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Essential role of protein kinase Cζ in the impairment of insulin-induced glucose transport in IRS-2-deficient brown adipocytes

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Abstract Insulin receptor substrate-2-deficient (IRS-2$^{-/-}$) mice develop type 2 diabetes. We have investigated the molecular mechanisms by which IRS-2$^{-/-}$ immortalized brown adipocytes showed an impaired response to insulin in inducing GLUT4 translocation and glucose uptake. IRS-2-associated phosphatidylinositol 3-kinase (PI 3-kinase) activity was blunted in IRS-2$^{-/-}$ cells, total PI 3-kinase activity being reduced by 30%. Downstream, activation of protein kinase C (PKC) ζ was abolished in IRS-2$^{-/-}$ cells. Reconstitution with retroviral IRS-2 restores IRS-2/PI 3-kinase/PKCζ signalling, as well as glucose uptake. Wild-type cells expressing a kinase-inactive mutant of PKCζ lack GLUT4 translocation and glucose uptake. Our results support the essential role played by PKCζ in the insulin resistance and impaired glucose uptake observed in IRS-2-deficient brown adipocytes.

Key words: Protein kinase C; Insulin receptor substrate-2; Glucose transport

1. Introduction

One of the major metabolic functions of insulin is to stimulate glucose uptake in adipose tissues and skeletal muscle [1,2]. In these tissues insulin-induced glucose transport is maintained through the translocation of the GLUT4 glucose transporter to the cell surface. Since impaired insulin action in peripheral tissues has been associated with defects in insulin signalling molecules leading to glucose transport, over the last years considerable work has been done to elucidate the molecular mechanisms regulating GLUT4 translocation. In this regard, it is now generally accepted that phosphatidylinositol 3-kinase (PI 3-kinase) activation via its interaction with insulin receptor substrates (IRSs) [3] plays an essential role [4–7]. In addition, downstream of PI 3-kinase two serine/threonine kinases (PKB/Akt and atypical protein kinase C (PKC) ζ/λ) have been implicated as major transducer proteins of glucose transport in response to insulin in different cell models [8–11]. However, recent findings indicating that insulin action as well as its signalling seem to be tissue-specific [12,13], make it difficult to assume that there is a unique pathway that mediates GLUT4 translocation and glucose transport in insulin target tissues.

Brown adipocytes have been extensively used to study molecular mechanisms of insulin action and inaction [14,15]. These cells display a large number of insulin receptors [16] and activate the insulin signalling cascade under physiological conditions [17]. Regarding glucose uptake, activation of both Akt [18] and PKCζ [19] by insulin has been shown to mediate this metabolic effect in primary cultures of fetal brown adipocytes. More importantly, beside its short-term effect on GLUT4 translocation, insulin, in the long term, increased GLUT4 and down-regulated GLUT1 gene expression in primary brown adipocytes, in a PI 3-kinase-dependent manner [15].

On the other hand, upstream of PI 3-kinase, IRS-2 seems to be crucial in mediating insulin-induced glucose uptake. Thus, insulin responsiveness in induced glucose uptake was almost impaired in immortalized brown pre-adipocytes derived from IRS-2-deficient mice. In addition, in those cells an insulin-induced effect on GLUT4 translocation to the plasma membrane was precluded [20]. Accordingly, we addressed the underlying molecular mechanisms by which IRS-2 mediates the insulin effect on glucose uptake in IRS-2-deficient brown adipocytes. Data shown herein propose that PI 3-kinase/PKCζ, but not the PI 3-kinase/Akt signalling pathway, is inhibited in IRS-2-deficient brown adipocytes upon insulin stimulation. In addition, wild-type cells expressing a kinase-inactive (KI) mutant of PKCζ lack insulin responsiveness in induced GLUT4 translocation and glucose uptake.

