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ARTÍCULO

Beta-cell hyperplasia induced by hepatic insulin resistance: Role of a liver-pancreatic endocrine axis

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ABSTRACT

Type 2 diabetes results from a combination of insulin resistance and impaired insulin secretion. To directly address the effects of hepatic insulin resistance in adult animals, we developed an inducible liver-specific IR knockout mouse (iLIRKO). Using this approach, we were able to induce variable IR deletion in a tissue-specific manner (liver mosaicism). These mice demonstrate progressive hepatic and extra hepatic insulin resistance, without liver dysfunction. Initially there is hyperinsulinemia and increased beta-cell mass in parallel to IR deletion by the liver. Our results with iLIRKO demonstrate a cause and effect relationship between progressive insulin resistance and the fold-increase of plasma insulin levels and beta-cell mass. Ultimately the beta cells undergo a failure in the insulin secretion that leads to uncontrolled diabetes. In this context, iLIRKO mice induced IGF-1 in parallel to IR (IR) deletion in the liver. This resulted in an increase of circulating IGF-1.

Concurrently, there was a huge increase of IR-A in the hyperplastic beta cells. More importantly, as assessed in mouse beta-cell lines, IR-A, but not IR-B, confers a proliferative capability to beta-cells in response to insulin or IGF-1 that may account for beta-cell hyperplasia induced by liver insulin resistance in iLIRKO mice. Thus, our results in iLIRKO mice suggest a liver-pancreatic endocrine axis, IGF-1 being a liver factor that might contribute together with insulin to compensatory pancreatic islet hyperplasia through IR-A.

Keywords: Diabetes; IGF-1; Signalling; Mouse; Proliferation.

RESUMEN

Hiperplasia de célula beta inducida por resistencia hepática a insulina: Papel de un eje hepato-pancreático

La diabetes tipo 2 es el resultado de una combinación de resistencia a insulina y un defecto en la secreción de la misma. Para determinar el papel de la resistencia a la insulina hepática en animales adultos, nuestro grupo generó un ratón para el receptor de insulina en hígado de forma inducible (iLIRKO). Utilizando esta tecnología fuimos capaces de obtener ratones con distinto grado de deleción del receptor de insulina hepático. Estos ratones mostraron una resistencia progresiva a la insulina inicialmente hepática pero que se extendió a tejidos extrahepáticos. Además, no se observó ningún tipo de patología hepática. Inicialmente se observó un incremento en los niveles de insulina circulantes y un aumento de la masa de célula beta pancreática que fue proporcional al grado de deleción del receptor de insulina en hígado. Finalmente, tiene lugar un fallo en la secreción de insulina por parte de la célula beta pancreática. En este contexto, el hígado de los animales iLIRKO es capaz de sintetizar IGF-1 de forma proporcional al grado de deleción del receptor de insulina en hígado. Además, tuvo lugar un importante incremento en la expresión de la isoforma A del receptor de insulina (IR-A) en las células beta. Más importante, como demostramos en células beta inmortalizadas, IR-A pero no IR-B confiere una gran capacidad proliferativa a las células beta en respuesta tanto a insulina como IGF-1, lo que puede explicar, al menos en parte, los

mecanismos involucrados en la hiperplasia de la célula beta pancreática desarrollada por los ratones iLIRKO.

Palabras clave: Diabetes; IGF-1; Señalización; Ratón; Proliferación.

1. INTRODUCTION

Type 2 diabetes results from a combination of insulin resistance and impaired insulin secretion. While it is not clear the primary defect in type 2 diabetes, insulin resistance is the most relevant pathophysiological feature in the prediabetic state (1, 2). Rodent studies have shown that insulin insensitivity in both classical and non-classical insulin target tissues can play a role in the control of glucose homeostasis (3). Several mouse models have been developed to study the role of insulin resistance in various tissues (4). While total whole body insulin resistance produced by generalized deletion of the IR does not produce any major effect in mouse development, these mice died one week after birth from severe ketosis (5, 6). Combined restoration of IR function in brain, liver, and pancreatic b cells rescues IR knockout mice from neonatal death, prevents diabetes in a majority of animals, and normalizes adipose tissue content, lifespan and reproductive function (7).

In a serie of studies using tissue-specific conditional knockout of IR, the liver, brain and beta cell have also been implicated as the key sites of insulin resistance in the development of type 2 diabetes (8-10). The liver-specific IR knockout (LIRKO) showed that hepatic insulin resistance is the most important in development of impaired glucose tolerance and fasting hyperglycemia. In addition, these mice also developed marked beta-cell hyperplasia and hyperinsulinemia and decreased insulin clearance. With aging, however, the diabetic state regressed, suggesting some form of compensatory mechanism, possibly linked with the development of liver damage related to appearance of hyperplastic nodules that might have altered glucose production by the liver, leading to regression of the diabetic state with aging (8). To better address the role of this important tissue in the pathogenesis of type 2 diabetes in the adult, we developed LIRKO mice in an inducible manner (iLIRKO).

2. MATERIALS AND METHODS

2.1. Animals

$IR^{(loxP/loxP)}$ C57Bl/6 mice were created by homologous recombination using an IR gene targeting vector with lox P sites flanking exon 4 as previously described (11). Transgenic mice expressing a *Cre* recombinase transgene under the control of the Mx1 promoter/enhancer were purchased from Jackson Laboratory (Bar Harbour, Maine, USA). The $IR^{(loxP/loxP)}$ mice were crossed with Mx-Cre mice to obtain inducible LIRKO (iLIRKO) mice. After the suckling period, iLIRKO mice were injected intraperitoneally with poly-Inositic-poly-Cytidilic acid (500 mg/injection) to induce the interferon alpha response and the consequent Mx1 promoter activation as previously described (12). All animal experimentation described in this manuscript was conducted according with accepted standards of human animal care published by the National Institutes of Health.

