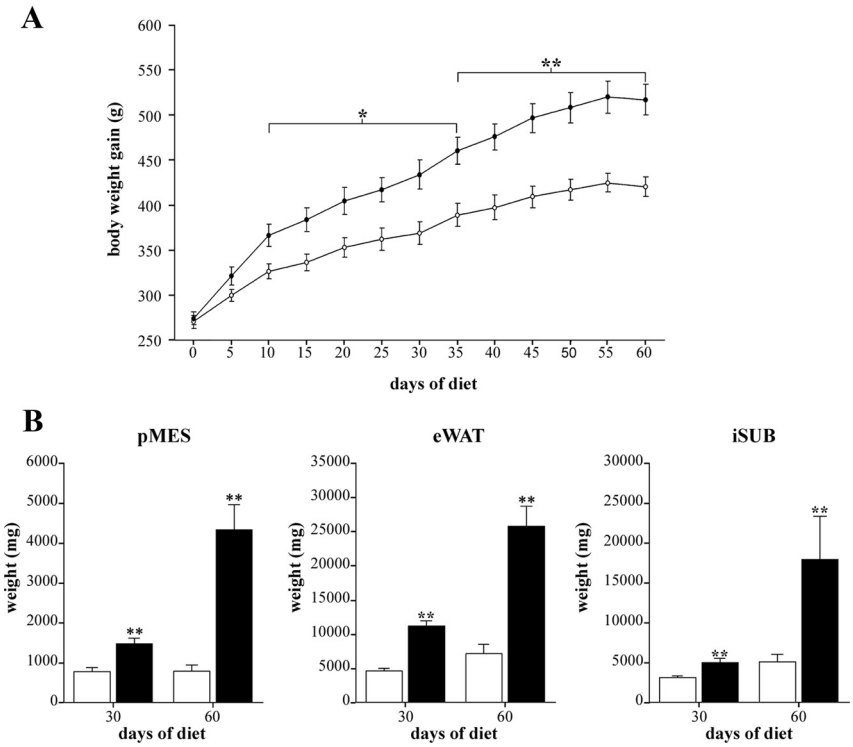

FIG. 1. Effect of the cafeteria diet on body and adipose tissue weight. Evolution of body weight in diet-induced obese and lean rats. Weight of three different adipose tissue depots after 30 or 60 d of the cafeteria diet (A, *white circles*, lean; *black circles*, obese; B, *white bars*, lean; *black bars*, obese). Data are expressed as mean ± SEM from four to 12 animals per group. *, $P < 0.05$ and **, $P < 0.01$ for obese *vs.* lean rats for the same diet period.

