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Liver-specific deletion of insulin receptor substrate 2 does not impair hepatic glucose and lipid metabolism in mice

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Abstract *Aims/hypothesis:* Hepatic insulin resistance is thought to be a critical component in the pathogenesis of type 2 diabetes but the role of intrinsic insulin signalling pathways in the regulation of hepatic metabolism remains controversial. Global gene targeting in mice and in vitro studies have suggested that IRS2 mediates the physiological effects of insulin in the liver. Reduced hepatic production of IRS2 is found in many cases of insulin resistance. To investigate the role of IRS2 in regulating liver function in vivo, we generated mice that specifically lack *Irs2* in the liver (*LivIrs2KO*). *Materials and methods:* Hepatic insulin signalling events were examined in *LivIrs2KO* mice by western blotting. Glucose homeostasis and insulin sensitivity were assessed by glucose tolerance tests and hyperinsulinaemic–euglycaemic clamp studies. The effects of high-fat feeding upon glucose homeostasis were also determined. Liver function tests were performed and expression of key metabolic genes in the liver was determined by RT-PCR. *Results:* Proximal insulin signalling events and forkhead box O1 and A2 function were normal in the liver of *LivIrs2KO* mice, which displayed minimal abnormalities in glucose and lipid homeostasis, hepatic gene

expression and liver function. In addition, hepatic lipid homeostasis and the metabolic response to a high-fat diet did not differ between *LivIrs2KO* and control mice. *Conclusions/interpretation:* Our findings suggest that liver IRS2 signalling, surprisingly, is not required for the long-term maintenance of glucose and lipid homeostasis, and that extra-hepatic IRS2-dependent mechanisms are involved in the regulation of these processes.

Keywords Insulin receptor substrate protein · Insulin resistance · Insulin signalling · Liver

Abbreviations *AlbCre* mice: albumin Cre recombinase transgenic mice · *Fasn*: fatty acid synthase · FOXA2: forkhead box A2 · FOXO1: forkhead box O1 · *G6pc*: glucose-6-phosphatase · *Gck*: glucokinase · GSK: glycogen synthase kinase · HGP: hepatic glucose production · *Insr*: insulin receptor · *Irs2lox* mice: mice with a floxed allele of *Irs2* · *LIRKO* mice: mice lacking *Insr* in the liver · *LivIrs2KO* mice: mice lacking *Irs2* in liver · MAPK: mitogen-activated protein kinase · PI3K: phosphatidylinositol 3-kinase · RNAi: RNA interference · ZBTB16: zinc finger and BTB domain containing protein 16

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Introduction

Type 2 diabetes is a complex metabolic disorder characterised by skeletal muscle and adipose tissue insulin resistance coupled with pancreatic beta cell dysfunction [1, 2]. A failure to appropriately regulate hepatic glucose and lipid metabolism are also key components, but the mechanisms underlying these abnormalities are incompletely understood [2]. The liver plays a critical role in fuel homeostasis, integrating the actions of anabolic and catabolic hormones with nutrient availability and requirements. Insulin may alter hepatic glucose production (HGP) and other aspects of metabolism through direct effects upon liver enzyme activity and gene expression, and through diverse indirect effects such as the regulation of glucagon production, suppression of gluconeogenic precursor production and via the central nervous system [3]. However,

the contribution of intrinsic hepatic insulin receptor (INSR) signalling to normal physiology and the pathogenesis of type 2 diabetes is an area of ongoing controversy [3], fuelled in part by observations using mouse models with different manipulations of the insulin signalling pathway [4–7].

Mice lacking *Insr* only in the liver (*LIRKO* mice) have marked insulin resistance and defects in hepatic glucose metabolism and gene expression, suggesting that direct hepatic INSR signalling is required for whole-body glucose homeostasis [7]. Hyperinsulinaemic clamp studies in these mice suggest that hepatic *Insr* is required for both direct and indirect effects of insulin upon HGP [8]. However, *LIRKO* mice developed significant liver disease and have abnormal insulin clearance, which may complicate this interpretation [7]. In contrast, acute anti-sense oligonucleotide-mediated knockdown of liver *Insr* expression did not impair insulin action upon HGP [5]. Similarly, transgenic rescue of *Insr* expression in the liver but not other tissues of *Insr* global null mice, suggested that indirect effects of insulin signalling predominate in the regulation of hepatic glucose metabolism [4]. However, methodological and physiological questions have been raised about these studies [3].