2.2. Genotyping of the $IR^{(loxP/loxP)}$ transgenic mice

Genotyping of the mice was performed by PCR. Tail DNA (100-200 ng) was amplified 30 cycles (40 seconds, 94° C; 40 seconds, 60° C; and 1 minute, 75° C) on a thermal cycler. Two primers flanking the *loxP* site behind exon 4 of the IR were used: the forward primer (5'-GATGTGCACCCCATGTCTG-3') and the reverse primer (5'-CTGAATAGCTGAGACCACAG-3'). A 320-bp band was obtained for the floxed allele or a 280-bp band for the wild-type allele.

2.3. Genotyping of the Mx1-Cre transgenic mice

Mice were genotyped by PCR. Tail DNA (100-200 ng) was amplified 35 cycles (1 minute, 94° C; 1 minute, 60° C; and 1 minute, 72° C) on a thermal cycler. To amplify the Mx1-Cre transgene (PCR product, 269 bp), primers SF-4 (5'-GCATAACCAGTGAAACAGCATTGCTG-3') and 69R (5'-GGACATGTTCAGGGATCGCCAGGCG-3') were used.

2.4. Western blot analysis

Tissues were homogenized as described (11). Western blot analyses of insulin signaling proteins were performed as described (13). The antibodies used were anti-IRβ (Ab-4) from Oncogene (San Diego, CA), anti-PEPCK antibody was kindly provided by Dr. DK. Granner (Vanderbilt University, TN), Anti-Glucokinase antibody was a generous gift of Dr. S. Lenzen (Hannover Medical School, Germany), anti-FAS antibody was purchased from BD Transduction Laboratories (San Diego, CA) anti-β-actin antibody was from Sigma-Aldrich Corp. (St. Louis, MO). Anti-IGF-1 antibody was purchased from Upstate Biotechnology (Lake Placid, NY). Anti-phospho-p70-S6-Kinase (Thr421/Ser424), anti-phospho-p44/p42-MAPK (Thr202/Tyr204), anti-phospho-Akt (Ser473) antibodies were purchased from Cell Signaling (Beverly, MA). For *in vivo* insulin signaling studies, mice were intraperitoneally injected with 1 U/kg body weight of human insulin (Novo Nordisk, Denmark). After 10 minutes, the tissues were removed and frozen in liquid nitrogen. Densitometric analysis of the autoradiograms was performed using a GS-710 Imaging Densitometer and the Quantity One software (Bio-Rad Laboratories, CA).

2.5. Analytical procedures

Insulin levels in serum were measured by radioimmunoassay (RIA) using mouse insulin as a standard (Linco Research, MO). IGF-1 levels in serum were measured by RIA using mouse IGF-1 as a standard (Diagnostic Systems Laboratories, TX). Glucose tolerance and insulin tolerance tests were performed as previously described (14).

2.6. Histological analysis

The immunohistochemical analysis of pancreata was preformed as described (14). β cell mass was evaluated by point counting morphometry (15, 16). Staining of liver sections with hematoxylin/eosin, periodic Acid-Schiff (PAS) and Masson reagents was performed using standard techniques.

2.7. Real-time quantitative PCR for IR isoforms

The pancreatic islets were isolated from 6-month-old control and iLIRKO mice as previously described (10). IR isoforms expression in isolated islets was analyzed by qPCR, using Taqman probes (Applied Biosystems, NJ). The comparative threshold cycle (Ct) method was used to calculate the relative expression. For gene expression quantification, the target genes values were normalized to the expression of the endogenous reference (18S). Thus, the amount of target, normalized to 18S and relative to the control is given by $2^{-\Delta\Delta Ct}$ [ΔCt = Ct (Target gene) - Ct (18S); $\Delta\Delta$Ct = ΔCt for any sample - ΔCt for the control].

2.8. Reconstitution of IRKO immortalized beta cells with IR A or B iosoforms b retroviral infection

IRKO beta cells were generated in our laboratory as previously described (17). From these cells we reconstituted and characterized the expression of the IR-A (Rec A) and IR-B (Rec B) generating new cell lines as previously described (18).

2.9. Measurement of glucose uptake in beta cells

Cells were cultured to 80% confluence in 10% FBS-DMEM and then serum and glucose starved for 4-6 h. After that, 10 nM Insulin or 10 nM IGF-1 were added to the wells for 30 min. Glucose uptake was measured by incubating cells with 2-deoxy-D-[1-^{3}H]glucose for the last 10 min, in triplicate dishes from six independent experiments as previously described (19).

2.10. Cell viability assays

Cells were plated in 12-multiwell plates and cultured in 10% FBS-DMEM until 40-50% of confluence was reached. Then cells were serum starved for 4 h, and then treated with 10nM Insulin, 10 nM IGF-1 or both during 24 hours. Then, the cells were washed with PBS and stained with violet crystal as described (17).

2.11. Proliferation studies in isolated pancreatic islets and cultured cells

DNA synthesis in isolated islets was estimated by determining BrdU incorporation by using the Cell Proliferation ELISA kit (Roche Diagnostics GmbH, Germany). In cultured beta cells the DNA synthesis was estimated by [^{3}H]-thymidine incorporation as previously described (20).

2.12. Statistical analysis

All values are expressed as mean +/– SEM. Statistical analyses were carried out using a two-tailed Student's unpaired *t* test, and the null hypothesis was rejected at the 0.05 level.

3. RESULTS

3.1. Progressive liver-specific IR deletion without liver dysfunction

The iLIRKO mice were generated as described in Materials and Methods. These mice showed variable IR deletion and were grouped into three groups (50, 25 and 0% of the normal receptor complement). Ablation of IR was tissue-specific, none of other tissues studied were affected (Figure 1).