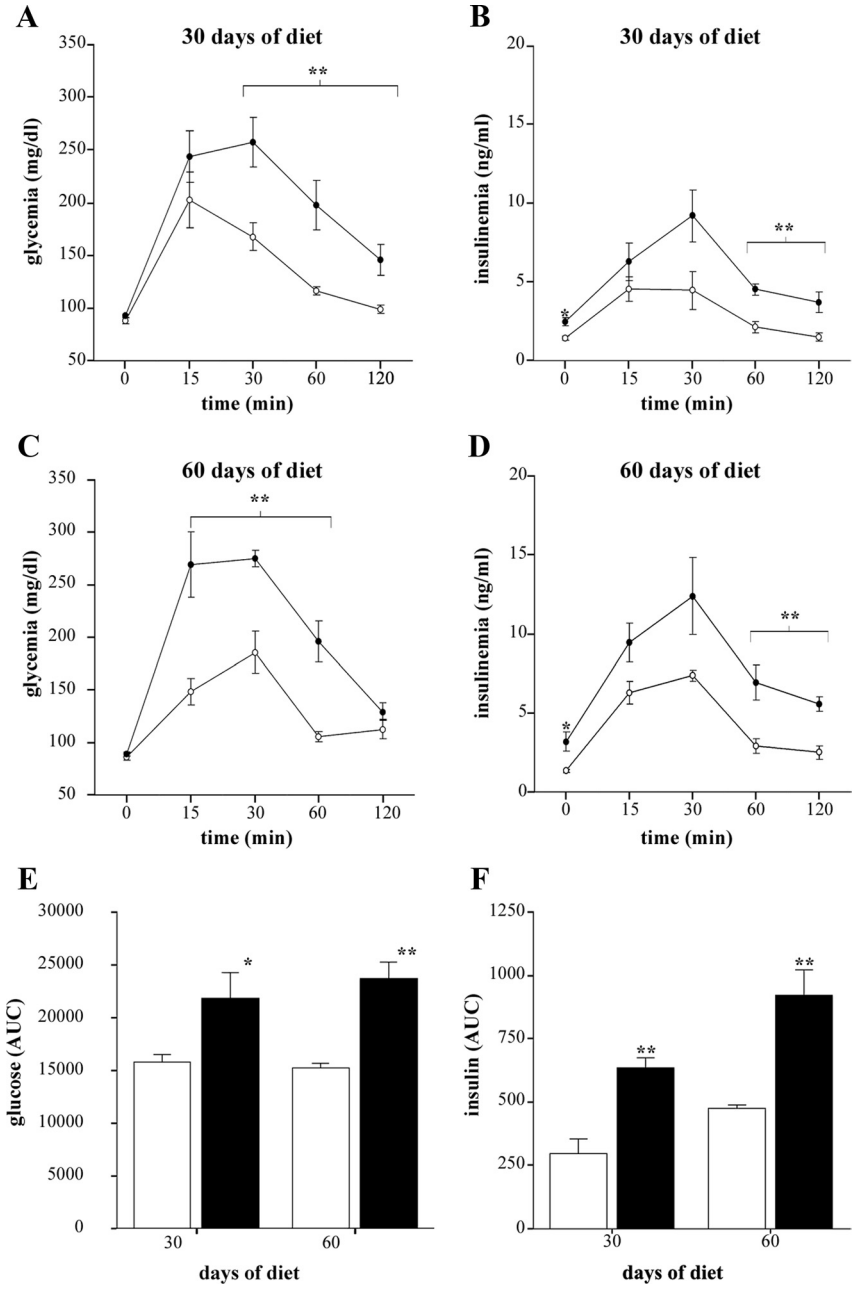

FIG. 2. Administration of the cafeteria diet alters glucose tolerance. Glycemia (A and C, *left panels*) and insulinemia (B and D, *right panels*) were determined during an ip glucose tolerance test, as described in *Materials and Methods*, in lean (*white circles*) and diet-induced obese (*black circles*) rats after 30 (A and B) or 60 (C and D) d of the diet. Areas under the curve (AUC) for glycemia and insulinemia were also calculated (E and F; *white bars,* lean; *black bars,* obese). Data are expressed as mean ± SEM from four to 10 animals per group. *, $P < 0.05$ and **, $P < 0.01$ for obese *vs.* lean rats for the same diet period.

sodium vanadate, and protease inhibitors (Sigma cocktail; Sigma) (400 μl of buffer were used per 200 μg of adipose tissue). After 30 min of incubation at 4 C, lysates were centrifuged at 14,000 rpm for 30 min at 4 C, and supernatants were recovered and kept at −80 C.

Western blot

Proteins were separated by SDS-PAGE and transferred to polyvinyl difluoride membranes by standard protocols. The membranes, previously blocked for 1 h in Tris-buffered saline buffer containing 0.05% Tween 20 and 5% skimmed milk, were first incubated overnight at 4 C with anti-IGFBP-3 antibody (1:1000; Upstate Biotechnology, Lake Placid, NY) or anti-actin antibody (1:500; Sigma-Aldrich). Then, after washing, an antirabbit peroxidase conjugate secondary antibody was added (1:5000; Amersham Biosciences, Buckinghamshire, UK) for 1 h at room temperature. The complex was visualized with enhanced chemiluminescence (Amersham Biosciences, Buckinghamshire, UK). Intensity values were obtained with Image Gauge 4.0 software (Fujifilm, Valhalla, NY).

Statistical analysis

All results are expressed as mean ± SEM. Differences between the experimental groups were evaluated by the Student's *t* test and ANOVA, with a Tukey's *post hoc* test, or by the nonparametric Mann-Whitney *U* test. $P < 0.05$ was considered significant.

Results

Characterization of the cafeteria model

Administration of a cafeteria diet induced a rapid increase in the animals' body weight. The difference in body weight between lean and diet-induced obese rats was observed after 10 d of diet and increased gradually with time (Fig. 1A). These results are similar to those obtained previously by our group and others using a similar diet (13, 18). After the diet-induced obesity period (30 and 60 d), animals were killed, allowing a gravimetric analysis of different adipose tissues (Fig. 1B). Specifically we measured three different white adipose tissue deposits: iSUB, eWAT, and pMES. The cafeteria diet increased the weight of all the depots analyzed, with this difference being already significant after 30 d of the diet and even more notable after 60 d.

As expected, the obesity induced by the cafeteria diet led to an impairment of glucose tolerance. Figure 2 shows that during an IGTT, diet-induced obese rats presented higher levels of glycemia and insulinemia, which were unable to return to baseline levels after 2 h. Moreover, impairment on the insulin sensitivity test has also been