Insulin receptor substrate proteins mediate the effects of the insulin receptor upon cellular and whole-body physiology [9], and therefore hepatic insulin resistance may result from defects in their function [10, 11]. Mice lacking *Irs1* display profound growth retardation and insulin resistance, but do not develop diabetes due to beta cell compensation [12]. In contrast, mice lacking *Irs2* develop diabetes due to a combination of insulin resistance and pancreatic beta cell dysfunction [12, 13]. Hyperinsulinaemic–euglycaemic clamp analysis of *Irs1* and *Irs2*-null mice demonstrated that *Irs2*-null mice have markedly impaired suppression of HGP and reduced stimulation of liver glycogen synthesis, while *Irs1*-null mice have insulin resistance predominantly in skeletal muscle and adipose tissue [14]. Reduced hepatic IRS2 production has also been observed in a number of in vivo models of insulin resistance, suggesting a role in this condition [10, 15]. Studies on transformed hepatic cell lines derived from *Irs2*- or *Insr*-null mice have also demonstrated that IRS2 is the predominant IRS protein through which insulin acts [16, 17]. Short-term adenoviral RNA interference (RNAi) strategies to cause acute knockdown of hepatic *Irs* expression have suggested that IRS2 also mediates the effects of insulin upon hepatic lipid metabolism [6]. Therefore to further understand the complex roles of IRS2 in liver metabolism, we generated a novel mouse line lacking *Irs2* specifically in hepatocytes (*LivIrs2KO* mice) and performed detailed analysis of glucose homeostasis and liver function in these mice.

Materials and methods

Mouse breeding and genotyping strategies for mice with a floxed allele of *Irs2* and for albumin Cre recombinase transgenic mice

Mice with a floxed allele of *Irs2* (*Irs2lox* mice) [18] were intercrossed with albumin Cre recombinase transgenic

mice (*AlbCre* mice) obtained from The Jackson Laboratory (Bar Harbor, ME, USA) [19] to generate compound heterozygote mice. Double heterozygote mice were crossed with *Irs2lox^{+/-}* mice to obtain wild-type, *Irs2lox^{+/+}*, *AlbCre* and *AlbCreIrs2lox^{+/+}* mice. Mice lacking *Irs2* in *AlbCre*-expressing cells were designated *LivIrs2KO* mice. Mice were maintained on a 12-h light/dark cycle with free access to water and standard mouse chow (4% fat, RM1; Special Diet Services, Witham, Essex, UK) and housed in specific-pathogen-free barrier facilities. For high-fat diet studies, mice were fed with a diet containing 45% fat, 20% protein and 35% carbohydrate (Special Diet Services) for 3 months. Mice were handled and all in vivo studies performed in accordance with the 1986 Home Office Animal Procedures Act (Home Office, London, UK). *LivIrs2KO* mice were studied on a mixed *129Sv/C57Bl/6* background with appropriate litter-mate controls. Wild-type, *Cre* transgenic and *Irs2lox^{+/+}* mice were phenotypically indistinguishable and balanced numbers of mice of these genotypes were used as controls. Genotyping of the mice was performed by PCR amplification of tail DNA as described previously [18].

Metabolic studies

Blood samples were collected from mice via tail vein bleeds or from cardiac puncture on terminally anaesthetised mice. Blood glucose, plasma insulin levels and leptin levels were determined as previously described [18]. Adiponectin was measured using a mouse adiponectin ELISA (R & D Systems, Minneapolis, MN, USA). NEFAs were measured using a NEFA kit (Roche Diagnostics, Lewes, UK). Serum and tissue triglyceride levels from fasted and fed animals were assayed using the GPO Trinder kit (Sigma-Aldrich, Dorset, UK). Albumin and liver function tests were measured using a Dimension RXL multi-channel analyzer (Dade-Behring, Milton Keynes, UK). Glucose and insulin tolerance tests were performed as previously described [18]. Body fat mass was determined by dual emission X-ray absorptiometry using a PIXImus densitometer (GE Healthcare, Chalfont St Giles, UK).

RNA isolation and real-time quantitative RT-PCR

RT-PCR was performed as previously described [18] using FAM/TAMRA-labelled fluorescent probes. RT primers listed in 5' to 3' orientation were: for glucokinase (*Gck*), forward: GGTGCTTTTGAGACCCGTTTT, reverse: GAGTGCTCAGGATGTTAAGGATCTG, probe: TGTCGCAGGTGGAGAGCGACTCTG; and for glucose-6-phosphatase (*G6pc*) forward: ACTCTTGCTATCTTTTCGAGGAAAGA, reverse: CCAACCACAAGATGACGTTCA, probe: AAAGCCAACGTATGGATTCCGGTGT. The relative amount of mRNA was calculated from an internal standard curve following normalisation to 18 S ribosomal RNA levels.