2. Materials and methods

2.1. Materials

Fetal calf serum, culture media and puromycin were obtained from Gibco (Gaithersburg, MD, USA). Insulin, hygromycin, albumin, polybrene and anti-mouse IgG-agarose were from Sigma (St. Louis, MO, USA). Protein A-agarose was from Roche Molecular Biochem-

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Abbreviations: IRS, insulin receptor substrate; PI, phosphatidylinositol

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icals. Anti-IRS-1 (06-248), anti-Tyr(P) (clone 4G10) (05-321), anti-IRS-2 (06-506) were purchased from Upstate Biotechnology (Lake Placid, NY, USA). Anti-phospho p70s6k (Ser424/Thr421 #9204S), anti-p70s6k (#9202), anti-phospho Akt (Ser473 #9271), anti-Akt (#9272), anti-phospho PKCζ (Thr410 #9378) and anti-phospho GSK-3α/β (Ser21/9 #9331) were purchased from Cell Signaling (Beverly, MA, USA). Anti-PKCζ antibody (c-20) (sc-216) and anti-Tyr(P) (Py20) (sc-508) were purchased from Santa Cruz Biotechnology (Palo Alto, CA, USA). [γ-^{32}P]ATP (3000 Ci/mmol) and 2-deoxy-D-[1-^{3}H]glucose (11.0 Ci/mmol) were from Amersham (Aylesbury, UK). All other reagents used were of the purest grade available.

2.2. Cell culture and retroviral infections

Brown adipocytes were obtained from interscapular brown adipose tissue of 3-day newborn mice from crosses of IRS-2$^{+/-}$ parental mice. Each brown adipose tissue from newborn genotypes, analyzed by polymerase chain reaction, was subjected separately to collagenase dispersion as previously described [21]. Viral Bosc packaging cells were transfected at 70% confluence by calcium phosphate coprecipitation with 3 μg/6-cm dish of the puromycin-resistant retroviral vector pBABE encoding SV40 large T antigen (kindly provided by J. de Caprio, Dana Farber Cancer Institute, Boston, MA, USA). Then, primary brown adipocytes were infected at 60% confluence with polybrene (4 μg/ml)-supplemented virus for 48 h and maintained in culture for 72 h, before selection with puromycin (1 μg/ml) for 1 week. Cells lines IRS-2$^{+/+}$, IRS-2$^{+/-}$ and IRS-2$^{-/-}$ were expanded, the expression of IRS-2 being assessed by Western blot. To avoid variations between littermates of the same genotype all the experiments were performed in two different cell preparations.

2.3. Infection with adenoviral vectors

Adenoviral vectors encoding KI-PKCζ or vector alone (mock) have been previously described [22]. Wild-type brown adipocytes were routinely infected with 10 MOI (multiplicity of infection or viral particles per cell) KI-PKCζ, which after 48 h of incubation increased by 1.6-fold basal levels of PKCζ (see below and [22]). Then, cells were serum-starved for 12–15 h and subsequently used for determination of PKCζ activity or glucose transport, upon insulin stimulation.

2.4. Immunoprecipitations

Quiescent brown adipocytes (after 20 h of serum deprivation) were treated without or with several doses of insulin as indicated, and lysed at 4°C in buffer containing 10 mM Tris–HCl, 5 mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM NaF, 100 μM Na_3VO_4, 1% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride, pH 7.6 (lysis buffer). After protein content determination, equal amounts of protein (500–600 μg) were immunoprecipitated at 4°C with the corresponding antibodies. The immune complexes were collected on protein A-agarose or anti-mouse IgG-agarose beads. Immunoprecipitates were washed with lysis buffer and analyzed by SDS–PAGE followed by Western blotting.

2.5. PI 3-kinase activity

PI 3-kinase activity was measured in the anti-IRS-1, anti-IRS-2 and anti-Tyr(P) immunoprecipitates by in vitro phosphorylation of phosphatidylinositol as previously described [19].

2.6. PKCζ activity

PKCζ activity was measured in anti-PKCζ immune complexes by in vitro phosphorylation of myelin basic protein (MBP) as previously described [19].

2.7. Subcellular fractionation

Cells were washed with ice-cold phosphate-buffered saline and scraped in homogenization buffer containing 20 mM Tris–HCl, pH 7.4, 2 mM EGTA, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluo-