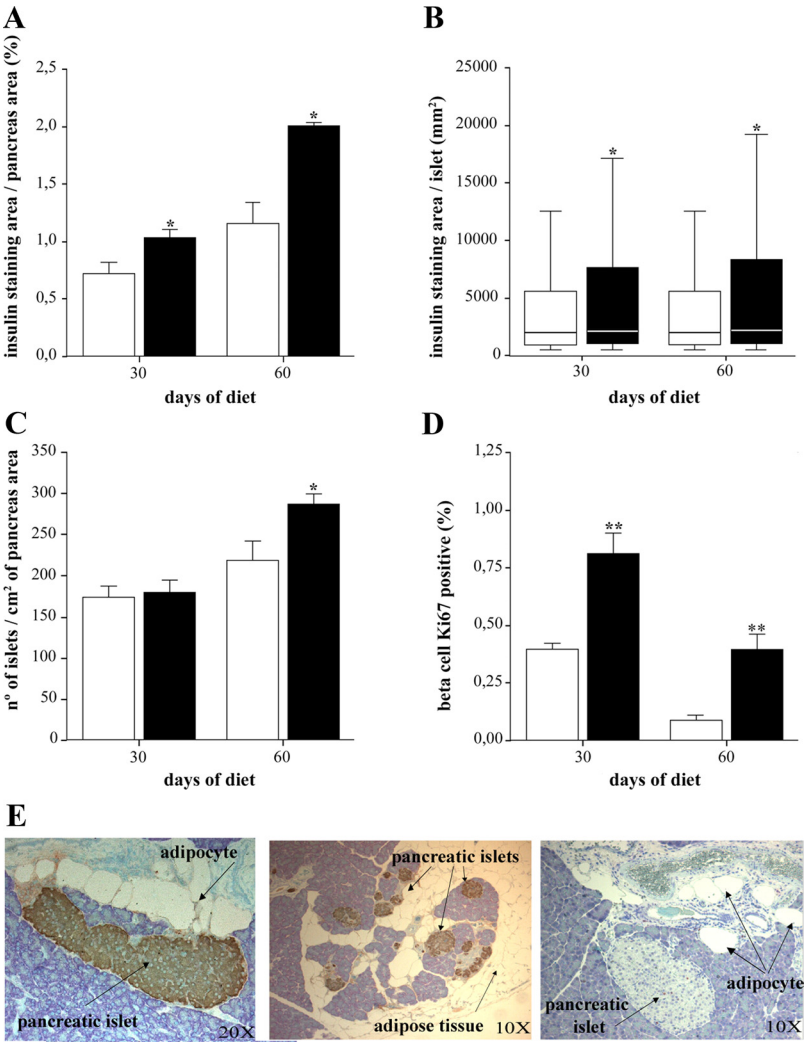

FIG. 3. The cafeteria diet increases β-cell proliferation. Pancreatic morphometry was performed as detailed in *Materials and Methods*. The percentage of insulin-stained area per whole pancreas area (A), islet size assessed by insulin-stained area per islet (B), number of islets per area of pancreas (C), and percentage of β-cell replication assessed by Ki67 staining (D) were measured in lean (*white bars*) and diet-induced obese (*black bars*) rats. E, Representative images of pancreatic-embedded section of obese rats after 60 d of cafeteria diet showing islets in close contact with adipocytes. The results are expressed as mean ± SEM (A, C, and D) or box plot (B) from four to seven animals per experimental group. *, $P < 0.05$ and **, $P < 0.01$ for obese *vs.* lean rats for the same diet period.

shown in this model (13). Finally, although no significant differences were observed in baseline glycemia, diet-induced obese rats had higher fasted plasma levels of insulin and serum levels of nonesterified fatty acids compared with lean ones (Supplemental Methods and Supplemental Fig. 1).

Diet-induced obese rats show higher β-cell replication rates

To determine whether an increase in β-cell mass was responsible for the hyperinsulinemia observed in diet-induced obese rats, a morphological study of the pancreas was carried out. The percentage of insulin area relative to total pancreas was used as an indicator of β-cell mass (7, 19). As shown in Fig. 3A, the cafeteria diet induced a significant increase in the insulin area of the pancreas in diet-induced obese rats compared with lean ones, after both 30 and 60 d of diet. Moreover, in diet-induced obese rats, a progressive increase in this parameter was observed over time.

Because a rise in β-cell mass was observed, we therefore decided to study the mechanisms involved. Our results clearly indicate that this mass expansion was due to an increase in both islet size (hyperplasia) and islet number (neogenesis) (Fig. 3, B and C). Thus, although the β-cell replication rates decreased with time in both dietary groups, the diet-induced obese rats always showed a higher rate compared with lean animals (Fig. 3D). This, in turn, led to an increase in islet size after 30 and 60 d of the cafeteria diet (Fig. 3B). In addition, after 60 d of diet, the number of islets per pancreas area was also elevated in diet-induced obese rats (Fig. 3C). Moreover, during the morphological characterization of the pancreas in our obesity model, we found a degree of infiltration of adipose tissue in the pancreas and some fat cells in close contact with pancreatic islets (Fig. 3E). This infiltration suggested a possible cross talk between adipose tissue and β-cells. We decided to study this further, focusing mainly on β-cell replication, one of the mechanisms that, as shown before, is important for the diet-induced mass expansion of β-cells.

The CM of pMES adipose tissue induced β-cell proliferation

To test whether the increase in β-cells was mediated through a factor secreted by adipose tissue, an *in vitro* experiment was set up. Specifically, proliferation of a β-cell line, INS1E cells, was analyzed when cultured in the presence of CM obtained from different adipose tissue depots: eWAT, iSUB, and pMES. Only in the latter were significant differences observed between the conditioned media from diet-induced obese and lean rats. A 2-fold increase in the INS1E proliferation rate was obtained in conditioned media prepared from pMES adipose tissues