Hyperinsulinaemic clamp studies, whole-body glucose turnover rate, and tissue glycogen content

Hyperinsulinaemic clamp studies were performed as previously described [20]. In brief, under general anaesthesia, an indwelling catheter was placed into the left femoral vein, subcutaneously tunnelled and externalised in the interscapular region. The animals were allowed to recover for 5 days and fasted for 6 h on the day of the experiment. For 3 h D-[3-³H]-glucose was infused at a rate of 11 mBq kg⁻¹min⁻¹ and insulin at a rate of 18 mU kg⁻¹min⁻¹. Euglycaemia was maintained by a variable infusion of 15% glucose. Whole blood was sampled from the tail every 10 min during the last hour for ³H-Glucose enrichment, which was biochemically determined as follows. Samples were de-proteinised by precipitation with equal volumes (125 µl) of 0.1 mol/l Ba(OH)₂ and 0.1 mol/l ZnSO₄, followed by centrifugation for 5 min. Total radioactivity in the supernatant (from D-[3-³H]-glucose and glycolysis-derived ³H₂O) was measured by scintillation counting. A corresponding aliquot was evaporated to dryness to estimate D-[3-³H]-glucose alone. Total glucose concentration was determined in a third aliquot by the glucose oxidase method (BioMerieux, Marcy l'Etoile, France). Glycogen content was determined in liver and quadriceps muscle using the amyloglucosidase method. In brief, tissues underwent alkaline hydrolysis at 55°C for 1 h (30 mg of liver in 200 µl 1 mol/l NaOH, 20 mg of muscle in 50 µl 1 mol/l NaOH). Equivalent volumes of 1 mol/l HCl were added for neutralisation and the samples spun at 340 g for 2 min. Aliquots of the glycogen-containing supernatant were incubated at 37°C for 1 h with α-amyloglucosidase (Roche Diagnostics) at a concentration of 0.01 mg/ml in 0.2 mol/l Na citrate buffer pH 4.8. The released glucose was determined by the glucose oxidase method (BioMerieux). Baseline hepatic glucose content was measured in an undigested aliquot and subtracted from the value obtained after α-amyloglucosidase incubation. Calculations of glucose turnover were made from parameters obtained during the last 60 min of the infusions under steady-state conditions as described previously [20].

Preparation of tissue extracts and immunoblot assays

Following an overnight fast, a bolus of insulin (5 IU) or vehicle was injected via the inferior vena cava of terminally anaesthetised mice. Tissues were removed and snap-frozen in liquid nitrogen and stored at -80°C until use. Tissues were homogenised in lysis buffer [18], solubilised for 30 min on ice and clarified by centrifugation. Supernatants were snap-frozen in aliquots and stored at -80°C. For Western blotting, 50 µg of total protein extract was immunoblotted with the indicated antibodies. For analysis of IRS protein levels and tyrosine phosphorylation or INSR β tyrosine phosphorylation, tissue extracts (2 mg of total protein) were immunoprecipitated for 2 h with the indicated antibodies. Immune complexes were collected with 100 µl of 50% slurry of protein-A or protein-G sepharose,

washed with lysis buffer and resolved on 7.5% SDS-PAGE and transferred to nitrocellulose. The blots were probed with polyclonal antibodies against IRS2 and IRS1 or monoclonal antibody to antiphosphotyrosine and enhanced chemiluminescence (Amersham, Little Chalfont, UK) detection performed. Nuclear preparations were performed by homogenisation of liver samples in nuclear buffer [21] followed by centrifugation over a cushion of nuclear buffer with 1.2 mol/l sucrose and 5% glycerol. Nuclear pellets were re-suspended in nuclear buffer with 0.3 mol/l sucrose and 10% glycerol, purity analysed under a haematocytometer and aliquots frozen. Nuclear preparations were subsequently lysed in lysis buffer, solubilised for 5 min on ice, clarified by centrifugation, after which 50 µg of nuclear extract was immunoblotted with the indicated antibodies. Blots were stripped and re-probed with an antibody against zinc finger and BTB domain containing protein 16 (ZBTB16) as a nuclear loading control.