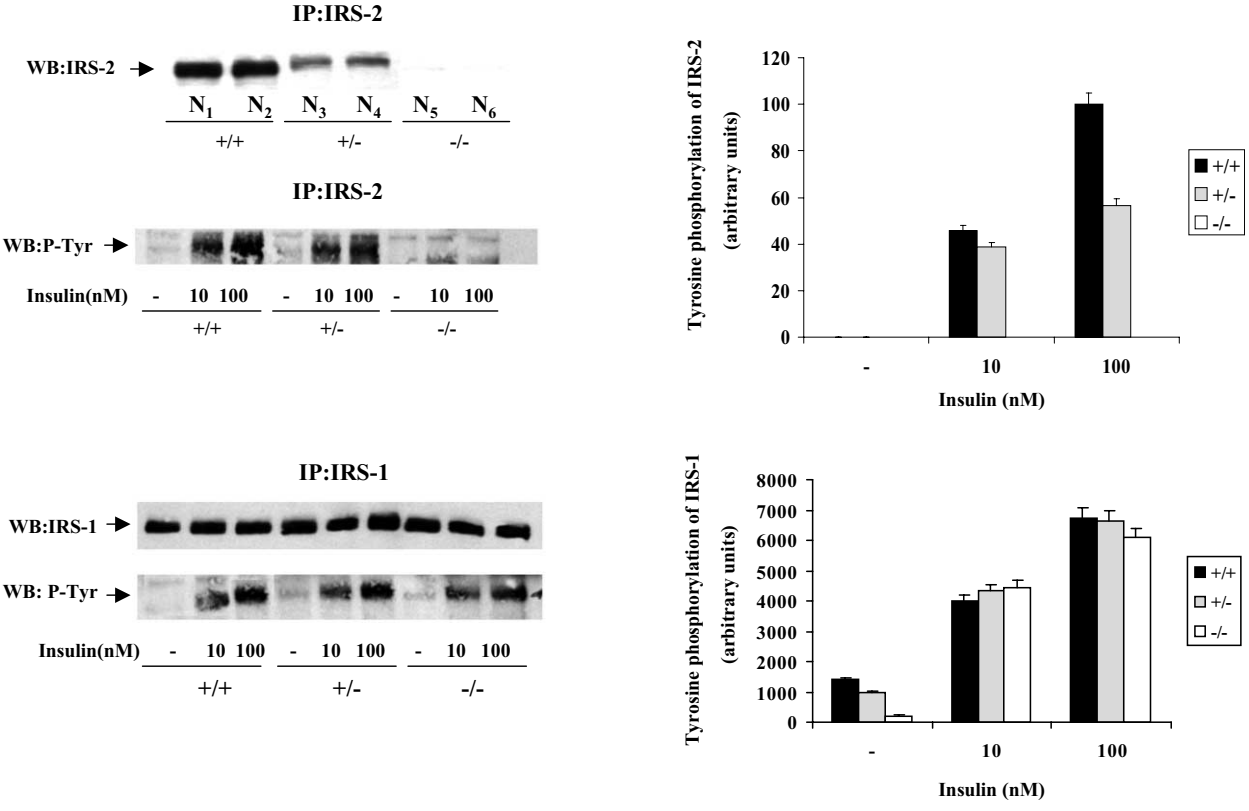

Fig. 1. IRS expression and tyrosine phosphorylation in wild-type, heterozygous and IRS-2-deficient brown adipocytes. Brown adipocyte cell lines were cultured under growing conditions. Total protein was subjected to SDS–PAGE and analyzed by Western blot with the anti-IRS-2 antibody. Two different cell lines from each genotype were analyzed. Serum-starved brown adipocytes (IRS-2$^{+/+}$, IRS-2$^{+/-}$ and IRS-2$^{-/-}$) were incubated for 5 min without or with various doses of insulin (10–100 nM). Cells were lysed and total protein was immunoprecipitated with anti-IRS-2 or anti-IRS-1 antibodies, subjected to SDS–PAGE and analyzed by Western blot with the anti-Tyr(P), anti-IRS-2 and anti-IRS-1 antibodies. The autoradiograms corresponding to three independent experiments were quantitated by scanning densitometry. Results are expressed as arbitrary units of IRS-1 and IRS-2 tyrosine phosphorylation and are means ± S.E.M.

