Peripheral insulin resistance in ILK-depleted mice by reduction of GLUT4 expression

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Abstract

The development of insulin resistance is characterized by the impairment of glucose uptake mediated by glucose transporter 4 (GLUT4). Extracellular matrix changes are induced when the metabolic dysregulation is sustained. The present work was devoted to analyze the possible link between the extracellular-to-intracellular mediator integrin-linked kinase (ILK) and the peripheral tissue modification that leads to glucose homeostasis impairment. Mice with general depletion of ILK in adulthood (cKD-ILK) maintained in a chow diet exhibited increased glycemia and insulinemia concurrently with a reduction of the expression and membrane presence of GLUT4 in the insulin-sensitive peripheral tissues compared with their wild-type littermates (WT). Tolerance tests and insulin sensitivity indexes confirmed the insulin resistance in cKD-ILK, suggesting a similar stage to prediabetes in humans. Under randomly fed conditions, no differences between cKD-ILK and WT were observed in the expression of insulin receptor (IR-B) and its substrate IRS-1 expressions. The IR-B isoform phosphorylated at tyrosines 1150/1151 was increased, but the AKT phosphorylation in serine 473 was reduced in cKD-ILK tissues. Similarly, ILK-blocked myotubes reduced their GLUT4 promoter activity and GLUT4 expression levels. On the other hand, the glucose uptake capacity in response to exogenous insulin was impaired when ILK was blocked *in vivo* and *in vitro*, although IR/IRS/AKT phosphorylation states were increased but not different between groups. We conclude that ILK depletion modifies the transcription of GLUT4, which results in reduced peripheral insulin sensitivity and glucose uptake, suggesting ILK as a molecular target and a prognostic biomarker of insulin resistance.

Key Words

- ▶ integrin-linked kinase
- ▶ Glut4
- ▶ striated muscle
- ▶ white adipose tissue
- ▶ myotubes
- ▶ insulin resistance
- ▶ glucose uptake

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Introduction

Type 2 diabetes and metabolic syndrome (MS) are characterized by high blood glucose levels as a consequence of inappropriate peripheral glucose uptake. The insulin-responsive facilitative glucose transporter GLUT4 is strongly expressed in striated muscle and white adipose tissue (WAT), which are responsible for glucose disposal in postprandial state (Shepherd & Kahn 1999); therefore, the total amount of GLUT4 expression has been directly related with whole-body glucose homeostasis by affecting glucose transport in these tissues (Kern *et al.* 1990, Shepherd & Kahn 1999, Matsui *et al.* 2006, Karnieli & Armoni 2008). Supporting this concept, transgenic GLUT4 knockdown or overexpression in animal models displayed, respectively, diminished or enhanced peripheral glucose utilization (Hansen *et al.* 1995, Zisman *et al.* 2000, Wallberg-Henriksson & Zierath 2001, Minokoshi *et al.* 2003, Atkinson *et al.* 2013). In fact, human studies have proposed that during type 2 diabetes, MS and aging, GLUT4 expression is reduced in peripheral tissue, which may play a role in increasing insulin resistance (Shepherd & Kahn 1999, Gaster *et al.* 2001, Zeyda & Stulnig 2009). Moreover, some medical therapies for type 2 diabetes-like glitazones are able to increase total GLUT4 levels and improve glucose uptake (Bähr *et al.* 1996, Hammarstedt *et al.* 2005). In addition, glucose uptake mechanism is regulated by the modulation of downstream effectors of insulin-mediated signaling, which promotes GLUT4 translocation from the reservoir vesicles to the plasma membrane (Carnagarin *et al.* 2015). Quantitative and qualitative changes in extracellular matrix (ECM) proteins are characteristic of sustained insulin resistance (Berria *et al.* 2006, Williams *et al.* 2015). ECM proteins bind to integrins, which are clustered in focal adhesion complexes connected to the cytoskeleton and are able to transmit morphological and gene expression changes (Wu & Dedhar 2001). Thus, it could be the case that ECM-focal adhesion changes may modulate glucose transporters. Indeed, *in vitro*, transgenic and type 2 diabetes animal models have shown integrin subunit beta 1 (ITGB1) as a modulator of GLUT4 (Guilherme & Czech 1998, Zong *et al.* 2009). As part of the ITGB1-focal adhesion complex, the integrin-linked kinase (ILK) has been suggested to modulate capillarization of the muscle from diet-induced insulin resistant mice (Kang *et al.* 2016). To elucidate the role of ILK in the insulin-sensitive peripheral tissues concerning the regulation of the whole-body glucose homeostasis, we studied the effect of ILK depletion over the expression of GLUT4, its integration

with insulin signaling and the consequent impact on the glucose uptake capacity.

Materials and methods

Conditional ILK knockdown mice (cKD-ILK)

All procedures involving animals were approved by the Institutional Animal Care and Use Committee of the University of Alcalá and conformed to Directive 2010/63/EU of the European Parliament. We have implemented cKD-ILK mice model previously (Serrano *et al.* 2013, Cano-Peñalver *et al.* 2014, 2015). Briefly, general inactivation of the *Ilk* gene was accomplished by crossing C57Bl/6 mice homozygous for the floxed *Ilk* allele, flanked by loxP sites (LOX) with homozygous BALB/cJ strain mice carrying a CMV-driven tamoxifen-inducible CreER (T) recombinase gene (CRE); three-month-old male CRE-LOX mice weighing 20–28 g were injected intraperitoneally (i.p.) with 1.5 mg of 4-hydroxytamoxifen (TX, Sigma-Aldrich) or vehicle (VH, corn oil/ethanol, 9:1, Sigma-Aldrich) once a day for five consecutive days. Three weeks after the injections, the tail DNA was genotyped by PCR. The TX-treated CRE-LOX mice displaying successful depletion of ILK were termed cKD-ILK and the VH-treated CRE-LOX were termed wild-type (WT) mice. Once the experiments were terminated, the mice were killed, and cardiac left ventricle, slow-twitch (red) fibers from the vastus lateralis, epididymal and mesenteric fat depots were dissected. See Supplementary methods for further details (see section on [supplementary data](#) given at the end of this article).