Antibodies

Sheep polyclonal antibodies to IRS1 and IRS2 were used as previously described [18]. Rabbit anti-IRS1, anti-IRS2, anti-phosphotyrosine (PY) (4G10) and anti-p85 antibodies were from Upstate Biotechnology (Dundee, UK). Anti-AKT, anti-pAKT^{Ser473}, anti-pp70S6K^{Thr389}, anti-glycogen synthase kinase (GSK)-3β, anti-pGSK3α/β^{Ser9/21}, anti-mitogen-activated protein kinase (MAPK), anti-pMAPK^{Thr202/Tyr204}, anti-forkhead box O1 (FOXO1) antibodies

Fig. 1 Liver-specific deletion of *Irs2* and signalling characteristics in *LivIrs2KO* mice. **a** Liver lysates from control (*Cont*) and *LivIrs2KO* mice were subjected to IRS2 immunoprecipitation and subsequent IRS2 immunoblotting. Results are representative of three independent experiments. **b** Muscle and fat lysates from control and *LivIrs2KO* mice were subjected to IRS2 immunoprecipitation and subsequent IRS2 immunoblotting. Results are representative of three mice of each genotype. **c** INSR β immunoprecipitates from liver lysates from saline (–) and insulin-stimulated (+) control and *LivIrs2KO* mice were immunoblotted with anti-phosphotyrosine (PY) or INSR β antibody. Results are representative of four mice of each genotype. **d** IRS1 immunoprecipitates from liver lysates from saline (–) and insulin-stimulated (+) control and *LivIrs2KO* mice were immunoblotted with 4G10 anti-phosphotyrosine (PY), anti-p85 or anti-IRS1 antibodies. Results are representative of four mice of each genotype. **e–j** Liver lysates from saline (–) and insulin-stimulated (+) control and *LivIrs2KO* mice were immunoblotted with anti-phospho AKT (*p-AKT*) or anti-total AKT antibodies (**e**), anti-phosphoGSK3 (*p-GSK3*) or anti-total GSK3 antibodies which recognise the α and β isoforms (**g**) and anti-phospho-MAPK (*p-MAPK*) or anti-total MAPK antibodies which recognise the p42/44 isoforms (**i**). Blots are representative of four mice of each genotype. Quantification of phosphorylation of AKT (**f**), GSK3 (**h**) and MAPK (**j**) on Western blotting was performed by scanning densitometry. Filled bars, saline; open bars, insulin-stimulated. Results shown are means±SEM (*n*=4). **k, l** Hepatic nuclear extracts were blotted for FOXO1 and FOXA2 in control and *LivIrs2KO* mice after saline (–) or insulin (+) stimulation (**k**), or after an overnight fast (*Fast*) and after 2 h of refeeding (*Fed*) (**l**). Blots were stripped and re-probed with an antibody against ZBTB16 protein as a nuclear loading control. Results are representative of four mice of each genotype for each condition

were from Cell Signalling Technology (Beverly, MA, USA) and anti-forkhead box A2 (FOXA2) antibody was a gift from R. I. Altaba (University of Geneva Medical School, Geneva, Switzerland). Anti-INSR β , and anti-p70^{S6K} antibodies were from Santa Cruz (Insight Biotechnology, Wembley, UK). Mouse anti-ZBTB16 was from Calbiochem-Novachem (San Diego, CA, USA). HRP-conjugated goat anti-rabbit antibody was from DAKO (Ely, UK) and HRP-conjugated sheep anti-mouse antibody was from Amersham Bioscience.

Statistical methods

All statistics were performed using GraphPad Prism4 software (Graphpad Software, San Diego, CA, USA) and paired and unpaired *t*-tests and two-way ANOVA with Bonferroni post-tests performed as appropriate. A *p* value of <0.05 was regarded as significant.

Results

Mice lacking *Irs2* in liver (*LivIrs2KO* mice)

LivIrs2KO mice were obtained with the expected Mendelian frequency and developed normally. To confirm the deletion of *Irs2* in liver, we performed Western blotting on IRS2 immunoprecipitates from *LivIrs2KO* and control mouse liver lysates. At 4 weeks of age, liver IRS2 protein was reduced by >95% with residual production likely to be occurring in non-hepatocyte cell types not expressing the *AlbCre* transgene (Fig. 1a). A similar level of deletion was seen in 12-week-old *LivIrs2KO* mice (data not shown). In contrast, no alteration of IRS2 production was detected in the muscle and fat of *LivIrs2KO* mice compared with control animals (Fig. 1b).