A critical issue in constitutive LIRKO was the appearance of some liver dysfunction. However, hematoxylin-eosin staining in liver sections revealed no dysplastic or hyperplastic nodules in 6 month- and 12 month-old iLIRKO mice as compared with their respective controls. In addition, Masson staining showed no increase in collagen infiltration or fibrosis in iLIRKO mice (Figure 2, upper panels). Regarding enzymes of hepatic glucose metabolism, we found two kind of evidence as compared with constitutive LIRKO mice. Thus, late IR deletion in iLIRKO mice resulted in reduced glucokinase (GK) protein expression and a dramatic loss of glycogen liver content, as shown by PAS-staining of liver sections, as previously

reported in early insulin IR deletion (8). However, no effect was observed on phosphoenol pyruvate carboxykinase (PEPCK) or fatty acid synthase (FAS) gene expression in 6 month-old iLIRKO mice as compared with controls (Figure 2, lower panels).

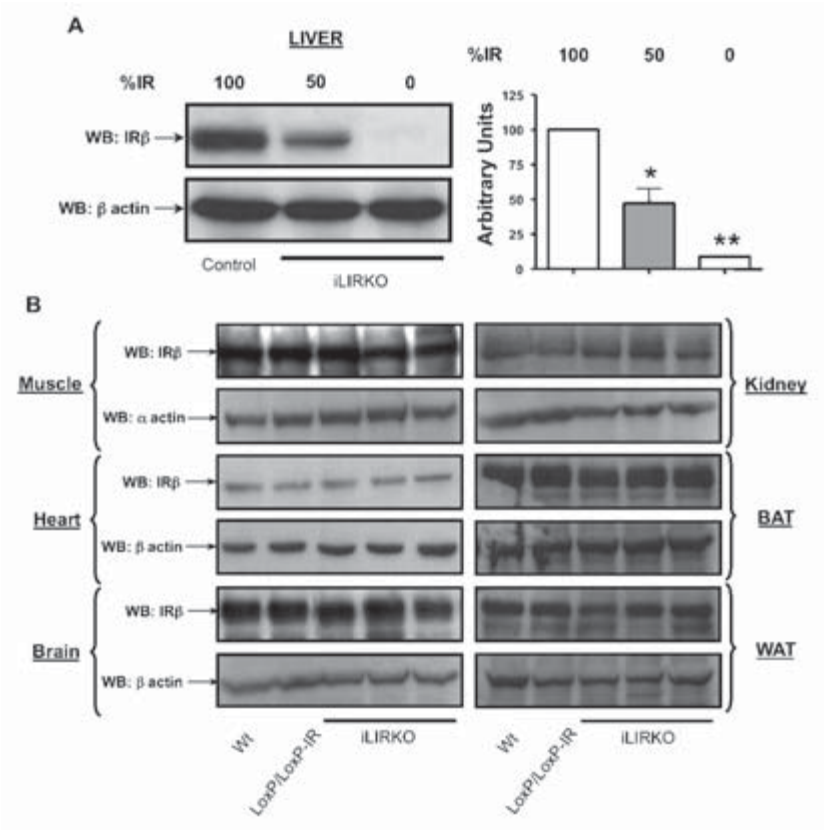

Figure 1. Induction of Liver-Specific IR Knockout. A) (Upper panel) Liver protein extracts from 6-month-old Control and iLIRKO mice were analyzed by Western blot. (Lower panel) The autoradiograms corresponding to four independent experiments were quantitated by scanning densitometry. **B)** Protein extracts of several tissues from 6-month-old Wt, $IR^{(loxP/loxP)}$ and iLIRKO mice were analyzed by Western blot. A representative experiment out of four is shown. Results are expressed as mean +/– SEM. **P < 0.005; *P < 0.05; iLIRKO vs. Control.

3.2. Progressive insulin resistance and glucose intolerance in inducible LIRKO

At 6 months of age iLIRKO mice demonstrated defects in insulin signaling proportional to the lost of IR expression. Thus, in the group with 50% of IR expression, there was a marked decreased in Akt, ERKs and p70-S6-kinase activation while in the group with complete IR deletion; there was a virtual absence of insulin activation of these enzymes. Interestingly, despite the fact that there was no IR lost in other tissues (Figure 1, panel B), there was a progressive impairment of insulin signaling in other peripheral tissues. Thus, there was progressive insulin impairment on Akt, ERKs and p70-S6-kinase signaling from 50 to 0% IR expression left by the liver, in brain,