Myotubes culture, transient transfections and pharmacological treatments

Mouse myoblasts cell line (C2C12; ATCC) were induced to differentiate into myotubes and transfected (Metafectene si+; Biontex, Munich, Germany) with a combination of 40 nM different small interfering RNAs against *Ilk* (si-ILK, Santa Cruz) or 20 nM scrambled siRNAs as control (CT, Thermo Fisher Scientific). The silencing process took 48 h, and cells were processed or treated after that time. To pharmacologically inhibit the ILK, myotubes were treated for 24 h with 3 μ M ILK inhibitor CPD-22 (Merck-Millipore) or vehicle (CT) (Mamuya *et al.* 2016). In some experiments, ILK-depleted myotubes were transfected overnight with the luciferase reporter plasmid for human

GLUT4 promoter (Knight *et al.* 2003). See Supplementary methods for further details.

Glucose, insulin and pyruvate tolerance tests (GTT, ITT and PTT): glycemia and insulinemia determinations

After 16 h fasting, mice were intraperitoneally (i.p.) injected with glucose (2 mg per g of body weight, Sigma-Aldrich) or sodium pyruvate (2 mg per g of body weight, Sigma-Aldrich). 4-h fasting mice were i.p. injected with insulin (0.75 U per kg of body weight, Actrapid, Novo Nordisk A/S). At different time lapses, blood glucose was measured via tail bleeding using a glucometer (Accu-Check Aviva; Roche).

Insulinemia was analyzed from submandibular vein plasma (Cloud-Clone Corp. ELISA kit, Houston, USA) (Ayala *et al.* 2010). To provide a reliable approach to formal measures of insulin resistance and sensitivity, the homeostasis model assessment of insulin resistance (HOMA-IR, fasting glycemia in mg/dL multiplied by fasting insulinemia in μ U/mL, divided to 405) and the quantitative insulin sensitivity check index (QUICKI, 1 divided to log fasting insulinemia in μ U/mL plus log fasting glycemia in mg/dL) values were calculated (Bowe *et al.* 2014). See Supplementary methods for further details.

Acute insulin stimulation *in vivo*

Four -hour fasted mice were i.p. injected with an exogenous insulin bolus (0.75 U per kg of body weight, Actrapid, Novo Nordisk A/S) or saline. After 30 min, mice were killed, and the cardiac left ventricle was rapidly excised for subsequent analysis.

Reverse transcription–quantitative polymerase chain reaction (RT-qPCR)

Total RNA from animal or cell samples was extracted with Trizol, and RT-qPCR was performed as described previously (Cano-Peñalver *et al.* 2014, 2015). For relative quantification, $2^{-\Delta\Delta CT}$ normalized gene expression method was used. See Supplementary methods for further details.

Protein extraction and immunoblot analysis

Immunoblots were performed as described previously (Cano-Peñalver *et al.* 2014, 2015). Tissues or cells were

homogenized, and equal amounts were separated on SDS-polyacrylamide gels, transferred to membranes, blocked and incubated with specific antibodies. Immunoblots were developed, and the densitometries were measured. See Supplementary methods for further details.

Differential centrifugation of subcellular fractions

The differential centrifugation was performed as described previously (Cano-Peñalver *et al.* 2014). Briefly, tissues from fed and insulin-treated mice were homogenized in isolation solution (250 mM sucrose and 10 mM Tris, and protease/phosphatase inhibitors, pH=7.5). The supernatants obtained in 2 serial centrifugations, at 4000g for 10 min at 4°C to remove cell debris and nuclear fragments, were centrifuged at 17,000g for 20 min at 4°C. The obtained pellet was suspended in lysis buffer (commented above) to facilitate homogenization and corresponds to the high-density plasma membrane (PM) fraction. The supernatants were spun to 200,000g for 60 min, and these pellets were suspended in the same volume of lysis buffer as PM and recorded as the low-density, vesicle-enriched intracellular membrane fraction (IM). 30 μ L aliquots of each fraction per sample were subjected to immunoblot analysis. The PM/IM densitometry comparatives were representative of subcellular cytoplasm (vesicular) vs plasmatic membranes GLUT4 location.

Glucose uptake assay

Glucose uptake was performed as described previously (Nedachi & Kanzaki 2006, Wang *et al.* 2012). Briefly, freshly excised cardiac left ventricle, ILK-depleted or inhibited myotubes were incubated in deprived DMEM with or without insulin (100 nM) for 15 min before adding 0.1 mM of the fluorescent D-glucose analog 2-[N(7nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-glucose (2-NBDG, Sigma-Aldrich); after 30 min incubation, free 2-NBDG was washed out 3 times with cold PBS. Rates of glucose uptake, determined as the intracellular fluorescence (VICTORX4, PerkinElmer), were calculated after subtraction of autofluorescence from negative control without 2-NBDG and expressed as arbitrary units (a.u.).

Statistics

The data shown are represented as the means $\pm$ S.E.M. of a variable number of experiments detailed in the figure legends. Student's *t*-test was used for 2 samples, and

1- or 2-way analysis of variance (ANOVA) was used for >2 samples, with a paired or unpaired design followed by a multiple comparison test. Values of $P < 0.05$ were considered statistically significant.