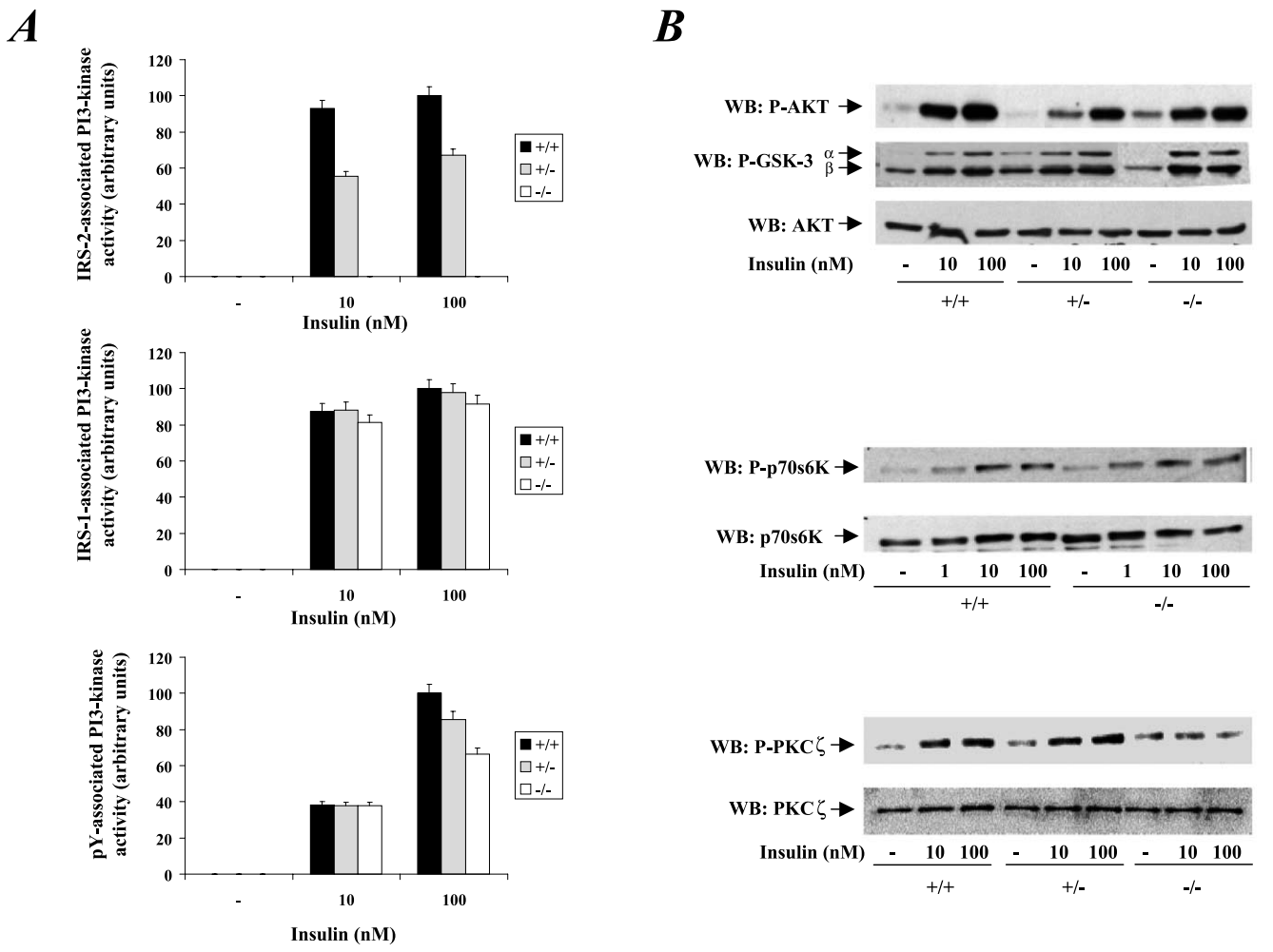

Fig. 2. PI 3-kinase activity and its downstream effectors in insulin-stimulated wild-type and IRS-2-deficient brown adipocytes. A: Serum-starved brown adipocytes (IRS-$2^{+/+}$, IRS-$2^{+/-}$ and IRS-$2^{-/-}$) were incubated for 5 min without or with various doses of insulin (10–100 nM). Cells were lysed and total protein was immunoprecipitated with anti-IRS-2, anti-IRS-1 and anti-Tyr(P) antibodies. The resulting immune complexes were washed and immediately used for an in vitro phosphatidylinositol kinase assay. The conversion of phosphatidylinositol to phosphatidylinositol phosphate in the presence of [γ-^{32}P]ATP was analyzed by thin-layer chromatography. The autoradiograms corresponding to three independent experiments were quantitated by scanning densitometry. Results are expressed as arbitrary units of PI 3-kinase and are means ± S.E.M. B: Lysates of insulin-stimulated cells were subjected to SDS–PAGE and analyzed by Western blot with the anti-phospho-Akt, anti-Akt, anti-phospho-GSK-3α/β, anti-phospho-p70s6k, anti-p70s6k, anti-phospho-PKCζ and anti-PKCζ antibodies. The results shown are representative of at least three independent experiments.