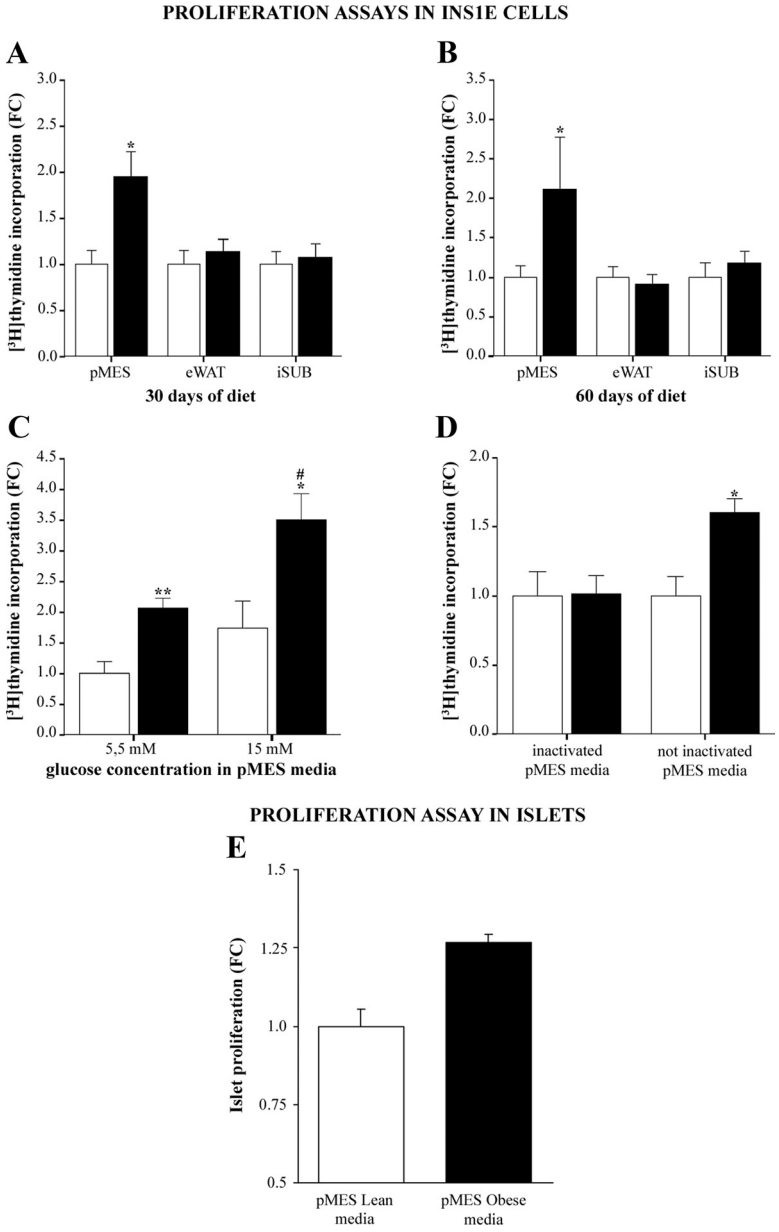

FIG. 4. β-Cell proliferation induced by conditioned media from different adipose tissue depots. Three adipose tissues (pMES, eWAT, and iSUB) obtained after 30 and 60 d of the cafeteria diet were used to produce the CM. Proliferation of INS1E cells was determined after being cultured in the different CM from lean (*white bars*) and diet-induced obese (*black bars*) rats after 30 (A) or 60 (B) d of diet. The effect of glucose concentration on the proliferation of INS1E cells cultured with pMES CM from lean (*white bars*) and diet-induced obese (*black bars*) rats after 30 d of diet (C) is shown. The effect on INS1E cell proliferation of heat inactivation of the pMES CM from lean (*white bars*) and diet-induced obese (*black bars*) rats after 30 d of diet (D) is shown. Proliferation in rat islets cultured with pMES CM from lean (*white bars*) and diet-induced obese (*black bars*) rats after 30 d of diet (E) is shown. In all cases the proliferation induced by conditioned media in INS1E cells is normalized with a control nonconditioned medium included in each experiment and standardized to the proliferation induced by the mean of the lean rats. In the glucose experiments, the results were normalized with the results at 5.5 mM concentration. Data are expressed as mean ± SEM of four to six independent experiments, each performed with conditioned media from different animals. *, $P < 0.05$ and **, $P < 0.01$ for diet-induced obese *vs.* lean rats; #, $P < 0.05$ for 15 *vs.* 5.5 mM glucose concentration.

from rats after 30 and 60 d of the cafeteria diet (Fig. 4, A and B). These results suggest that only in the pMES depot does the cafeteria diet induce changes that lead to a new secretion pattern of adipokines with greater proliferative impact on β-cells.

Some growth factors such as IGF-I and GH are known to have an effect on β-cell proliferation in a glucose-dependent manner (20, 21). To elucidate whether glucose also influences the proliferation induced by the CM from pMES, the INS1E proliferation experiments were performed at two different glucose concentrations (5.5 and 15 mM). As shown in Fig. 4C, glucose alone showed a tendency to increase proliferation as concentration rose, this being significant for conditioned media from diet-induced obese rats. However, the effect of the cafeteria-diet CM compared with the control medium was not potentiated by glucose because a 2-fold increase was observed at both low and high glucose concentrations (Fig. 4C).

To determine whether the factor(s) secreted by pMES adipose tissue and responsible for the higher proliferation rate was a protein, we then conducted heat inactivation experiments with the CM. The inactivation by heat of the CM completely abolished the observed effects on proliferation (Fig. 4D), *i.e.* no differences were observed in the INS1E replication rate between the two experimental groups. Therefore, the chemical nature of these factors is probably protein related.

Finally, the greater proliferative properties of pMES from diet-induced obese rats were also tested in control islets, using the same protocols as in INS1E cells. Islets were cultured with CM obtained from pMES of diet-induced obese and lean rats, and, similar to what we observed in INS1E experiments, a higher proliferation rate was observed in the conditioned media from diet-induced obese rats compared with lean ones (Fig. 4E).