Results

We previously reported a mice model in which the sixth and seventh exons of the *Ilk* gene were able to be excised during young adulthood once they are injected with TX (Serrano *et al.* 2013, Cano-Peñalver *et al.* 2014, 2015). Three weeks later, the adult mice displaying successful ILK depletion (cKD-ILK) and control littermates (VH-treated, WT) were used.

cKD-ILK and WT mice were fed with a normal chow diet, and their body weight gains and food intake were normal within the monitoring period (body weights in g at the time of the experiments, mean $\pm$ S.E.M.: WT = 26.3 ± 1.1 ; cKD-ILK = 28.3 ± 1.8). Figure 1A shows that blood glucose levels, in either randomly fed or after 16 h fasting conditions, were significantly higher in cKD-ILK when compared to WT. CRE-driven mice models have been extensively used in functional metabolism analyses (Zong *et al.* 2009), but some changes in metabolism have been reported immediately after TX injections (Hesselbarth *et al.* 2015); in our parental CRE-mice 3 weeks after the TX administration stated, we confirmed the lack of a direct effect of TX on glycemia (16 h fasting glycemia, mg/dL, mean $\pm$ S.E.M.: CRE + VH = 64.2 ± 3.5 ; CRE + TX = 61.0 ± 2.3). Figure 1B shows that insulin levels were higher in cKD-ILK under fed but not under fasting conditions compared with WT. We next examined changes in plasma glucose and insulin concentrations after the i.p. glucose administration during GTT. Figure 1C shows similar profiles of blood glucose time courses in both groups, and the AUC from GTT curves were not different (mean $\pm$ S.E.M.: WT = 458 ± 72 ; cKD-ILK = 416 ± 63), similar as observed in other insulin resistance models (Zisman *et al.* 2000, Wang *et al.* 2013); however, Fig. 1D shows that insulinemia values and AUC during GTT were higher in cKD-ILK (mean $\pm$ S.E.M., $*P < 0.05$ vs WT: WT = 2089 ± 823 ; cKD-ILK = 4848 ± 1496). To further confirm that cKD-ILK mice are insulin resistant, we analyzed the changes in plasma glucose after the i.p. insulin injection during ITT in both groups. Insulin bolus did not decrease the bloodstream glucose in cKD-ILK as efficiently as in WT (Fig. 1E), and cKD-ILK AUC below the baseline was lower (mean $\pm$ S.E.M., $*P < 0.05$ vs WT: WT = -164 ± 30 ; cKD-ILK = -117 ± 4). cKD-ILK show

Figure 1

Effect of ILK depletion in blood glucose homeostasis of conditional ILK knockdown (cKD-ILK) and wild-type (WT) mice. (A) Blood glucose levels and (B) insulin levels from randomly fed or 16-h fasted mice. (C) Blood glucose levels and (D) insulin levels during i.p. glucose tolerance test (GTT). (E) Blood glucose levels during i.p. insulin tolerance test (ITT). (F) Blood glucose levels during i.p. pyruvate tolerance test (PTT). Curves data are represented as WT (black circles) and cKD-ILK (open squares). Data are shown as mean $\pm$ S.E.M. $N = 6-9$; $*P < 0.05$ vs the same time WT fasting, $*P < 0.05$ vs fasting animals, $^{\$}P < 0.05$ vs WT fed.

higher HOMA-IR values (mean $\pm$ S.E.M., $*P < 0.05$ vs WT: WT = 1.08 ± 0.12 ; cKD-ILK = 1.47 ± 0.12) and lower QUICKI values (mean $\pm$ S.E.M., $*P < 0.05$ vs WT: WT = 0.386 ± 0.007 ; cKD-ILK = 0.358 ± 0.005) compared to WT.

Insulin resistance can be characterized by either decreased glucose uptake in peripheral tissue and/or enhanced hepatic glucose production. To assess whether an underlying hepatic insulin resistance appeared in cKD-ILK, we examined changes in plasma glucose after an i.p. pyruvate administration during PTT (Fig. 1F), where blood glucose time courses and AUC were not different between groups (mean $\pm$ S.E.M.: WT = 254 ± 82 ; cKD-ILK = 142 ± 34).

The primary peripheral tissue responsible for the postprandial glucose disposal in a physiological context is the striated muscle. Thus, we studied whether the ILK depletion was affecting the expression of the

glucose transporter GLUT4 in cardiac left ventricle and vastus lateralis.

The glucose enters to the striated muscle cells mainly via GLUT1 and GLUT4, where GLUT4 is the insulin-responsive glucose transporter. Figure 2A shows reduced *Glut4* mRNA levels in cKD-ILK cardiac tissue from both fasting and randomly fed mice.

Figure 2

GLUT4 expression and membrane presence in the cardiac tissue of conditional ILK knockdown (cKD-ILK) and wild-type (WT) mice. Cardiac left ventricles from fasting or randomly fed WT and cKD-ILK were processed. *Glut4* (A) and *Glut1* (B) mRNA levels fold change normalized against *Actb*. Representative immunoblot and densitometric analysis of GLUT4 (total extract) normalized against ACTIN (C). Membrane-enriched subcellular cardiac fractions were obtained by differential centrifugation and subjected to immunoblotting against GLUT4. Representative immunoblot and densitometric analysis of plasmatic membrane (PM) GLUT4 (D) or intracellular plasmatic membrane (IM) GLUT4 (E), normalized against total protein load using Ponceau membrane dye. PM/IM densitometries ratios (F). Data are shown as mean $\pm$ S.E.M. $N=7-10$. * $P<0.05$ vs WT.