Analysis of insulin signalling pathways in *LivIrs2KO* mice

INSR β and IRS1 expression and tyrosine phosphorylation in response to in vivo insulin stimulation were equivalent in control and *LivIrs2KO* mice, suggesting there was no compensation by these events for the loss of IRS2 (Fig. 1c,d). Insulin-stimulated recruitment of the p85 adaptor subunit of phosphatidylinositol 3-kinase (PI3K) to IRS1 immunoprecipitates was also unchanged in *LivIrs2KO* mice (Fig. 1d). These findings suggest that proximal elements of the IRS1 limb of the insulin signalling cascade were normal in *LivIrs2KO* mice, but it has been suggested that there is differential signalling via IRS1 and IRS2 to AKT and GSK-3 β . We detected no alteration in expression of AKT or GSK3 α/β in the *LivIrs2KO* mice (Fig. 1e,g). Both AKT and GSK3 β basal phosphorylation was mildly elevated in *LivIrs2KO* mice, but there was no defect in insulin-stimulated phosphorylation of either of these kinases (Fig. 1e–h). MAPK expression and phosphorylation were

not perturbed in *LivIrs2KO* mice (Fig. 1i,j), and no alterations in p70^{S6K} and PDK-1 expression and phosphorylation were detected in these mice (data not shown).

IRS1 and IRS2 have been suggested to have different signalling potential to forkhead transcription factors, which are major transcriptional mediators of insulin action [22]. We therefore examined the nuclear localisation of FOXO1 and FOXA2 upon insulin stimulation, as well as the effects of a physiological stimulus, the fast/fed transition, by isolating nuclear protein preparations from liver lysates and subjecting them to Western blotting for both of these factors. In response to insulin stimulation, we detected an equivalent translocation of either FOXO1 or FOXA2 from the nucleus in *LivIrs2KO* mice compared to controls (Fig. 1k). Likewise, FOXO1 translocated from the nucleus after a 2-h period of feeding following a 16-h overnight fast in both *LivIrs2KO* and control mice (Fig. 1l). However, we were unable to demonstrate a convincing translocation of FOXA2 under these conditions in mice of either genotype (Fig. 1), while analysis of AKT phosphorylation in the same animals demonstrated an equivalent increase in pAKT^{Ser473} upon re-feeding in *LivIrs2KO* and control mice (data not shown). These findings suggest that there is no defect in FOXO1 or FOXA2 function in *LivIrs2KO* mice.

Glucose homeostasis in *LivIrs2KO* mice

Our failure to detect significant abnormalities in proximal insulin signalling events in *LivIrs2KO* mice prompted us to examine in detail glucose and lipid homeostasis in these mice. Fasting blood glucose levels in 3-month-old male *LivIrs2KO* mice were indistinguishable from those seen in control mice (Fig. 2a). Random fed blood glucose levels in male *LivIrs2KO* mice at this age were also comparable to values in control mice (data not shown). Glucose tolerance was also normal in *LivIrs2KO* mice (Fig. 2b). Analysis of fasting insulin levels and insulin sensitivity demonstrated that there was a non-significant increase in insulin and mild non-significant reduction in glucose clearance in an insulin tolerance test (Fig. 2c,d). At 6 months of age, no deterioration in fasted and fed blood glucose levels and fasting insulin levels was seen in *LivIrs2KO* mice (data not shown).

The surprisingly modest abnormalities in glucose metabolism in *LivIrs2KO* mice prompted us to undertake more detailed analysis by performing hyperinsulinaemic-euglycaemic clamps. Whole-body glucose disposal was mildly reduced in *LivIrs2KO* mice (Fig. 2e). This impairment was associated with a small reduction in whole-body glycogen synthesis, which accounted for the impaired whole-body glucose utilisation (Fig. 2h). However, endogenous glucose production and glycolysis rates were not different between *LivIrs2KO* and control mice (Fig. 2f,g). These findings suggest that *LivIrs2KO* mice have a very mild defect in whole-body insulin sensitivity, which is unlikely to be detected by glucose and insulin tolerance tests, and also does not impact on long-term glucose homeostasis.