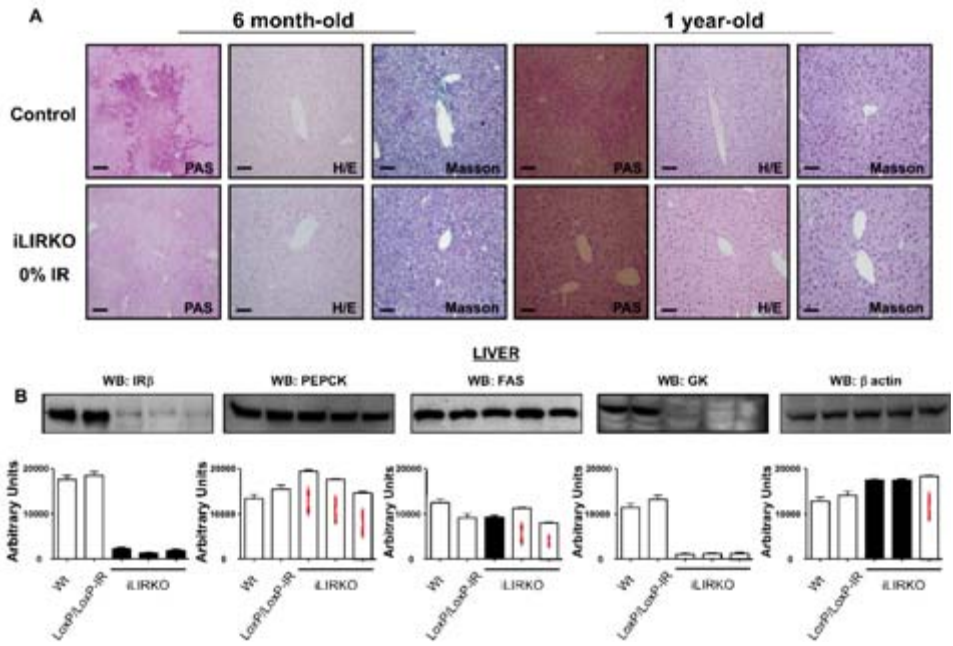

Figure 2. Liver Histology and Metabolic Gene Expression in iLIRKO mice. A) Periodic Acid-Schiff reagent (PAS), Hematoxylin/Eosin and Masson staining of liver sections from random-fed, 6-month- and 1-year-old male control (upper panel) and iLIRKO (lower panel) mice. A representative experiment out of four is shown. Magnification 20X. **B)** Protein liver extracts from 6-month-old Wt, $IR^{(loxP/loxP)}$ and iLIRKO mice were analyzed by Western blot. A representative experiment out of five is shown. Histograms show the densitometric analysis.

skeletal muscle or brown adipose tissue (BAT) (Figure 3). Insulin signaling in both the liver and these extra hepatic tissues was similarly impaired in 1 year-old iLIRKO mice, (Figure 4). These results indicate that iLIRKO mouse develops primary insulin resistance in the liver, and this is associated with secondary insulin resistance in extra hepatic tissues which persists throughout the animal life.

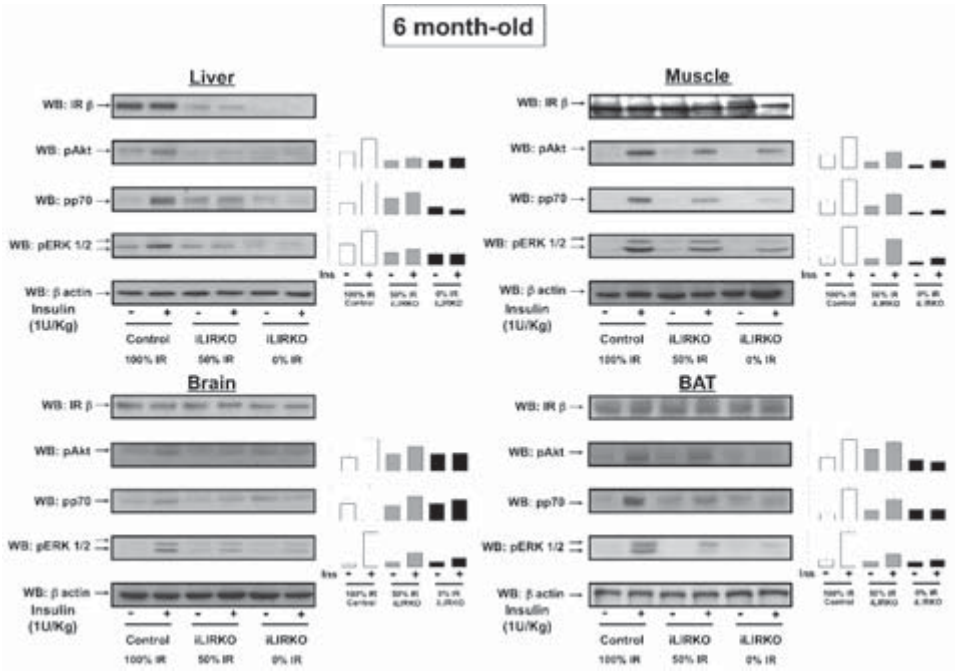

Figure 3. iLIRKO Shows Insulin Resistance in the Liver and Extra Hepatic Tissues. *In vivo* insulin signaling studies were performed in 6-month-old Control and iLIRKO mice by intraperitoneal injection of 1 U/kg body weight of human insulin. After 10 minutes, the tissues were removed and analyzed by Western blot. A representative experiment out of four is shown. Histograms show the densitometric analysis.

Post-weaning IR deletion induced progressive insulin resistance in iLIRKO mice from 2- to 6-month-old and this persisted at 1 year of age (Figure 5, upper panels). More importantly, the mice with no IR expression showed progressive glucose intolerance, and even developed fasting hyperglycemia at 1 year of age (Figure 5, lower panels).

3.3. Progressive beta-cell hyperplasia and failure of insulin secretion with aging

iLIRKO showed progressive beta-cell hyperplasia as compared with controls. More importantly, the level of hyperplasia was correlated to the level of IR expression remaining in the liver (Figure 6, panel B). The iLIRKO mice also developed progressive hyperinsulinemia as compared with controls and paralleled with the level of IR deletion by the liver (Figure 6, panel B). This beta-cell expansion persisted throughout life as shown in Figure 6, panel D. In parallel, plas-