No differences were observed in *Glut1* mRNA levels between groups in randomly fed mice (Fig. 2B). Figure 2C shows reduced GLUT4 protein expression levels in randomly fed cKD-ILK total cardiac tissue extract when compared with WT. The magnitude of GLUT4 subcellular presence in a postprandial state was analyzed by determining its content in the cardiac PM and IM extracts from randomly fed mice. When compared with WT, PM and IM fractions from cKD-ILK showed lower amounts of GLUT4 (Fig. 2D and E), because the total GLUT4 decreased as described above. The PM/IM densitometry rates represented in Fig. 2F, which quantify the vesicular to the plasmatic membrane relocation of GLUT4, were similar in both groups. We confirmed cardiac ILK depletion in randomly fed cKD-ILK mice (Fig. 3A and B) and its consequently reduced activity, determined as the phosphorylation state in downstream substrates GSK3B on serine 9 and AKT on serine 473 (Fig. 3C and D) (Troussard *et al.* 2003, Edwards *et al.* 2005, García-Jérez *et al.* 2015). Since AKT is shared as a downstream component of the IR/IRS-1 network during the GLUT4 modulation (Carnagarin *et al.* 2015), we also studied the AKT phosphorylation state on threonine 308, which was unchanged between groups (Fig. 3E). Similar AKT phosphorylation patterns were observed in fasting cKD-ILK cardiac tissue (Supplementary Fig. 1C and D).

The cardiac ILK depletion in randomly fed mice was not affecting the expression levels of both IR isoforms IR-A and IR-B or IRS-1 (Fig. 4A, B and C). Although the phosphorylation of IR B on tyr1150/1151 was not particularly affected in fasting mice (Supplementary Fig. 1A), it was significantly upregulated in randomly fed cKD-ILK when compared to WT (Fig. 4D), in accordance with the increased insulin production observed in Fig. 1B. Phosphorylation of IRS on different sites may affect its interaction with IR-B leading to a modulation of the signal transduction. In order to analyze a known site to participate in this dynamic process (Weigert *et al.* 2008), we observed no changes in the phosphorylation state of IRS-1 on serine 302 between groups in randomly fed (Fig. 4E) or fasting conditions (Supplementary Fig. 1B).

GLUT4 content was also analyzed in the vastus lateralis muscle portion rich in red, slow-twitch fibers, which present higher basal GLUT4 expression than the rest of the fibers (Gaster *et al.* 2001). As observed in cardiac tissue, cKD-ILK skeletal muscle have reduced ILK levels (Fig. 5A and Supplementary Fig. 2A) and GLUT4 expression under both fasting and fed states (Fig. 5B and Supplementary Fig. 2B). The phosphorylated isoforms

Figure 3

ILK expression and activity in the cardiac tissue of conditional ILK knockdown (cKD-ILK) and wild-type (WT) randomly fed mice. Cardiac left ventricle from randomly fed WT and cKD-ILK were processed. (A) *Ilk* mRNA levels fold change normalized against *Actb*. Representative immunoblot and densitometric analysis of (B) ILK and (C) GSK3B phosphorylated at serine 9 (P-GSK), AKT phosphorylated at serine 473 (D) and AKT phosphorylated at threonine 308 (E), normalized against total ACTIN, GSK3B (GSK) or AKT, respectively. Data are shown as mean $\pm$ S.E.M. $N=7-10$. * $P<0.05$ vs WT.

levels of GSK3B on serine 9 and AKT on serine 473 were reduced in randomly fed cKD-ILK, in accordance with the reduced ILK content (Fig. 5C and D). Similar to cardiac

tissue, the vastus mRNA levels of *Glut1*, *Ir-a* and *Ir-b* were not different between randomly fed animal groups (Supplementary Fig. 2C, D and E). Besides the striated

Figure 4

Insulin receptor (IR)/IR substrate-1 (IRS-1)-signaling pathway in the cardiac tissue of conditional ILK knockdown (cKD-ILK) and wild-type (WT) randomly fed mice. Randomly fed WT and cKD-ILK (A) *Ir-a*, (B) *Ir-b* and (C) *Irs-1* mRNA levels fold change normalized against *Actb*. Representative immunoblot and densitometric analysis of total cardiac tissue extracts IR beta chain (IR-B) phosphorylated on tyrosine 1150/1151 (D) and IRS-1 phosphorylated on serine 302 (E), normalized against total IR-B or IRS-1, respectively. Data are shown as mean $\pm$ S.E.M. $N=7-10$. * $P<0.05$ vs WT.

Figure 5

ILK expression and activity and GLUT4 expression in the skeletal muscle of conditional ILK knockdown (cKD-ILK) and wild-type (WT) mice. Vastus lateralis segment rich in red fibers from randomly fed WT and cKD-ILK were processed. Representative immunoblot and densitometric analysis of (A) ILK and (B) GLUT4 normalized against total ACTIN, and (C) GSK3B phosphorylated at serine 9 (P-GSK) and (D) AKT phosphorylated at serine 473, normalized against total GSK3B (GSK) or AKT. Data are shown as mean $\pm$ S.E.M. $N=7-10$. * $P<0.05$ vs WT.

muscle, the reduction of GLUT4 levels in adipose tissue observed in altered metabolic states leads to a systemic glucose clearance impairment (Shepherd & Kahn 1999, Armoni *et al.* 2007). Similar to the other insulin-sensitive peripheral tissues studied above, *Ilk* and *Glut4* mRNA levels in the mesenteric and epididymal visceral WAT depots from cKD-ILK were reduced compared with WT (Fig. 6A, B, C and D). The phosphorylation state of GSK3B on serine 9 and AKT on serine 473 was reduced in randomly fed cKD-ILK as a consequence of ILK depletion (Fig. 6E and F). No changes were observed in *Glut1* mRNA levels (Supplementary Fig. 2F and G).