Identification of the proliferative factors in pMES adipose tissue from diet-induced obese rats

To identify the factor(s) secreted by the pMES adipose tissue from diet-induced obese rats, which is able to increase the proliferation rate of pancreatic β-cells, a transcriptomic approach was used to analyze the pMES adipose

Gene Name	Gene Title	Public ID	FC	Effect on proliferation
Grifin	galectin-related inter-fiber protein	NM_057187	3,55	
Lep	leptin	NM_013076	3,01	+
Obp3	alpha-2u globulin PGCL4	AB039825	2,23	
Fgf18	fibroblast growth factor 18	NM_019199	2,09	+
Crlf1	cytokine receptor-like factor 1	AA925924	1,88	
Mmp23	matrix metallopeptidase 23	A1548892	1,88	
Mfap5	microfibrillar associated protein 5	BI283094	1,75	
Sfrp4	secreted frizzled-related protein 4	AF140346	1,64	-
Cgref1	cell growth regulator with EF hand domain 1	U66470	1,61	-
Ghr	growth hormone receptor	AI17077	1,54	+/-
Tex264	testis expressed gene 264	AW252660	1,54	
Jam3	junctional adhesion molecule 3	AA998207	-1,51	
Ccl19	chemokine (C-C motif) ligand 19	AA996885	-1,54	
Egfl7	EGF-like domain 7	AF223678	-1,57	-
Ptn	pleiotrophin	NM_017066	-1,58	+
Eltd1	EGF, latrophilin and seven transmembrane domain containing 1	NM_022294	-1,63	
Clu	clusterin	AF314657	-1,67	+
RT1-Db1	RT1 class II, locus Dd 1	BI279526	-1,67	
Mpzl1	myelin protein zero-like 1	BM384017	-1,71	
Aqp1	aquaporin	AA891661	-1,92	
Igfbp3	insulin-like growth factor binding protein 3	AI713966	-2,59	-
Gdf10	growth differentiation factor 10	NM_024375	-2,62	-
Cp	ceruloplasmin	NM_012532	-2,94	
RT1-Aw2	RT1 class Ib, locus Aw2	LA0362	-3,24	
Ccl11	chemokine (C-C motif) ligand 11	NM_019205	-3,96	
RT1-Bb	RT1 class II, locus Bb	BM389513	-17,49	

FIG. 5. List of secreted genes differentially expressed between diet-induced obese and lean animals. The table shows the genes that codify for secreted proteins selected from the microarray list of genes differentially expressed in pMES adipose tissue between the diet-induced obese and lean rats after 30 d of the cafeteria diet. The heat diagram on the left of the table represents the expression level of each gene, increased (*red*) or reduced (*blue*), in each microarray. In the table the genes implicated in proliferation processes are shown in a *gray shading*, and their effect on proliferation, *i.e.* induction (+) or inhibition (−), is also indicated. FC (fold change), Ratio of expression between the group of obese and lean rats.

tissue gene expression of the two experimental groups. The comparison of gene expression levels between the two groups resulted in the identification of 213 differentially expressed genes. Of these, 104 were induced and 109 repressed by the cafeteria diet. The complete list of these genes is shown in Supplemental Table 2. Because the protein(s) responsible for the proliferative effects has to be secreted to the medium to exert its functions, we then used the SignalP 3.0 Server of the ExPASy Proteomics Server web (22) to select from the complete list of differentially expressed genes those whose protein was secreted (Fig. 5). Of the genes identified, 15 were up-regulated and 11 down-regulated, and when plotted using a heat diagram, it was clearly possible to differentiate between the diet-induced obese and lean groups (Fig. 5).

After bibliographic research from the abovementioned list, we selected those genes whose function could explain the proliferative effects on β-cells induced by the pMES adipose tissue from diet-induced obese rats (Fig. 5, last column). The expression of these genes was verified using quantitative RT-PCR to check whether they behave similarly to what we observed in the array analysis. Their expression was also checked in eWAT adipose tissue. Because the proliferative effect was observed only in pMES conditioned media, the candidate gene(s) must show significant differences only in its expression levels in pMES and not in eWAT. Five of the 10 studied genes failed to confirm the microarray results, meaning that no significant differences were observed in the pMES gene expression between diet-induced obese and lean rats (Supplemental Fig. 2, A–E). Another two genes, clusterin (*Clu*) and leptin (*Lep*) (Supplemental Fig. 2, F and G), did show differential pMES expression between diet-induced obese and lean groups, confirming the microarray data. However, a similar pattern was observed in the eWAT expression, invalidating the possibility of these being the candidate gene. Another of the candidate genes, *Ghr*, codes for three different GH receptors, which are obtained by differential splicing (23): *Ghr*, GH receptor; *Ghrtr*, a truncated version; and *Ghbp*, growth hormone binding protein. Therefore, we decided to analyze these three transcripts by quantitative RT-PCR (Supplemental Fig. 2, H–J). Of the three, only *Ghbp*, a secreted protein, was of interest. Although an increase in the expression of *Ghr* and *Ghrtr* transcripts was observed in the obese pMES, *Ghbp* expression did not differ between the diet-induced obese and lean rats. Therefore, *Ghbp* was not the candidate gene. Finally, the expression of the insulin-like growth factor binding protein (*Igfbp3*) and secreted frizzled-related protein 4 (*Sfrp4*) genes was analyzed. Similarly to what was observed in the microarray analysis, this revealed a decrease in *Igfbp3* (Fig. 6A) and an increase in *Sfrp4* (Supplemental Fig. 2K). Furthermore, these differences were identified only in pMES adipose tissue, whereas no significant changes were observed in eWAT or iSUB adipose tissue. The latest depot was added to the analysis to confirm the hypothesis of these being the candidate genes.