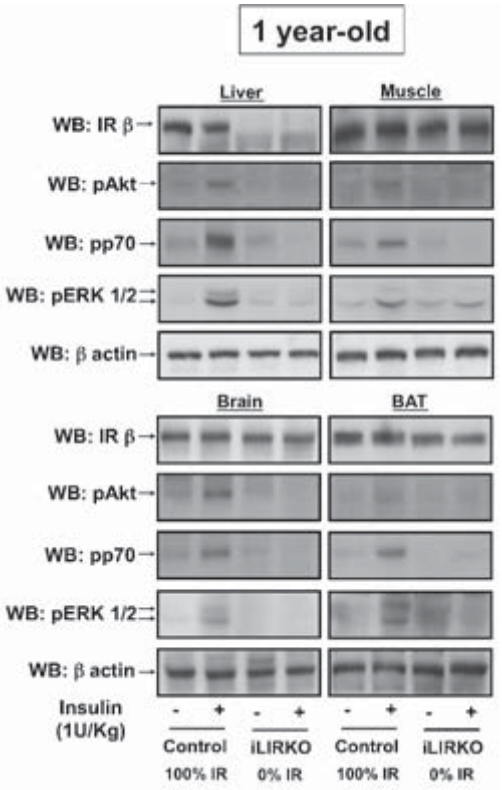

Figure 4. Generalized Insulin Resistance persists in aged iLIRKO. *In vivo* insulin signaling studies were performed in 1-year-old Control and iLIRKO mice by intraperitoneal injection of 1 U/kg body weight of human insulin. After 10 minutes, the tissues were removed and analyzed by Western blot. A representative experiment out of four is shown. Histograms show the densitometric analysis.

ma insulin increased by 2.5-fold in 1-year-old mice as compared with their controls (Figure 6, panel D), however, this increase was much lower than those at 6 months of age (3-fold, panel D), suggesting a failure in insulin secretion with aging.

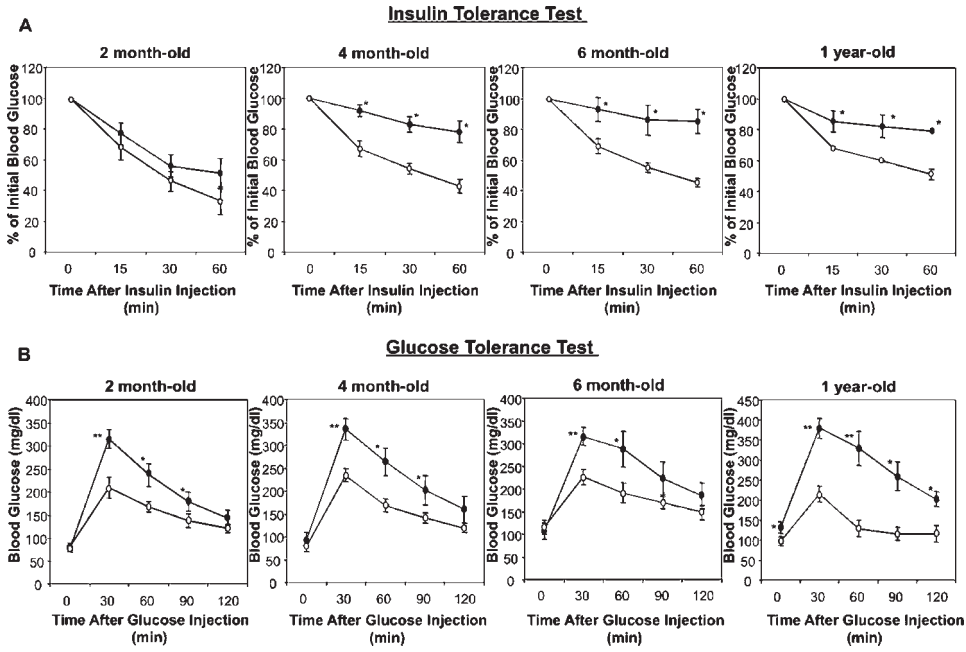

Figure 5. Progressive Insulin Resistance and Glucose Intolerance in iLIRKO mice. A) Insulin tolerance tests were performed on random-fed, 2-, 4-, 6-month- and 1-year-old male Control (white circles) and iLIRKO (black circles) mice. Animals were injected intraperitoneally with 1 U/kg body weight of human insulin. Blood glucose was measured immediately before injection and 15, 30, and 60 min after the injection. Results expressed as percentage of initial blood glucose concentration are means +/– SEM (n = 10-20). **B)** Glucose tolerance tests were performed on 2-, 4-, 6-month- and 1-year-old Control (white circles) and iLIRKO (black circles) mice that had been fasted for 16 h. Animals were injected intraperitoneally with 2 g/kg body weight of glucose. Blood glucose was measured immediately before injection and 30, 60, 90 and 120 min after the injection. Results are expressed as mean +/– SEM (n = 10–20). **P < 0.005; *P < 0.05; iLIRKO vs. Control.

3.4. Progressive hepatic expression and plasma concentration of IGF-1 in iLIRKO mice

Previous studies have suggested the presence of a circulating islet growth factor in insulin resistant states, independent of glucose and obesity (21). To address this important issue, we conducted western blot analysis with anti-IGF-1 antibody in the liver. Thus, iLIRKO induced expression depending on the level of IR deletion by the liver (Figure 6, panel A). In parallel, iLIRKO mice induced IGFBP1 and IGFBP3 also depending on the level of IR deletion by the liver. These data were confirmed in 1 year-old mice (Figure 6, panel C). Noteworthy, iLIRKO mice bearing no liver IR expression significantly induced plasma IGF-1 in 6 month- and 1 year-old mice as compared with controls (Figure 6, panels B and D respectively).