In order to challenge the insulin response of the IR-B/IRS-1/AKT network from the cKD-ILK peripheral tissues, four -hour fasted mice were injected with a high dose of insulin (0.75 U/kg, i.p.) (Zong *et al.* 2009), and cardiac left ventricles were isolated and processed 30min later. The IR-B and AKT phosphorylation states, in tyrosines 1150/1151 and serine 473, respectively, were highly increased in insulin-treated mice compared to fasting,

Figure 6

ILK expression and activity and GLUT4 expression in the visceral white adipose tissue (WAT). Mesenteric and epididymal WAT depots from randomly fed WT and cKD-ILK were processed. *Ilk* and *Glut4* mRNA levels fold change from mesenteric WAT (A and C) or epididymal WAT (B and D), normalized against *Actb*. Representative immunoblot and densitometric analysis of (E) GSK3B phosphorylated at serine 9 (P-GSK) and (F) AKT phosphorylated at serine 473, normalized against total GSK3B (GSK) or AKT, respectively, from mesenteric WAT. Data are shown as mean $\pm$ S.E.M. $N=7-10$. * $P<0.05$ vs WT.

non-treated animals, but no differences between insulin-treated WT and cKD-ILK groups were observed (Fig. 7A and B). We analyzed the GLUT4 PM/IM ratios in the cardiac tissue from these insulin-stimulated animals. Figure 7C confirms that the intracellular translocation of GLUT4 to the plasmatic membrane after the insulin bolus was not affected by the lack of ILK, as we observed in randomly fed mice (Fig. 2F). Finally, to test whether ILK depletion observed in the tissue affects insulin sensitivity during the glucose transport, we studied the uptake capacity of the glucose-based fluorescent probe 2-NBDG in either *ex vivo* basal or short-term insulin-stimulated tissue (100nM).

Figure 7

The insulin receptor (IR)/AKT/GLUT4 membrane translocation response and glucose uptake capacity in the cardiac tissue of conditional ILK knockdown (cKD-ILK) and wild-type (WT) mice after a short-term exogenous insulin bolus. 4-h fasting WT and cKD-ILK mice were subjected to a single bolus of insulin (0.75 μ g/kg b.w., i.p.) or vehicle. 30 min later, cardiac left ventricles were processed. Representative immunoblot and densitometric analysis of total cardiac tissue extracts IR beta chain (IR-B) phosphorylated in tyr 1150/1151 (A) and AKT phosphorylated at serine 473 (B), normalized against total IR-B or AKT, respectively. Membrane-enriched subcellular cardiac fractions were obtained from insulin-treated mice by differential centrifugation and subjected to immunoblotting against GLUT4. Representative immunoblot of plasmatic membrane (PM) or intracellular plasmatic membrane (IM) GLUT4, and analysis of PM/IM densitometries ratios, normalized against total protein load using Ponceau membrane dye (C). Cardiac explants from fasting mice were incubated *ex vivo* with insulin (100 nM) for 15 min, and 2-NBDG (0.1 mM) was added for 30 min more. The intracellular 2-NBDG fluorescence fold change was determined (D). Data are shown as mean $\pm$ S.E.M. $N=7-10$. * $P<0.05$ vs non-treated WT, # $P<0.05$ vs no treatment, \$ $P<0.05$ vs WT + insulin.

Figure 7D shows that under non-stimulated conditions, there were no differences between groups. However, the 2-NBDG uptake after 45 min of insulin stimulation was lower in cKD-ILK than in WT tissues, confirming the reduced levels of total GLUT4 observed in cKD-ILK (Fig. 2C).

To better study the ILK-dependent GLUT4 downregulation and its consequences into the glucose uptake, we performed long-term blockade of ILK in myotubes. Supplementary Figure 3 shows the myoblast cell line C2C12 differentiation to myotubes, with increased levels of the myotube differentiation markers MYOG (Bolado-Carrancio *et al.* 2014) and GLUT4, as already observed in other works (Tortorella & Pilch 2002).

To deplete ILK expression, differentiated myotubes were silenced for 48 h with specific siRNAs against ILK prior to process the samples. Figure 8A shows that successfully ILK-depleted myotubes have reduced ILK activity in these conditions, shown as decreased levels of GSK3B phosphorylated in ser 9. Figure 8B shows that long-term ILK-blocked myotubes exhibit decreased GLUT4 protein levels. The ILK-dependent transcriptional downregulation of *Glut4* gene was confirmed by transfecting a reporter plasmid for human GLUT4 promoter in ILK-depleted myotubes for 24 h after depletion (Knight *et al.* 2003). Figure 8C shows the decrease of the reporter activity in the ILK-depleted myotubes. Besides reducing ILK content, we challenged its pharmacological blockade by treating differentiated myotubes with ILK inhibitor CPD22 for 24 h (Mamuya *et al.* 2016). Figure 8D shows no changes in the ILK expression when the myotubes are treated with CPD22. However, GSK3B phosphorylation levels are reduced (Fig. 8E). Figure 8F shows reduced GLUT4 in CPD22-treated myotubes.