Fig. 2 Glucose homeostasis in *Livlrs2KO* mice. **a** Fasting blood glucose levels were determined on male control and *Livlrs2KO* mice at 3 months of age. Data represent the mean $\pm$ SEM for six to eight animals of each genotype. **b** Glucose tolerance tests were performed on male control (closed squares) and *Livlrs2KO* mice (open squares) at 3 months of age. Data represent the mean $\pm$ SEM for six to eight animals of each genotype. **c** Fasting blood insulin levels were measured on male control and *Livlrs2KO* mice at 3 months of age. Data represent the mean $\pm$ SEM for six to eight animals of each

genotype. **d** Insulin tolerance tests (0.75 IU/kg) were performed on 3-month-old male control (closed squares) and *Livlrs2KO* mice (open squares). Results represent blood glucose concentration as a percentage of starting value at time 0 and are expressed as mean $\pm$ SEM ($n=8$). **e–h** Hyperinsulinaemic–euglycaemic clamps were performed on conscious mice of the indicated genotypes to determine glucose turnover (**e**), endogenous glucose production (**f**), glycolysis (**g**) and glycogen synthesis rates (**h**). Data represent the mean $\pm$ SEM for eight to nine animals of each genotype. * $p<0.05$

Lipid homeostasis and response to high-fat diet in *LivIrs2KO* mice

IRS2 signalling may play a predominant role in hepatic lipid metabolism rather than glucose homeostasis [6], and therefore we assessed a number of parameters of whole-body lipid homeostasis in *LivIrs2KO* mice on normal chow. In both the fasted and fed states NEFA were comparable in *LivIrs2KO* and control mice (Fig. 3a). No differences in serum and liver tissue triglyceride levels were

found in *LivIrs2KO* and control mice, either in the fed or fasted conditions (Fig. 3b,c and data not shown). We also found no differences in fasting leptin levels between *LivIrs2KO* and control animals (Fig. 3d). Serum adiponectin levels were non-significantly elevated in *LivIrs2KO* mice compared with control animals (control, 6.98 ± 0.94 $\mu\text{g/ml}$ vs *LivIrs2KO*, 9.3 ± 1.1 $\mu\text{g/ml}$, $n=17$, $p=\text{NS}$). Dual emission X-ray absorptiometry scanning at 6 months of age did not reveal alterations in fat mass in *LivIrs2KO* mice (data not shown). Taken together, these findings suggest that defects

Fig. 3 Assessment of lipid metabolism and high-fat feeding in *LivIrs2KO* mice. **a** Fasted and fed serum NEFA levels were determined in 12-week-old male control and *LivIrs2KO* mice. Data represent the mean $\pm$ SEM for five animals of each genotype. **b** Fed serum triglycerides (TG) in 12-week-old male control and *LivIrs2KO* mice. Data represent the mean $\pm$ SEM for six animals of each genotype. **c** Triglyceride levels in liver extracts from fed 12-week-old male control and *LivIrs2KO* mice. Data represent the mean $\pm$ SEM for six animals of each genotype. **d** Serum

leptin levels in fasting 6-month-old male control and *LivIrs2KO* mice. Data represent the mean $\pm$ SEM for five animals of each genotype. **e** Fasting blood glucose levels in 6-month-old male mice of the indicated genotypes after 3 months on a high-fat diet. Data represent the mean $\pm$ SEM for four to six animals of each genotype. **f** Glucose tolerance tests were performed on 6-month-old male control (closed squares) and *LivIrs2KO* mice (open squares) after 3 months on a high-fat diet. Data represent the mean $\pm$ SEM for six animals of each genotype. * $p < 0.05$

in hepatic lipid homeostasis and fat storage in *LivIrs2KO* mice are minimal.

Hepatic insulin resistance is involved in the development of the metabolic syndrome induced by increased fat intake [23]. Therefore, we next assessed the effects of a high-fat diet upon glucose homeostasis in *LivIrs2KO* mice. Control and *LivIrs2KO* male mice fed a diet consisting of 45% fat for 3 months gained on average 25% more weight than mice on normal chow (data not shown). However, while fasting blood glucose levels in *LivIrs2KO* mice were mildly higher than in control mice (Fig. 3e), glucose tolerance was not significantly different (Fig. 3f). Fasting insulin levels and serum and liver triglyceride levels were similar in both control and *LivIrs2KO* mice subjected to high-fat diet (data not shown). These findings suggest that a high-fat diet does not result in greater insulin resistance in *LivIrs2KO* mice than in control animals.