ride, 10 mM β-mercaptoethanol, 10 μg/ml aprotinin and 10 μg/ml leupeptin. After 10 min incubation cells were homogenized with 30 strokes of a Dounce homogenizer using a tight-fitting pestle. Nuclei were collected by centrifugation at 500×*g* for 5 min, and the low speed supernatant was centrifuged at 100 000×*g* for 30 min. The high speed supernatant constituted the internal membrane fraction. The pellet was washed three times and extracted in ice-cold homogenization buffer containing 1% Triton X-100 for 60 min. The Triton-soluble component (plasma membrane fraction) was separated from the Triton-insoluble material (cytoskeletal fraction) by centrifugation at 100 000×*g* for 15 min. Plasma membrane fractions were kept at −70°C before protein quantification and Western blotting with antibodies against GLUT4 and β-actin.

2.8. Measurement of glucose transport

Quiescent cells after 20 h of serum deprivation in 5 mM glucose DMEM medium were treated with or without insulin for 10 min. Glucose transport was measured as 2-deoxy-D[1-^{3}H]glucose uptake in triplicate dishes from three independent experiments as previously described [19].

2.9. Protein determination

Protein determination was performed by the Bradford dye method [23], using the Bio-Rad reagent and bovine serum albumin as the standard.

3. Results and discussion

3.1. The tyrosine phosphorylation cascade in wild-type and IRS-2-deficient brown adipocytes

We have generated immortalized brown adipocyte cell lines derived from individual brown adipose tissue of 3-day-old newborn wild-type, heterozygous and IRS-2-deficient mice. As shown in Fig. 1, cell lines derived from homozygous mice (IRS-$2^{-/-}$; N5 and N6) had no immunoreactive IRS-2 protein, whereas cell lines derived from heterozygous mice (IRS-$2^{+/-}$; N3 and N4) showed a 50% reduction in IRS-2 expression as compared to the cell lines derived from the wild-type mice (IRS-$2^{+/+}$; N1 and N2). To assess the tyrosine phosphorylation of both IRS-1 and IRS-2 in the three cell types, brown adipocytes were serum-starved and stimulated with insulin (10–100 nM). Then, cell lysates were immunopre-