In order to challenge the insulin response in ILK-suppressed cells, we stimulated for 40 min with a high dose of insulin (100 nM) in ILK-blocked or ILK-depleted myotubes similarly as we previously did in cKD-ILK mice cardiac tissue (Fig. 7). Figure 9 shows that the insulin stimulation increased the phosphorylation of IR-B on tyrosines 1150/1151 (Fig. 9A), IRS-1 on serine 302 (Fig. 9B) and AKT on serine 473/threonine 308 (Fig. 9C and D) in both control and CPD22-treated myotubes compared with non-insulin-stimulated myotubes. However, no differences were observed under insulin-stimulated conditions between control and CPD22-treated myotubes. Similar results were obtained in siRNA-based ILK-depleted myotubes (Fig. 9E and F). Finally, to test whether long-term ILK depletion or pharmacological inhibition affects insulin sensitivity during the glucose transport, we studied the uptake capacity of the glucose-based fluorescent probe 2-NBDG in either basal or short-term insulin-stimulated myotubes. Figure 10 shows that ILK-suppressed cells have similar loads of intracellular 2-NBDG as control cells. However, the 2-NBDG uptake after 45 min of insulin stimulation was lower in ILK-blocked or ILK-depleted myotubes than in controls.

Discussion

The factors that contribute to the sustained postprandial hyperglycemia include disrupted insulin secretion, insufficient inhibition of hepatic glucose production

Figure 8

ILK expression and activity and GLUT4 promoter activity in long-term ILK-blocked myotubes. Differentiated myotubes from C2C12 myoblasts were transfected with specific siRNAs against ILK (si-ILK) for 48 h, or treated with 3 μ M of the ILK inhibitor CPD22 (CPD22) for 24 h. On parallel, other differentiated myotubes were transfected with scramble siRNAs or treated with vehicle as the respective controls (CT). Representative immunoblot and densitometric analysis of (A) GSK3B phosphorylated at serine 9 (P-GSK) normalized against total GSK3B (GSK) and (B) GLUT4 normalized against GAPDH from CT and si-ILK myotubes. Representative ILK immunoblots are shown to demonstrate successful ILK depletions. (C) CT and si-ILK myotubes were transfected with a luciferase-based GLUT4 promoter activity reporter. The luciferase activity was normalized against total protein content. Representative immunoblot and densitometric analysis of (D) ILK normalized against GAPDH, (E) GSK3B phosphorylated at serine 9 (P-GSK) normalized against total GSK3B (GSK) and (F) GLUT4 normalized against GAPDH from CT and CPD22-treated myotubes. Data are shown as mean $\pm$ S.E.M. $N = 7-10$. * $P < 0.05$ vs CT.

and/or defective glucose uptake by the peripheral tissues (Graham & Kahn 2007). Adult cKD-ILK in a non-pathological context exhibits a slight but significant

Figure 9

The insulin receptor (IR)/IR substrate-1 (IRS-1)/AKT-signaling pathway in long-term ILK-blocked myotubes after a short-term stimulus of insulin. Differentiated myotubes from C2C12 myoblasts were transfected with specific siRNAs against ILK (si-ILK) for 48 h, or treated with 3 μ M of the ILK inhibitor CPD22 (CPD22) for 24 h. On parallel, other differentiated myotubes were transfected with scramble siRNAs or treated with vehicle as the respective controls. Afterward, cells were treated with insulin (100 nM) or vehicle for 40 min. Representative immunoblot and densitometric analysis of IR beta chain (IR-B) phosphorylated in tyr 1150/1151 (A), IRS-1 phosphorylated in serine 302 (B), AKT phosphorylated at serine 473 (C) and AKT phosphorylated at threonine 308 (D), normalized against total IR-B, IRS-1 or AKT, respectively, from CT- and CPD22-treated myotubes. Representative immunoblot and densitometric analysis of IR beta chain (IR-B) phosphorylated in tyr 1150/1151 (E) and AKT phosphorylated at serine 473 (F), normalized against total IR-B or AKT, respectively, from CT and si-ILK myotubes. Representative ILK immunoblots are shown to demonstrate successful ILK depletions. Data are shown as mean $\pm$ S.E.M. $N = 7-10$. * $P < 0.05$ vs non-treated control.

increase in blood glucose levels under randomly fed or fasting conditions, as well as a compensatory increased insulin secretion, postprandial or during GTT. All these data suggest that the partial depletion of ILK does not change the pancreatic beta cell activity, but indicate a possible peripheral tissue insulin resistance in cKD-ILK, which was further confirmed by increased HOMA-IR index, decreased QUICKI values and minor response to an

Figure 10

Basal and insulin-stimulated glucose uptake in long-term ILK-blocked myotubes. Differentiated myotubes were transfected with (A) specific siRNAs against ILK (si-ILK) for 48 h or (B) treated with 3 μ M of the ILK inhibitor CPD22 for 24 h (CPD-22). On parallel, other differentiated myotubes were transfected with scramble siRNAs or treated with vehicle as the respective controls. Cells were incubated with or without insulin (100 nM) for 15 min, and 2-NBDG (0.1 mM) was added for 30 min more and the intracellular 2-NBDG fluorescence fold change was determined. Data are shown as mean $\pm$ S.E.M. $N=7-10$. * $P<0.05$ vs non-treated control, # $P<0.05$ vs no treatment, \$ $P<0.05$ vs control+insulin.

exogenous bolus of insulin during ITT. It is important to take into consideration that our mice model, kept under physiological conditions, exhibits moderate differences in glycemia and insulinemia profiles, basally or under i.p. GTT or ITT stimulations, as other transgenic models that were also defined as insulin resistant without being forced to type 2 diabetes/MS conditions (e.g. a high fat diet-based model) (Zisman *et al.* 2000, Wang *et al.* 2013).

Hepatic gluconeogenesis is the primary source of endogenous glucose production (Rui 2014), and specifically small mammals such as mice are reliant on gluconeogenesis to supply their high-glucose demands (Kowalski & Bruce 2014). Our results can exclude any disturbance in the liver glucose homeostasis of cKD-ILK, because no differences in the PTT curves were observed when compared to WT. Moreover, we dissociated the effect of the diet or obesity on the cKD-ILK phenotype. Our descriptive work was intended to be performed in healthy adult mice in basal state, thus we may discard any upstream ILK pathological changes associated to type 2 diabetes and MS, such as differences in the peripheral tissues ECM content (Berria *et al.* 2006, Pasarica *et al.* 2009, Kang *et al.* 2011, Williams *et al.* 2015).