3.5. Increase of IR-A in pancreatic islets: beta cell lines expressing IR-A, but not IR-B, show increased proliferation in response to insulin or IGF-1

The IR expression in pancreatic islets was significantly increased in iLIRKO mice (Figure 7, panel A). More importantly, the percentage of IR-A from total IR dramatically increased in these mice (Figure 7, panel A). In order to investigate a possible role of this change in the pattern of expression of the IR isoforms in the beta-cell hyperplasia, we performed experiments of BrdU incorporation in pancreatic islets of 6-month-old control and iLIRKO mice in presence of 10 nM insulin or 10 nM IGF-1. The results showed that the islets of iLIRKO mice were significantly more sensitive to the IGF-1-induced proliferation than those control islets. Previous data indicated that IR-A was 2-fold more sensitive than IR-B in response to insulin regarding glycogen synthesis and also mitogenesis (22). To assess those results in mouse beta cells, we have generated beta cell lines bearing IR (IRLoxP), lacking IR (IRKO), expressing exclusively IR-A (Rec A), or alternatively expressing IR-B (Rec B) (Figure 7, panel C). Moreover, the generated cell lines were completely functional as assesed by IR and IGF-1R phosphorylation experiments (Figure 7, panel D). The lack of IR significantly decreased basal glucose uptake in beta cells. Reconstitution with IR-A, but not with IR-B, restored basal glucose

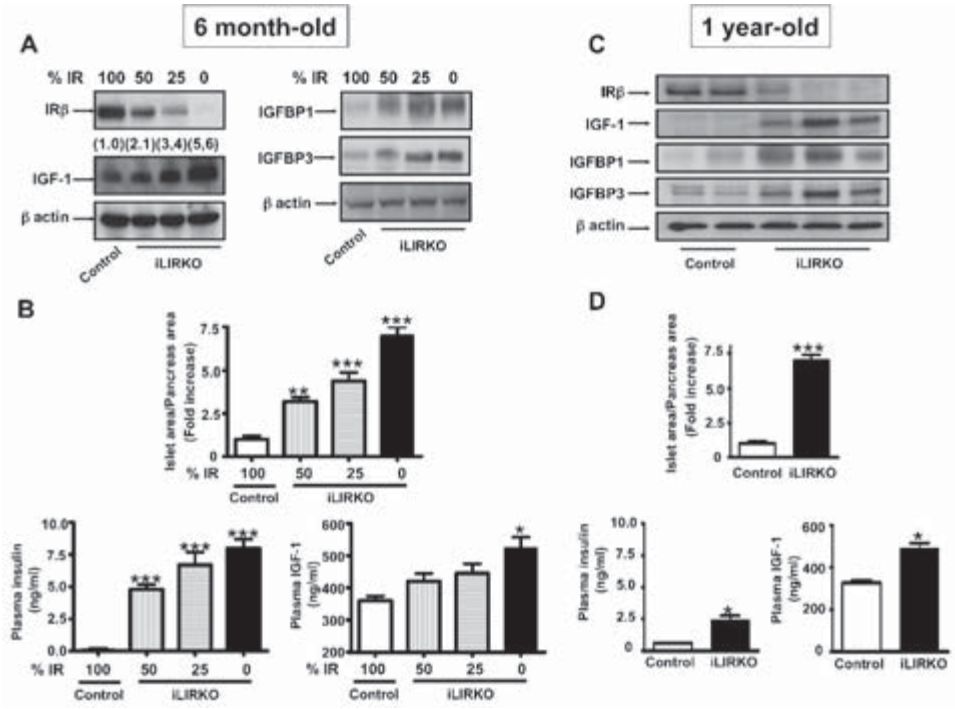

Figure 6. Progressive IGF-1 Liver Expression, Beta-Cell Hyperplasia and Insulin Secretion Defect with Aging in iLIRKO. A) Liver extracts from 6-month-old Control and iLIRKO mice with different IR deletion were analyzed by Western blot. The autoradiograms of IGF-1 were quantified by scanning densitometry from four independent experiments. **B)** (Upper panel) Beta cell mass was determined in 6-month-old Control (white bars) and iLIRKO mice with different grade of IR deletion. Results were shown as fold increase of Control beta cell mass. Data shown are from five independent experiments. (Lower-Left panel) Plasma insulin content was measured in 6-month-old male Control (white bars) and iLIRKO mice with different grade of IR deletion. Values are expressed as mean +/– SEM (n = 4). (Lower-Right panel) Plasma IGF-1 content was measured in 6-month-old male Control (white bars) and iLIRKO mice with different grade of IR deletion by RIA. Values are expressed as mean +/– SEM (n = 4). **C)** Liver extracts from 1-year-old Control and iLIRKO mice were analyzed by Western blot. A representative experiment out of four is shown. **D)** (Upper panel) Beta cell mass was evaluated by point counting morphometry in 1-year-old Control and iLIRKO mice. Results were shown as fold increase of Control beta cell mass. (Lower-Left panel) Plasma insulin content was measured in 1-year-old male Control and iLIRKO mice by RIA. Values are expressed as mean ± SEM (n = 4). (Lower-Right panel) Plasma IGF-1 content was measured in 1-year-old male Control and iLIRKO mice by RIA. Values are expressed as mean ± SEM (n=4).*P < 0.05; **P < 0.005; ***P < 0.001 iLIRKO vs Control.

uptake to levels higher than those observed in control cells (Figure 7, panel E). In addition, insulin or IGF-1 enhanced cell viability in control cells. In the same way, IGF-1 induced cell viability in beta cells lacking IR. Rec A but not Rec B cells, increased cell viability in response to either insulin or IGF-1 (Figure 7, panel F). More importantly, insulin or IGF-1 increased proliferation, as estimated by thymidine incorporation, in control beta cells. However, beta cells lacking IR did not respond to IGF-1. Finally, insulin and IGF-1 induced proliferation in Rec A but not in Rec B cells (Figure 7, panel G).