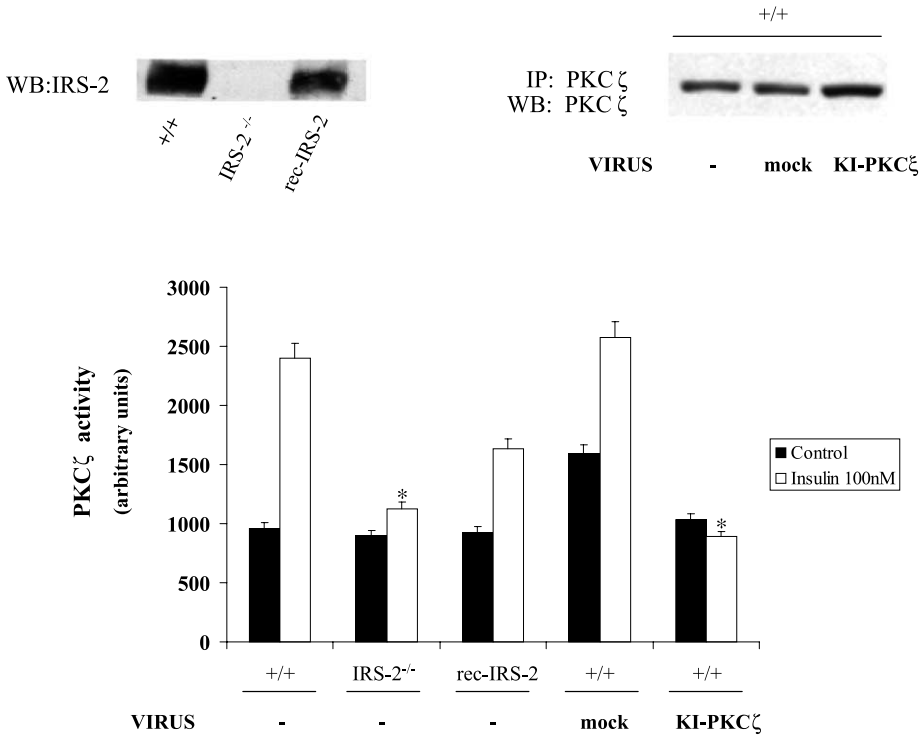

Fig. 3. PKCζ activity is severely decreased in insulin-stimulated IRS-2-deficient brown adipocytes. Upper panel (left): IRS-$2^{-/-}$ brown adipocytes were infected with a retroviral vector encoding IRS-2. Hygromycin-resistant clones were expanded and cell lysates were analyzed by Western blot with anti-IRS-2 antibody. (right) Wild-type cells were infected with adenoviruses encoding KI-PKCζ (10 MOI) or vector alone (mock) for 48 h. Total cell lysates were analyzed by Western blot with anti-PKCζ antibody. Lower panel: Cells were serum-starved for 12–15 h and further stimulated as described in Fig. 2. Cell lysates were immunoprecipitated with the anti-PKCζ antibody and assayed for PKCζ activity. The autoradiograms of MBP phosphorylation were quantitated by scanning densitometry. Results are normalized with the amount of total PKCζ and are expressed as arbitrary units of PKCζ activity. Statistical analysis of means ± S.E.M. of four to six determinations is indicated, $*P < 0.05$.

cipitated with the corresponding anti-IRS-1 and anti-IRS-2 antibodies and the resulting immunocomplexes were analyzed by Western blot with the anti-Tyr(P) antibody. Wild-type brown adipocytes showed a marked increase in tyrosine phosphorylation of IRS-2 upon insulin stimulation, the maximal effect being elicited at 100 nM insulin concentration (Fig. 1). As expected, no tyrosine-phosphorylated IRS-2 was found in IRS-$2^{-/-}$ cells, a 50% reduction of IRS-2 phosphorylation being observed in IRS-$2^{+/-}$ cell lines. Conversely, both IRS-1 expression and its tyrosine phosphorylation upon insulin stimulation did not change among the three cell types studied. Likewise, insulin receptor β-chain tyrosine phosphorylation was not affected by the lack of IRS-2 (results not shown). Our data clearly suggest that the lack of IRS-2 did not result in an increased IRS-1 expression nor in an enhanced tyrosine phosphorylation, upon insulin stimulation. This lack of compensation contrasts with the signalling in IRS-1-deficient brown adipocytes, where we observed an increased content and tyrosine phosphorylation of IRS-2 in response to insulin [24,25]

3.2. *Insulin effect on PI 3-kinase activity and its downstream effectors in wild-type and IRS-2-deficient brown adipocytes*

In insulin-stimulated primary brown adipocytes, PI 3-kinase is activated by an interaction of the p85 regulatory subunit with tyrosine-phosphorylated IRS-1 and IRS-2 [17]. To study whether this interaction is affected by the absence of IRS-2, we measured IRS-2- and IRS-1-associated PI 3-kinase activity in the three cell types stimulated with various doses of insulin. IRS-2-associated PI 3-kinase activity was completely blunted in IRS-$2^{-/-}$ brown adipocytes (Fig. 2A). Conversely, in these cells IRS-1-associated PI 3-kinase activity upon insulin stimulation was similar to that of the wild-type. In order to assess the relative contributions of IRS proteins to the total activation of PI 3-kinase, we measured this activity in anti-Tyr(P) immunoprecipitates. As shown in Fig. 2A, the amount of total PI 3-kinase recovered in IRS-2-deficient cells was reduced by 30% as compared to the wild-type. These data confirm the results seen above showing the lack of compensatory signalling through IRS-1/PI 3-kinase.

Next, we analyzed how the lack of IRS-2 worked on PI 3-kinase downstream signalling. Neither Akt phosphorylation (measured by direct Western blot analysis with the anti-phospho-Akt Ser473 antibody) nor GSK-3 phosphorylation (α and β isoforms) was altered in IRS-$2^{-/-}$ cells, upon insulin stimulation. Likewise, p70S6k phosphorylation remained unchanged in insulin-stimulated IRS-$2^{-/-}$ cells as compared to the wild-type. However, the phosphorylation of PKCζ (measured by Western blot analysis with the anti-phospho-PKCζ-Thr410 antibody), which was stimulated by insulin in primary [19] and immortalized wild-type brown adipocytes, was completely precluded in IRS-2-deficient cells (Fig. 2B). These data are entirely consistent with those results observed in L6hIR muscle cells overexpressing a peptide corresponding to the KRLB domain of IRS-2 [26]. In fact, these cells did not stimulate IRS-2 phosphorylation and PKCζ activity in response to