IGFBP-3 mRNA and protein are decreased in cafeteria rats

IGFBP-3 is able to modulate the actions of IGF in both the circulation and the extracellular environment (24). Because IGF is one of the regulators of β-cell plasticity (12), we decided to study further the role of IGFBP3 in the obesity-induced increase in β-cell replication. Our results in diet-induced obese rats showed a down-regulation of *Igfbp3* expression in pMES adipose tissue. The IGFBP-3 protein was also decreased in the pMES adipose tissue of diet-induced obese rats compared with lean ones (Fig. 6B), whereas no differences were observed in the eWAT depot

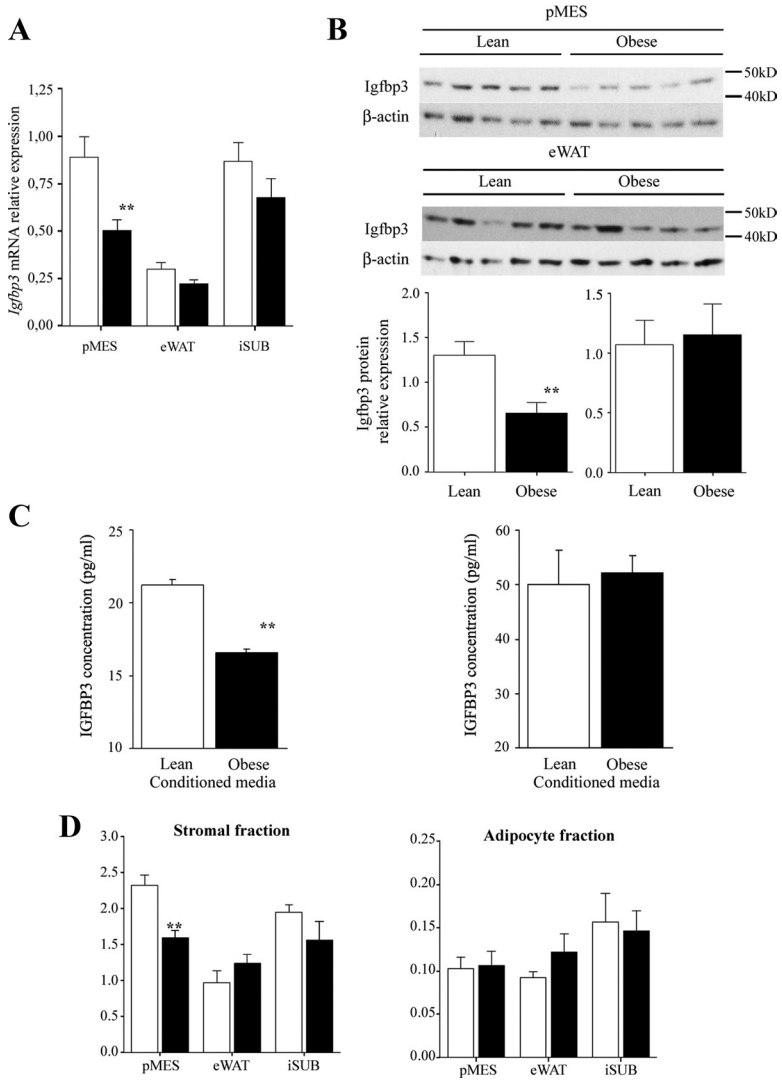

FIG. 6. IGFBP-3 levels in adipose tissue and CM of diet-induced obese and lean animals. Levels of mRNA expression of *Igfbp3* (A) in pMES, eWAT, and, iSUB adipose tissues from lean (*white bars*) and diet-induced obese (*black bars*) rats after 30 d of diet. The expression of each gene was assessed by quantitative PCR and normalized with the constitutive expression of the gene *Tbp* (TATA binding protein). Protein expression of IGFBP-3 in pMES and eWAT (B) adipose tissue was analyzed by Western blot. Representative immunoblots and densitometric analysis of three independent experiments are shown (five animals per experimental group). The IGFBP-3 protein expression was normalized by the constitutive expression of β-actin (lean, *white bars*; diet-induced obese rats, *black bars*). Levels of IGFBP-3 (C) in the conditioned media from lean (*white bars*) and diet-induced obese (*black bars*) pMES. Igfbp3 mRNA expression in stromal fraction and isolated adipocytes from pMES, eWAT, and iSUB adipose depots of lean (*white bars*) and diet-induced obese (*black bars*) rats after 30 d of diet (D) is shown. The expression of each gene was determined by quantitative PCR and normalized with the constitutive expression of the gene *Tbp*. The results are the mean ± SEM from seven to nine animals per experimental group. **, $P < 0.01$ for the gene expression of diet-induced obese *vs.* lean rats in the same adipose depot.

(Fig. 6B). Thus, the effects on gene expression are correlated with changes in protein levels. IGFBP-3 levels were also measured in the CM, and as shown in Fig. 6C, a significant decrease was observed in the CM from pMES adipose tissue. By contrast, no changes were detected in eWAT.

Adipose tissue consists mainly of adipocytes and a stromal fraction, which contains preadipocytes, macrophages, and stem cells, among other types, and plays a key role in the pathogenesis of obesity. *Igfbp3* is mainly expressed in the stromal fraction (data not shown). When we analyzed whether the decrease of *Igfbp3* in diet-induced obese rats took place in both fractions, the results surprisingly showed that *Igfbp3* decreased only in the stromal fraction, with no differences being found in the adipocyte fraction (Fig. 6D).