4. DISCUSSION

Insulin promotes both metabolism and growth in the liver. Constitutive ablation of IR in liver resulted in both metabolic changes and a reduction in liver size by about 50% (8). Likewise, mice in which there is variable IR deletion (cellular mosaicism) exhibit different degrees of growth retardation and metabolic abnormalities depending on the extent of IR deletion (23). Those findings suggest that insulin regulates growth independently of metabolism and that the IR number is an important determinant of the insulin action specificity. Using the approach to generate LIRKO mice in an inducible manner, we were able to induce variable IR deletion (liver mosaicism). As a result, iLIRKO induced progressive insulin resistance and glucose intolerance without growth retardation. Under our experimental conditions, the insulin-induced effect on growth and metabolic regulation are mutually independent. Thus, while the late IR deletion gave rise to irreversible insulin resistance and progressive glucose intolerance for over one year, no liver damage was observed.

Previous tissue specific knockout and tissue specific reconstitution studies concluded that the progression of insulin resistance to diabetes with fasting hyperglycemia requires defects in tissues other than liver (7, 8). More importantly, acute IR deletion by the liver although impaired insulin signaling did not induce insulin resistance or hyperinsulinemia (24). Our results show that liver-specific disruption of IR could produce impaired hepatic and extra hepatic

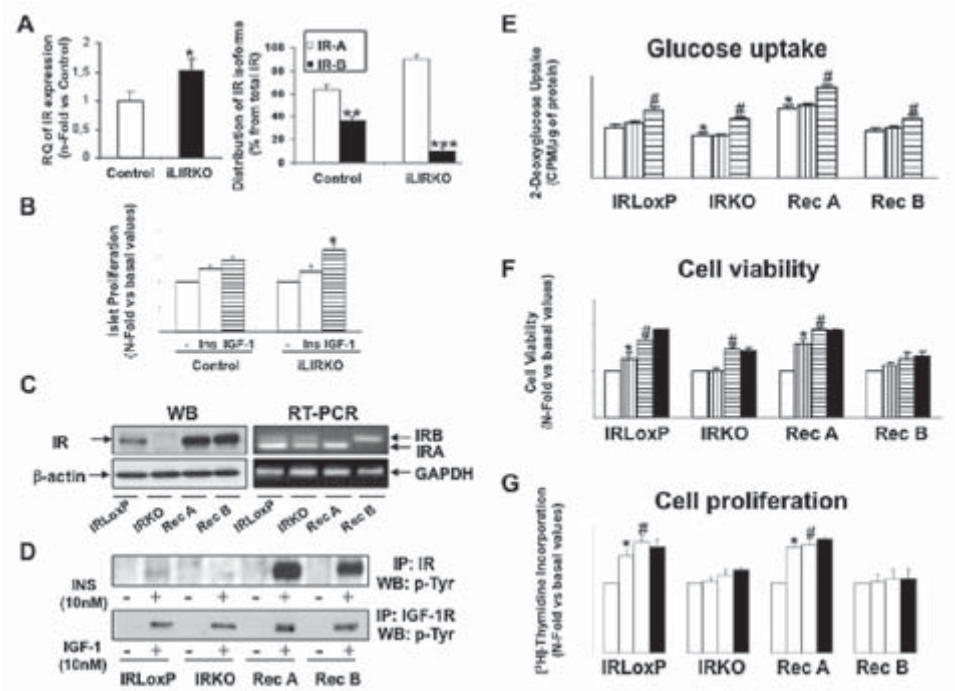

Figure 7. Increase of IR-A in pancreatic islets: Rec A but not Rec B cells show increased proliferation in response to insulin or IGF-1. A) mRNA levels of IR and the isoforms distribution were analyzed by qPCR in 6-month-old Control and iLIRKO mice. Values are expressed as mean ± SEM (n = 4). *P < 0.05; **P < 0.005; ***P < 0.001 iLIRKO vs Control. **B)** Pancreatic islets proliferation was assesed by BrdU incorporation in 6-month-old Control and iLIRKO mice. Values are expressed as mean ± SEM (n = 4). *P < 0.05. **C)** IR expression was analyzed by Western Blot and RT-PCR in IRLoxP, IRKO, Rec A and Rec B beta cells. A representative experiment out of four is shown. **D)** Functional assessment of IR reconstitution was carried out by phosphorylation experiments. **E)** Glucose uptake induced by Insulin (vertical striped bars) or IGF-1 (horizontal striped bars) was measured in each cell line. Statistical significance was carried out by Student's t test by comparison of IRKO and Rec A with IRLoxP beta cells respectively (*, P < 0.05). **F)** Cell viability was measured in each cell line by violet crystal staining after a 24h treatment with 10 nM Insulin (vertical striped bars), 10 nM IGF-1 (horizontal striped bars) or both (black bars). Statistical significance was carried out by Student's t test by comparison of basal conditions with insulin-stimulated conditions of each cell line (*, P < 0.05) or basal conditions with IGF-1-stimulated conditions of each cell line (#, P < 0.05). **G)** Cell proliferation was measured in each cell line by Thymidine incorporation after a 24h treatment with 10 nM Insulin (vertical striped bars), 10 nM IGF-1 (horizontal striped bars) or both (black bars). Statistical significance was carried out by Student's t test by comparison of basal conditions with insulin-stimulated conditions of each cell line (*, P < 0.05) or basal conditions with IGF-1-stimulated conditions of each cell line (#, P < 0.05).

insulin signaling and that the severity of this resistance depends on the level of IR deletion; iLIRKO mice suffer early insulin resistance, as a primary defect, directly related to the ablation of IR, but also have impaired insulin signaling in extra hepatic tissues. Thus, iLIRKO mice induce late insulin resistance, as a secondary effect. Given the fact that IR expression in those tissues was not affected, this effect may be likely due to IR desensitisation due to prolonged hyperinsulinemia. Our results demonstrate that a primary defect in the liver triggers a secondary insulin resistance in extra hepatic tissues. Accordingly, the progression to diabetes only requires defects in the liver as observed in iLIRKO mice.