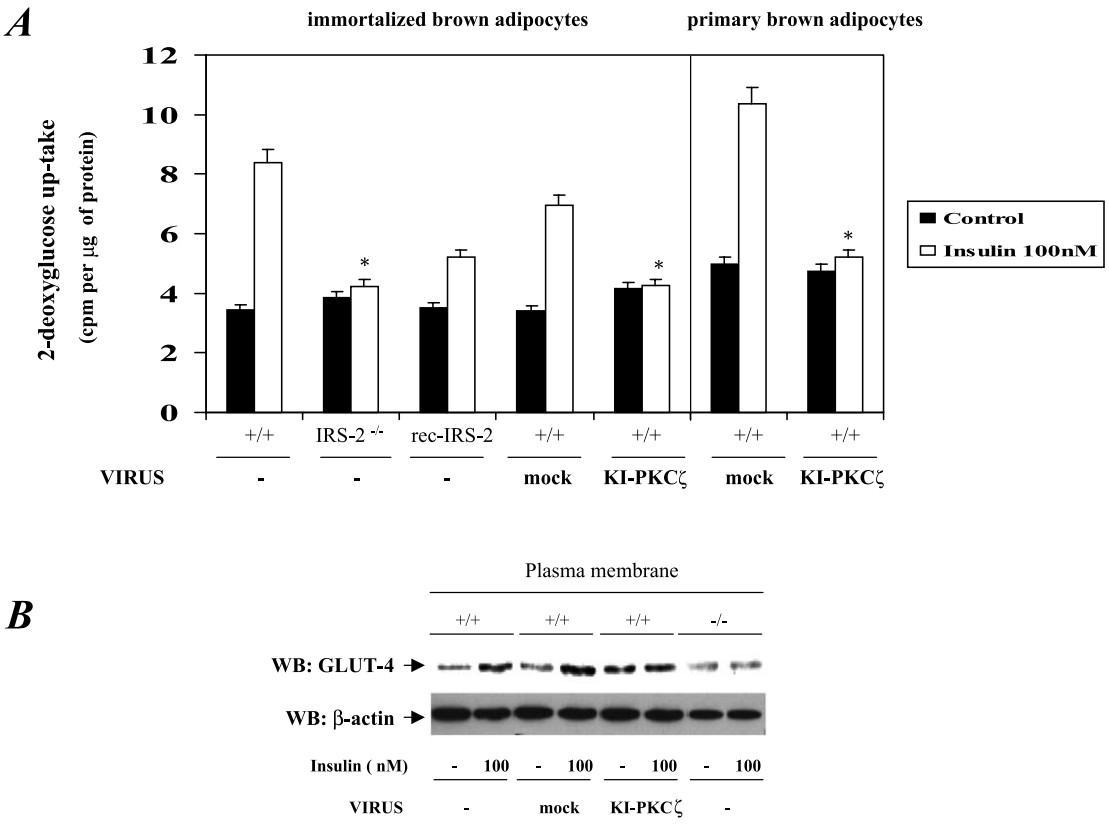

Fig. 4. Effect of the lack of IRS-2 or adenovirus encoding KI-PKCζ on basal and insulin-stimulated glucose uptake in brown adipocytes. A: Brown adipocytes (IRS-$2^{+/+}$, IRS-$2^{-/-}$ and rec-IRS-2) were serum-starved for 12–15 h in 5 mM glucose DMEM medium and further stimulated with 100 nM insulin. Wild-type or primary brown adipocytes were infected with 10 MOI of the adenoviruses encoding KI-PKCζ or vector alone (mock). After 48 h, cells were serum-starved in 5 mM glucose DMEM medium for 12–15 h and further stimulated with 100 nM insulin. Glucose uptake was measured as described in Section 2. Results are shown as the average ± S.E.M. ($n \geq 4$). Unpaired Student's *t*-tests were used for analysis of differences between various cell lines; $^{*}P < 0.05$ is considered significant. B: Cells were treated as described above and collected for subcellular fractionation. 10 μg of internal and plasma membrane proteins of each condition were subjected to SDS–PAGE and analyzed by Western blot with the anti-GLUT4 and β-actin antibodies. A representative experiment is shown. The autoradiograms corresponding to three independent experiments were quantitated by scanning densitometry. Results are expressed as arbitrary units of GLUT4 in the plasma membrane and are means ± S.E.M.

insulin. Furthermore, the impairment of IRS-2/PKCζ signalling resulted in a reduced phosphorylation of Akt and GSK-3α and β, with the outcome of an impaired glycogen synthesis in response to insulin. However, our data in brown adipocytes suggest a diverging signalling downstream of IRS-2/PI 3-kinase through PKCζ or alternatively through Akt/GSK-3α and β.