In order to analyze the intracellular mechanism involved during the increased insulin resistance observed in cKD-ILK, we focused our study in the GLUT4 levels, which play a key role in regulating whole-body glucose utilization by peripheral tissues. Striated muscle is responsible for the majority of the postprandial glucose disposal (Carnagarin *et al.* 2015), especially in the cardiac and red fibers-rich skeletal muscle tissues, where GLUT4 content is higher than white fiber-rich skeletal muscle

(Kern *et al.* 1990, Camps *et al.* 1992, Zorzano *et al.* 1997, Gaster *et al.* 2000). Although in a physiological state less glucose is transported into WAT compared with striated muscle (Shepherd & Kahn 1999), it has been reported that specific reduction of GLUT4 protein levels in WAT may affect glucose homeostasis regulated by the crosstalk between WAT and striated muscle leading to an impairment of insulin action on these tissues (Abel *et al.* 2001). Moreover, type 2 diabetes subjects and animal models have shown a decrease in adipose GLUT4 content, altering adipose tissue function and the systemic glucose metabolism (Shepherd & Kahn 1999, Karnieli & Armoni 2008). Our results demonstrate for the first time that whole-body ILK depletion exhibits a direct relationship between the peripheral tissues downregulating GLUT4 expression and the resulting glucose homeostasis alteration.

Previous publications that used both muscle-specific and adipose-specific GLUT4-KO mice (Zisman *et al.* 2000, Wallberg-Henriksson & Zierath 2001, Minokoshi *et al.* 2003, Kotani *et al.* 2004) support the direct relationship between the reduction of GLUT4 content in peripheral tissues and the impairment of whole-body glycemia and insulin sensitivity that we observed in cKD-ILK. Nevertheless, the relationship between GLUT4 expression and insulin resistance remains controversial in animal models and human studies, probably due to different model and tissue-dependence approaches used (Camps *et al.* 1992, Hansen *et al.* 1995, Shepherd & Kahn 1999, Zisman *et al.* 2000, Gaster *et al.* 2001, Minokoshi *et al.* 2003, Desrois *et al.* 2004, Graham & Kahn 2007, Karnieli & Armoni 2008, Zeyda & Stulnig 2009).

The relationship between the impairment of glucose homeostasis and defective ECM crosstalk with the cells has been evidenced in insulin-resistant KO mice models for ITGB1 or focal adhesion intracellular scaffold proteins as focal adhesion kinase (Bisht *et al.* 2008, Zong *et al.* 2009). On the other hand, a recent study by Kang and coworkers (Kang *et al.* 2016) shows that noninducible skeletal muscle-specific ILK KO mice maintained glucose homeostasis during a basal chow-fed diet. Differences in the origin and settings of the mice models used by Kang and coworkers could explain the contrast with our results: their CRE-mediated recombination strain (HSA-Cre79 from Jackson Laboratories) bred with the loxP-flanked *Ilk* strain produced a muscle-specific ablation of ILK at birth, whereas in our model, we induce a ubiquitous ILK depletion during the adulthood. It is possible that differences about glucose metabolism of muscle-specific vs total inducible ILK invalidation models are due to crosstalk between the different peripheral tissues.

Nevertheless, to support our *in vivo* observations, we designed parallel *in vitro* experiments using GLUT 4-expressing myotubes, where ILK is abundantly expressed (Huang *et al.* 2000). When ILK was partially silenced with siRNAs or pharmacologically inhibited, the promoter activity, mRNA and protein levels of GLUT4 were concomitantly decreased, and the result was the glucose uptake impairment after short-term insulin stimulation. Supporting part of our myotubes studies, reduced GLUT4 levels and glucose transport have been reported in 3T3L1 adipocytes with siRNAs-based ILK depletion (Tang *et al.* 2006). Together, these *in vitro* ILK blockade studies confirm and extend the cKD-ILK observations in the peripheral tissues during the glucose disposal.

The expression of other glucose transporter such as GLUT1 and the insulin receptor isoforms IR-A and IR-B was not affected in our ILK-depleted models. Interestingly, we previously reported in cKD-ILK similar defective expression and function of the vasopressin-dependent transporter aquaporin 2 (AQP2), which shares response similarities with GLUT4 (Planells-Cases & Ferrer-Montiel 2007, Kim *et al.* 2011) without affecting the vasopressin receptor or AQP3 levels (Cano-Peñalver *et al.* 2014, Mamuya *et al.* 2016).

Insulin binding to IR/IRS-1 activates the phosphorylation of AKT in both serine 473 and threonine 308 residues (Carnagarin *et al.* 2015), which is also an ILK downstream effector (Troussard *et al.* 2003, Edwards *et al.* 2005, García-Jérez *et al.* 2015). In order to elucidate whether ILK depletion was affecting the IR/IRS-1/AKT-mediated insulin response, we studied the behavior of this pathway under the stimulation of different insulin states *in vivo* and *in vitro*. First, the IR phosphorylation levels in fasting cKD-ILK were not different to their controls, but fed cKD-ILK mice have increased IR phosphorylation. This can be translated as a compensation to the slightly hyperinsulinemia observed in the randomly fed cKD-ILK. To better understand this feedback phenomenon, we studied the state of its immediate substrate IRS-1 which temporarily modulates the autologous and heterologous feedback mechanisms that mediates the IR and/or the AKT activity to terminate the insulin-mediated modulation of GLUT4 (Weigert *et al.* 2008, Carnagarin *et al.* 2015). The IRS-1 expression and its phosphorylation on serine 302, one of the residues implicated in the feedback regulation, were unaffected in the ILK-depleted animals, probably due to the complex dynamics of its multisites phosphorylation during hyperinsulinemia (Weigert *et al.* 2008).