Insulin resistance is associated with hyperinsulinemia and leads to beta-cell hyperplasia. Thus, early IR deletion by the liver in constitutive LIRKO induced beta-cell hyperplasia (8). However, in mice in which was created muscle-specific insulin resistance by conditional inactivation of the IR (MIRKO mice, 11), no islet hyperplasia was found in response to the isolated muscle insulin resistance. More importantly, insulin levels are elevated in most states of insulin resistance, including the LIRKO mouse, but are not elevated in MIRKO mouse. Our results with iLIRKO demonstrate a direct relationship between the level of IR deletion by the liver and the fold-increase of plasma insulin levels and also beta-cell mass. Finally, constitutive LIRKO developed hypersecretion of insulin for more than one year. However, in iLIRKO mice compensatory hyperinsulinemia in 1 year-old mice was much lower than in 6 month-old mice. Thus, a failure in the beta cells insulin secretion seems to occur, given the fact of the inhibition of the insulin clearance by the liver, the major site of insulin degradation in an IR-dependent manner (25). More importantly, iLIRKO suggests that owing to severe insulin resistance and prolonged hypersecretion of insulin, the beta cells ultimately undergo a failure in insulin secretion.

Previous evidence demonstrated that insulin signaling is essential for beta-cell growth (10, 26). Just recently, IR double null allele in the liver and in the beta cells failed to induce beta-cell hyperplasia in response to severe insulin resistance. In fact, the persistence of robust hyperplasia in six month-old hypoglycemic LIRKO mice supports the concept of a glucose-independent circulating islet growth factor (27). In this context, iLIRKO mice induced IGF-1

expression in parallel to IR deletion in the liver. This resulted in a persistent increase of plasma IGF-1, consistent with the fact that the liver is the main source of circulating IGF-1 (28). Previous data demonstrated that IGF-1R in the beta-cell is not crucial for islet β-cell mass growth, but participates in control of differentiated function (29). In fact, we have found a relationship among beta-cell hyperplasia, hyperinsulinemia and an increase of genes related to beta-cell proliferation and differentiation in iLIRKO mice. Thus, IGF-1 signaling might be involved in the induction of genes related to glucose-stimulated insulin secretion among others. However, IGFBPs regulates the IGF-1 systemic effect in a complex manner (30). More importantly, iLIRKO mice induced IGFBP1 in parallel to IGF-1 depending on the level of IR deletion by the liver. These results are entirely consistent with the insulin-mediated effect on the IGFBP1 gene expression by the liver cells (31). IGFBP1, which is mainly produced by the liver in the adult mice, inhibits IGF-dependent cellular growth *in vivo* (30, 32, 33). In addition, adult transgenic mice showed glucose intolerance and fasting hyperglycemia and hyperinsulinemia. However, the hyperinsulinemia observed in IGFBP1 transgenic mice can not be attributable to insulin resistance alone (34, 35). In fact, adult transgenic mice showed larger and more numerous beta-cell islets and also an increased beta-cell proliferation (36). Finally, iLIRKO mice showed an increase in the IR expression in the pancreatic islets. More importantly, an increase in the proportion of IR-A versus IR-B in the total percentage of IR was observed in pancreatic islets from iLIRKO mice. On this regard, it was previously reported that IGF-2 and also IGF-1 showed a high affinity to IR-A (37). In addition, BrdU incorporation experiments performed in pancreatic islets isolated from 6-month-old control and iLIRKO mice revealed that the islets from iLIRKO mice were significantly more sensitive to the IGF-1-induced proliferation. Moreover, as assessed in mouse beta-cell lines, occurrence mostly of IR-A in pancreatic islets from iLIRKO mice might imply firstly, an increase in the rate of glucose uptake, a mitogenic signal in beta cells independently of insulin (17) and secondly, an enhanced proliferation in response to either insulin or IGF-1. Taking together, IR-A, but not IR-B, confers a proliferative capability to beta-cells in response to insulin or IGF-1 that may account for beta-cell hyperplasia induced by liver insulin resistance in iLIRKO mice.

In conclusion, our results showed that a defect in the liver leads to an overall insulin resistance. Given the fact that insulin resistance states course with compensatory beta-cell hyperplasia, iLIRKO studies have pointed out a cause and effect relationship between progressive insulin resistance and beta-cell hyperplasia. Ultimately, the beta cells could undergo a failure in the insulin secretion that leads to uncontrolled diabetes. Thus, in these mice, isolated hepatic insulin resistance is sufficient to recapitulate the progressive pathogenesis of type 2 diabetes. In this context, iLIRKO mice induced IGF-1 expression in parallel to IR deletion in the liver. This resulted in a persistent increase of circulating IGF-1. Concurrently, there was a huge increase of IR-A in the total percentage of IR in the hyperplastic pancreatic islets from iLIRKO mice. Thus, our results in iLIRKO mice suggest a liver-pancreatic endocrine axis, IGF-1 being a liver factor that might contribute to compensatory beta-cell hyperplasia through IR-A.

5. ACKNOWLEDGEMENTS

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Abreviations: iLIRKO, Inducible Liver-specific IR KnockOut mouse. **IGF-I,** Insulin-like Growth Factor type I. **PEPCK,** Phosphoenol pyruvate carboxykinase. **FAS,** Fatty acid synthase. **GK,** Glucokinase. **IR,** Insulin Receptor. **IR-A,** insulin receptor A isoform. **IR-B,** insulin receptor B isoform. **BAT,** brown adipose tissue. **WAT,** white adipose tissue.