Influence of IGFBP-3 on β cell proliferation

Because the IGFBP-3 protein was decreased only in the pMES adipose tissue of diet-induced obese rats, we sought to confirm the role that it might play in the increase in β-cell replication. In rat islets, an antibody against IGFBP-3, which blocks this protein's effects, was added to the pMES CM from lean and diet-induced obese rats. In both cases the blockage of IGFBP-3 resulted in an increase in β-cell proliferation, whereas the addition of an isotype-matched irrelevant antibody had no action (Fig. 7A). Moreover, the addition of IGFBP-3 recombinant protein to the pMES conditioned media obtained from these animals decreased the proliferation rate (Fig. 7A). The physiological effects of IGFBP-3 have been shown to be caused by dependent and independent IGF-I mechanisms (24). To discriminate between these two possibilities, an IGF-I receptor blocking antibody was added to the CM. The blockage of this pathway completely abolished the increase in proliferation induced by the decrease in IGFBP-3 (Fig. 7B). Summing up, the data presented clearly show that a decrease in Igfbp3 expression participates in the adaptation of β-cell mass to the higher insulin demands in obesity.

Discussion

Our data indicate that there is cross talk between adipose tissue and β-cells and that this helps the organism to adapt to the new physiological needs linked to the onset of obesity. However, this cross talk does not happen with just any white adipose depot because only the obesity-induced changes in the adipose tissue that sur-

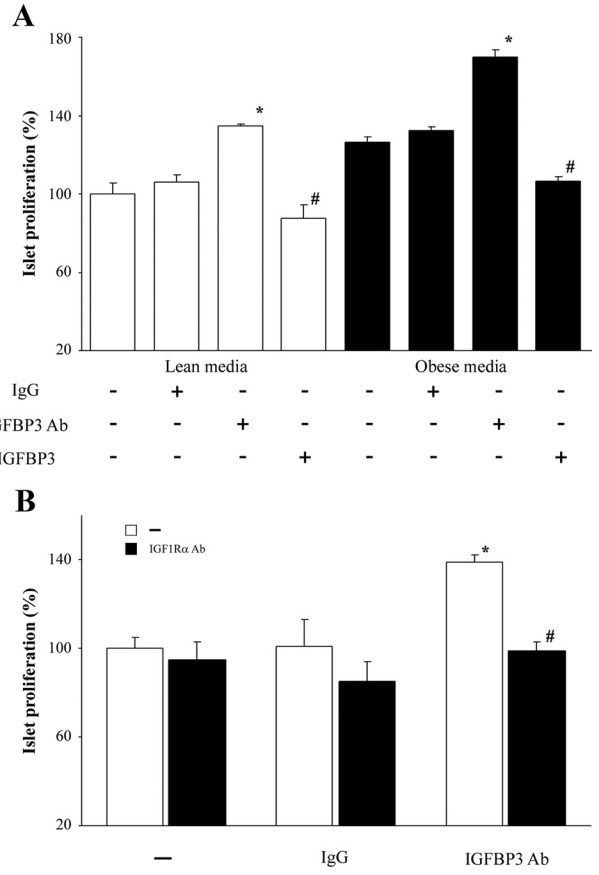

FIG. 7. Role of IGFBP-3 and IGF-I in the pMES CM effect on islet proliferation. The proliferation of islets was measured by BrdU incorporation, as described in *Materials and Methods*, in conditioned media from lean and diet-induced obese pMES (A) in the absence (−) or presence (+) of an isotype-matched irrelevant antibody (IgG) or an IGFBP-3 antibody (IGFBP3 Ab) and IGFBP-3 recombinant protein. Similarly, in conditioned media from lean pMES (B), islet proliferation was measured in the absence (−), or presence of an isotype-matched irrelevant antibody (IgG) or IGFBP-3 Ab and in each conditioned in the absence (*white bars*) and presence of an IGF-I receptor blocking antibody (IGFR1α Ab) (*black bars*). The results are the mean ± SEM of three independent experiments, each performed with conditioned media from different animals. In all cases the proliferation induced by the different media was normalized to the nonconditioned medium included in each experiment. *, $P < 0.05$ for medium with IGFBP-3 antibody *vs.* IgG conditioned media; #, $P < 0.05$ for medium with either IGFBP-3 protein or IGFR1α antibody *vs.* media with IGFBP-3 antibody.

rounds the pancreas affect β-cell plasticity. Specifically, a decrease in IGFBP-3 was discovered, which together with other factors and signals participates in the stimulation of β-cell replication. Thus, this increase leads to a rise in β-cell mass, which helps in coping with insulin resistance.

It should be noted that, similar to our results, it has been shown that the proliferative properties of conditioned media from adipose tissue increase with obesity (12). This greater mitogenic capacity seems to respond to changes in the secretion profile induced by adipocyte hypertrophy (12). In line with this, we also observed that the size of adipocytes from the peripancreatic depot nearly doubled in the diet-induced obese animals compared with lean ones (Supplemental Methods and Supplemental Fig. 3). The preadipocytes from the same depot are normally the main target for these proliferative actions. However, it has been suggested that these signals could act on other cell types; for example, they have been reported to be involved in the cardiovascular complications linked to obesity (25) and in the development of mammary gland cancer (26).