Liver function in *LivIrs2KO* mice

Since *LIRKO* mice have been reported to display progressive liver dysfunction [7], we performed liver function tests in *LivIrs2KO* mice. At 3 months of age, albumin and bilirubin levels were indistinguishable between male control and *LivIrs2KO* (Fig. 4a). Aspartate amino-transferase

and alanine amino-transferase levels were both elevated in *LivIrs2KO* mice, while alkaline phosphatase levels were not (Fig. 4b). *LivIrs2KO* mice remained healthy beyond 12 months of age and did not develop clinical evidence of progressive liver dysfunction such as oedema or morphological abnormalities in the liver (data not shown).

Hepatic gene and protein expression in *LivIrs2KO* mice

Insulin regulates hepatic metabolism partly by regulating the expression of key metabolic genes in glucose and lipid metabolism. RT-PCR analysis of liver mRNA expression revealed that in the fasted state, *Gck* expression was mildly reduced in *LivIrs2KO* mice, although in the fed state no difference was found (Fig. 4c). *G6pc* expression was mildly increased in fasted *LivIrs2KO* mice, but in the fed state no difference was found (Fig. 4d). In contrast RNA expression of a panel of other hepatic metabolic genes (notably *Pck1*, *Fasn*, *Srebf1a* and *Ic*, *Acaca*, *Ppara* and *Pparg1*, *Cpt1a*, *Ppargc1b* and *Igf1*) were unaltered in *LivIrs2KO* mice either in the fasted or fed state (data not shown). Therefore deletion of *Irs2* in the liver had no significant effect on a number of insulin-regulated transcriptional events.

Fig. 4 Liver function tests and gene expression in *LivIrs2KO* mice. **a** Serum albumin (left) and bilirubin (right) levels in fasting 12-week-old male control and *LivIrs2KO* mice. Data represent the mean±SEM for six animals of each genotype. **b** Serum aspartate amino-transferase (AST), alanine amino-transferase (ALT) and alkaline phosphatase (ALP) levels in fasting 12-week-old male control

and *LivIrs2KO* mice. Data represent the mean±SEM for six animals of each genotype. Hepatic glucokinase (*Gck*) (**c**) and glucose-6-phosphatase (*G6pc*) (**d**) mRNA expression in fasted and fed 12-week-old male control (closed bars) and *LivIrs2KO* (open bars) mice was determined by RT-PCR. Data represent the mean±SEM for five animals of each genotype. **p*<0.05

Discussion

LivIrs2KO mice displayed no marked alteration in insulin receptor, IRS1 and downstream PI3K-dependent signalling events in the liver. These observations contrast with findings in mice with global deletion of *Irs2* and in *Irs2*-null transformed hepatic cell lines derived from neonatal mice. In both these models there are abnormalities in signalling events downstream of the insulin receptor. From our studies, however, it is clear that the chronic deletion of *Irs2* in the liver appears to have minimal effects upon proximal insulin signalling events in vivo. The transcription factors FOXO1 and FOXA2 are thought to play distinct roles in the regulation of hepatic carbohydrate and lipid metabolism. FOXO1, which predominantly regulates gluconeogenesis, has been reported to be phosphorylated only by the IRS2 signalling limb while FOXA2, which regulates fatty acid oxidation, was phosphorylated in response to both IRS1 and IRS2 signalling pathways [22]. However, these in vitro findings were not confirmed by our studies. In the absence of liver *Irs2*, FOXO1 translocation both in response to insulin and re-feeding was normal. FOXA2 translocation in response to insulin was also comparable to that seen in control animals. Therefore, these results do not support the idea that IRS2 preferentially signals to FOXO1.

Consistent with the normal hepatic insulin signalling events, *LivIrs2KO* mice on a regular diet displayed minimal abnormalities of glucose homeostasis up to and beyond 6 months of age, which contrasts with the marked and progressive diabetes observed in *Irs2* global null mice [12]. Although we did detect decreased expression of *Gck* and increased expression of *G6pc* in the livers of fasted *LivIrs2KO* mice, suggesting some minor dysregulation of insulin-regulated gene expression in the fasted state, we did not find abnormalities in the expression of these enzymes in the fed state, suggesting that they are appropriately regulated by insulin. Interestingly, *Irs2* expression is high in the fasted state and markedly reduced by feeding and by increased insulin levels [10], and perhaps IRS2 in the liver serves to predominantly regulate the expression of particular genes in the fasted state. Taken together, these findings suggest that IRS2-dependent pathways are not essential for the long-term regulation of glucose homeostasis.