3.3. *PKCζ activity mediates insulin-induced glucose transport in brown adipocytes*

The fact that IRS-2-deficient cells did not respond to insulin regarding PKCζ phosphorylation in the threonine 410 residue prompted us to measure its enzymatic activity. As shown in Fig. 3 (lower panel), insulin induced PKCζ activity in wild-type brown adipocytes, this effect being totally blunted in the absence of IRS-2. To assess these data, we reconstituted IRS-2 expression in deficient cells by means of retroviral gene transfer. The Western blot depicted in Fig. 3 (upper left panel) shows that the reconstitution of IRS-2 expression represents 50–60% of the level seen in wild-type cells. IRS-2-reconstituted brown adipocytes (rec-IRS-2) activated PKCζ in parallel with the level of IRS-2 protein expressed, upon insulin stimulation. In addition, we expressed a KI mutant of PKCζ [22] in wild-type cells by means of adenoviral gene transfer (Fig. 3, upper right panel). Whereas PKCζ activity was induced by insulin in control cells (mock-infected), KI-PKCζ-infected cells completely lack the insulin effect in activating this enzyme (Fig. 3, lower panel). Taken together, these results indicate that IRS-2 mediates the activation of PKCζ by insulin in brown adipocytes, IRS-1 being unable to compensate this response.

Our laboratory has recently demonstrated an essential contribution of PKCζ to the insulin action regarding glucose transport in brown adipocyte primary cultures [19]. Based on that, we measured glucose uptake in insulin-stimulated wild-type, IRS-$2^{-/-}$ and rec-IRS-2 brown adipocytes. The results shown in Fig. 4A indicate that insulin-induced glucose transport in brown adipocytes parallels PKCζ activation. Whereas wild-type cells induced a 2.5-fold increase in glucose transport upon insulin stimulation, IRS-2-deficient cells were unable to respond to the hormone. Moreover, the reconstitution of IRS-2-deficient brown adipocytes recovered the activation of glucose transport upon insulin treatment in parallel to the recovery of both IRS-2 and PKCζ enzymatic activity. These data show that IRS-2 is an essential player in stimulating glucose transport in response to insulin in brown adipo-

cytes, as was previously reported in brown pre-adipocytes [20]. However, it has recently been demonstrated that either exercise or insulin-induced glucose transport in the skeletal muscle of IRS-2 knockout mice is not altered [27], illustrating the tissue specificity of the insulin action.

The above-described findings suggest that alterations in IRS-2-mediated PKCζ activation could result in the inhibition of insulin-stimulated glucose uptake. To directly address this point, we infected both immortalized and primary wild-type brown adipocytes with adenoviruses encoding KI-PKCζ. Then, cells were serum-starved, stimulated with 100 nM insulin and 2-deoxyglucose uptake was measured. As shown in Fig. 4A, insulin induced glucose transport in wild-type cell lines as well as in primary brown adipocytes. This effect was totally abolished in either established or primary cells infected with KI-PKCζ, but not in the mock cells. Finally, we addressed whether the decrease in insulin-stimulated glucose transport was caused by impaired GLUT4 translocation from the internal stores to the plasma membrane fraction. For this purpose, IRS-2-deficient and wild-type cells (mock or infected with KI-PKCζ) were stimulated with 100 nM insulin and subjected to subcellular fractionation as described in Section 2. The Western blot depicted in Fig. 4B revealed that insulin failed to translocate GLUT4 to the plasma membrane in brown adipocytes lacking IRS-2 and in wild-type cells expressing KI-PKCζ, but not in the wild-type or mock cells. These data emphasize the essential role played by IRS-2 through PKCζ in mediating GLUT4 translocation to the plasma membrane in response to insulin, and are fairly consistent with those previously reported in rat adipose cells [28]. More importantly, this association between IRS-2/PKCζ and GLUT4 translocation/glucose transport in response to insulin in brown adipocytes allows us to understand the defective IRS-2 phosphorylation and an impaired PKCζ activation in skeletal muscle from obese patients showing insulin resistance and glucose intolerance [29].

In conclusion, our data strongly support the essential role played by PKCζ in the insulin resistance and impaired glucose uptake observed in IRS-2-deficient brown adipocytes.

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