In a recent paper, it was shown that conditioned media from human adipocytes were able to increase Ins-1 proliferation (27), with WNT factors being identified as responsible for this effect. Our results provide additional data regarding this interesting link between obesity and β-cell mass regulation. Not only did we identify a new adipokine able to modulate β-cell proliferation, but we also demonstrate that these mitotic properties are induced by obesity only in a particular adipose depot, the pMES. These results are perhaps not as surprising as they may seem at first. On the one hand, the specific role of pMES adipose tissue can be understood by considering the well-established heterogeneity between the different adipose tissue depots (11). In fact, each adipose tissue displays a unique expression profile and a specific secretion pattern (28). Furthermore, it is also clear that obesity can modify the function of adipocytes and their pattern of secretion (29). In particular, the visceral adipose tissue depot, of which pMES is a part, has been linked to many diseases associated with obesity, including type 2 diabetes (30, 31). Therefore, obesity, probably through adipocyte hypertrophy, changes the adipokine profile in every adipose tissue depot, but only in the pMES are these changes able to stimulate β-cell proliferation. This makes sense physiologically because these actions take place locally, in a paracrine way, and it is the adipose tissue that surrounds the pancreas that is most accessible to islets.

The search for the adipokines responsible for this pMES-mediated effect on β-cell replication led to the identification of two proteins: sFRP4 and IGFBP-3. sFRP4 belongs to the secreted frizzled-related proteins that modulate WNT signaling (32), and both sFRP4 and IGFBP-3 are described mainly as inhibitors of proliferation (24, 32). In our expression analysis, sFRP4 was up-regulated in pMES adipose tissue from diet-induced obese rats, whereas IGFBP-3 was down-regulated. Only this decrease of an antiproliferative factor in pMES tissue could explain the higher mitotic properties of its CM, which is why we finally considered IGFBP-3 as the best possible candidate to explain the effects of conditioned media from obese rats. We therefore studied it more extensively.

These studies demonstrated that a decrease in IGFBP-3 expression and secretion is, at least in part, responsible for

mediating the proliferation of pancreatic β-cells. IGFBP-3 is the most abundant IGF binding protein and, in the cellular milieu, IGFBP-3 inhibits the action of IGF-I and IGF-II by preventing their interaction with type I IGF receptors (24). It is therefore reasonable to assume that the observed decrease in IGFBP-3 secretion to the pMES CM enhances the action of IGF. In fact, we have shown that the blockage of the IGF-I pathway completely abolished the increase in β-cell proliferation induced by the decrease of IGFBP-3. It has also been shown (24) that IGFBP-3 proteolysis is an additional mechanism to modify IGFBP-3 affinity to IGF. However, no proteolysis was observed in the Western blots of pMES IGFBP-3 (Supplemental Fig. 4). Moreover, the use of an antibody array revealed an increase of the protein, tissue inhibitor of matrix metalloproteinase 1, in the pMES secretome (Rebuffat, S., personal communication); this protein is an inhibitor of IGFBP-3 proteolysis (33). Therefore, taken together, the data indicate that the observed decrease in IGFBP-3 secretion is probably due to a down-regulation of its expression in the pMES depot. IGF proteins are well-known mitogens whose effects have been reported in β-cells in both *in vitro* (21, 34) and *in vivo* models (35, 36). Thus, the modulation of IGF-I action by the secretion of IGFBP-3 could be one of the physiological mechanisms by which β-cell plasticity can be finely tuned through the control of β-cell replication.

IGF and their binding proteins were first characterized as liver-derived endocrine factors. However, subsequent studies have shown that they are also synthesized and secreted by many nonhepatic tissues, including adipose tissue, in which they carry out major autocrine and paracrine functions (37). One recent paper (38) showed that the genetic depletion of IGFBP-3 modulates hepatic carbohydrate and lipid metabolism. However, circulating and local effects of IGFBP-3 may be quite different, and additional studies using tissue-specific knockout are needed. Here we studied the possible involvement of adipocyte-derived IGFBP-3 in the control of β-cell proliferation during the pathogenesis of obesity. Additional studies are currently underway to unravel the molecular mechanisms by which IGFBP-3 controls β-cell replication. We measured serum IGFBP-3 and IGF-I levels, observing no differences between diet-induced obese and lean rats (data not shown). These results suggest, first, that the contribution of visceral adipose tissue surrounding the pancreas to circulating IGFBP-3 levels is small compared with liver or other adipose pads, and second, that the action of IGFBP-3 in β-cell replication probably occurs locally in a paracrine manner. This paracrine action seems feasible because the only adipose depot from which the CM showed more proliferative properties with obesity was the pMES, located around the pancreas. The adipocyte infiltration observed in the pancreas of obese animals, which has been described in previous studies (39, 40), further supports a possible paracrine action of adipose tissue in β-cell physiology, supporting the idea of a cross talk between these two tissues.

In summary, our results show that changes in the adipokine secretion profile of the adipose tissue surrounding the pancreas due to diet-induced obesity is able to increase the rate of β-cell replication. IGFBP-3 is the secreted protein that participates in this paracrine action, through an IGF-I-dependent mechanism. Taken together these results demonstrate a new mechanism for controlling β-cell plasticity.

Acknowledgments

We thank M. Julià for excellent technical assistance.

Address all correspondence and requests for reprints to: Albert Barbera, Ph.D., Institut d'Investigations Biomediques August Pi i Sunyer, Rosselló 149-153, E-08036 Barcelona, Spain. E-mail: abarbera@clinic.ub.es.

This work was supported by the Spanish Ministerio de Educación y Ciencia (SAF 2006/07382), the European Union (UE-PEOPLE-2007-3-1-IAPP), and the Generalitat de Catalunya (Spain) (SGR2009/1426). The Centro de Investigación Biomédica en Red de Diabetes y Enfermedades Metabólicas is an initiative of the Instituto de Salud Carlos III. N.P. was a recipient of a grant from the Fondo de Investigacions Sanitarias (Instituto de Salud Carlos III, Spain).

Disclosure Summary: The authors have nothing to disclose.

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