Besides GLUT4 protein expression reduction, the disruption of GLUT4 translocation to the plasma

membrane in striated muscle and WAT could both be translated as glucose uptake impairment (Huang & Czech 2007). As we already observed with the trafficking vesicles of AQP2 (Mamuya *et al.* 2016), ILK may modulate the trafficking of the GLUT4-containing vesicles to the membrane via the actin cytoskeletal remodeling. Both the PM and IM presence of GLUT4 in fed cKD-ILK tissues were downregulated, and interestingly we observed no differences in the translocation capacity between fed WT and cKD-ILK, represented as the PM/IM ratios, probably due to an insulin-dependent counteracting effect over the ILK depletion. In the randomly fed cKD-ILK, the phosphorylation of AKT on serine 473 was decreased but not the threonine 308. Taking into consideration that ILK downregulates the serine 473 phosphorylation (Troussard *et al.* 2003, Edwards *et al.* 2005, García-Jérez *et al.* 2015), we suggest that the slightly increased insulinemia observed in fed cKD-ILK probably was not able to compensate the reduced serine phosphorylation, but the reciprocal relation that exists between the two activating phosphorylation sites of AKT, (serine 473 and threonine 308) (Troussard *et al.* 2003, Vadlakonda *et al.* 2013) may explain the unaffected GLUT4 translocation to the membrane observed in these animal.

Furthermore, we challenged the insulin-mediated pathway *in vivo* and *in vitro* with an exogenous insulin bolus. Notably, ILK blockade was unable to abrogate the positively increased IR-B/IRS-1/AKT phosphorylations and the GLUT4 translocation to the plasmatic membranes by the insulin bolus, probably because the actin filament remodeling induced by insulin counteracted the ILK depletion effect on cytoskeletal organization (Khayat *et al.* 2000). Taking into consideration these results, we suggest that the downregulation of GLUT4 expression is the definitive factor that reduces the glucose uptake *in vivo* and *in vitro*; nevertheless, further studies are required to investigate the role of ILK as an indirect modulator of insulin signaling pathway.

In this context, it is important to notice that besides the short-term AKT-mediated translocation to the membrane of the vesicles containing GLUT4, the long-term AKT modulation has been documented to reduce GLUT4 expression (Flores-Riveros *et al.* 1993, Taha *et al.* 1999, Matsui *et al.* 2006). Moreover, the insulin-resistant, muscle-specific ITGB1 KO showed lower levels of AKT phosphorylation on serine 473, concomitant with reduced ILK expression (Zong *et al.* 2009). Taking all these literature into consideration, it is possible that ILK blockade chronically inactivates AKT and shares with the exposed models some of the mechanisms that lead to reduced

Glut4 gene transcription. If that is the case, ILK-dependent reduction of AKT phosphorylation may decrease GLUT4 promoter activity by several transcriptional factors; some candidates are the AKT-dependent myocyte enhancer factor 2 (MEF2) and myogenic differentiation protein (MYOD) (Xu & Wu 2000, Santalucía *et al.* 2001, Serra *et al.* 2007). Another candidate is forkhead transcription factor (FOXO1), which is negatively regulated by AKT and either represses or activates transcription of the *Glut4* gene depending on the cell type (Matsuzaki *et al.* 2003, Armoni *et al.* 2007, Ni *et al.* 2007). Since AKT, MEF2 and MYOD also participate in the regulation of GLUT4 during contraction-induced glucose clearance in striated muscle (Sakamoto *et al.* 2002), (Richter & Hargreaves 2013), further research needs to be achieved to analyze the ILK-mediated transcriptional regulation in this or other contexts.

On the other hand, our results show that ILK blockade decreases GSK3B phosphorylation on serine 9, which means an activation of the kinase. The increased GSK3B activation has been reported to reduce insulin sensitivity and/or glycogenesis, which reduce the ratios of glucose uptake (Nikoulina *et al.* 2002, Henriksen *et al.* 2003, Carnagarin *et al.* 2015) and this probably may be part of the mechanism followed in the cKD-ILK tissues. Moreover, the striated muscle-specific ITGB1 KO shows lower phosphorylation levels of GSK3B and reduced skeletal muscle glucose uptake and glycogen synthesis (Zong *et al.* 2009).

In conclusion, our results demonstrated that global ILK depletion in insulin-sensitive peripheral tissues has a negative impact on glucose homeostasis because of the reduced GLUT4 expression that finally impairs the glucose uptake. Based on the relevance of ILK in the regulation of glucose metabolism, we consider that the development of a pharmacological modulator of ILK could be useful to improve metabolic disorders therapies. Moreover, the slight prediabetes observed in our model indicates the importance of whole-body ILK in the settlement of the disease, pointing to its activity as a potential biomarker during type 2 diabetes and MS progression.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JOE-16-0662>.

Declaration of interest

The authors declare there is no duality of interest associated with this manuscript.

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Author contribution statement

S D F, M R P and D R P conceived, designed and supervised the study and analyzed the data. M H V, M G, A L and A G J performed experiments. M H V and M G interpreted data and reviewed the experimental settings. L C, J A and J A R contributed with intellectual expertise. S D F and M H V wrote the manuscript. S D F, D R P and M R P are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. All authors contributed to reviewing and editing the manuscript, and approved its final version.

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