Recent studies have suggested that hepatic IRS2 is particularly involved in the regulation of lipid metabolism. RNAi knockdown of *Irs2* resulted in increased levels of the transcription factor SREBF1c and this was associated with mildly increased serum NEFA and triglyceride levels and mild hepatic steatosis [6]. Others have demonstrated a reciprocal relationship between IRS2 function and the expression of *Srebflc* [10, 11]. We found that hepatic deficiency of IRS2 had no effects upon circulating NEFA and triglyceride or on hepatic triglyceride levels. Likewise, we found no alterations in the expression of key hepatic transcription factors and enzymes involved in the regulation of lipid metabolism or of leptin levels or of total body fat stores. We also found no differences in glucose and

lipid homeostasis between *LivIrs2KO* and control mice while on a high-fat diet. These findings suggest that hepatic IRS2-dependent signalling events are not required for the long-term in vivo maintenance of hepatic lipid homeostasis.

The differences between our observations and those of others may be explained by a number of methodological and physiological factors. Differences between global and conditional deletion may reflect developmental events and are potentially related to both the timing of *Irs2* deletion as well as to the contribution of other tissues to the phenotype. *AlbCre* mice are reported to delete floxed alleles completely and this may occur at several weeks of age post-natally [19]. We found complete deletion of *Irs2* by 4 weeks of age and our subsequent physiological studies were performed on mice at older ages. Studies in immortalised cell lines do not reflect the in vivo physiological situation; moreover, the hepatocyte cell lines reported were derived from neonatal mice with germ-line deletion of either *Insr* or *Irs2* [16, 17]. For the studies involving the disruption of *Irs2* expression by RNAi, the knockdown was both relatively short-term lasting no more than 5 days and was incomplete, with a persistent 40% residual *Irs2* expression evident [6]. In contrast, our studies involve long-term complete deletion of *Irs2*, which may permit compensatory mechanisms to develop. However, we saw no increased expression of insulin signalling components in the liver and no alterations of *Irs2* expression in other tissues. Furthermore, in the *Irs2* RNAi knock-down there was an alteration in adipose tissue function, which was not apparent in our studies, but may explain the abnormalities seen in hepatic lipid homeostasis. The mechanism for this effect was not determined but it is clear that systemic as opposed to tissue-specific delivery of RNAi may abrogate gene expression in other tissues in addition to the liver [5]. Unfortunately, *Irs2* expression in adipose tissue was not reported in the liver *Irs2* RNAi model. Since insulin positively regulates lipogenesis, disruption of insulin receptor function via down-regulation of IRS signalling in the liver alone might be expected to reduce rather than increase lipogenesis. Indeed, this is seen in *LIRKO* mice and in mice with knock-down of liver *Insr* expression by anti-sense oligonucleotide [5, 7]. The mechanism of increased lipogenesis in *Irs2* RNAi knock-down mice is probably due to extra-hepatic effects or to residual insulin signalling in the liver. Our findings also call into question the suggestion that there is a physiologically relevant reciprocal relationship between IRS2 and regulators of lipid homeostasis such as SREBF1c [10, 11]. These findings were largely derived from in vitro models and in vivo models with systemic deletion of *Irs2* or with complex multi-tissue metabolic abnormalities such as *ob/ob* and lipodystrophic mice, and this may explain the differences between our observations and those of others.

Recent data from two distinct mouse models have suggested that insulin regulates hepatic metabolism largely through indirect means rather than through the liver insulin receptor and this provides a physiological explanation why *LivIrs2KO* mice have a minimal phenotype [4, 5]. While

these recent findings are at odds with those generated by studies on the *LIRKO* mice, this latter model has marked defects in hepatic function and insulin clearance that impinge upon insulin action in other tissues and may explain the differences in phenotype. Our observations in *LivIrs2 KO* mice are consistent with the idea that insulin signalling pathways are not required for the direct regulation of hepatic metabolism. While we have not abrogated other insulin receptor signalling pathways through this manipulation, hepatic *Irs2* is not required for the long-term maintenance of many aspects of hepatic function. Recently we showed that mice lacking *Irs2* only in the central nervous system develop insulin resistance and impaired glucose homeostasis, and we are exploring hepatic metabolism in this model, as it is likely that central nervous system IRS2 pathways contribute to the regulation of hepatic metabolism [18]. These findings together with those of others suggest that in mice, central nervous system pathways and other indirect effects may be the predominant mechanisms by which insulin signalling regulates hepatic metabolism.

In summary, through the generation of *LivIrs2KO* mice we have demonstrated that IRS2 signalling events in the liver are not required for the regulation of hepatic metabolism.

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