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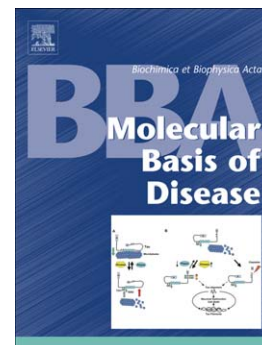
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**Alterations of insulin signalling in type 2 diabetes: a review of the current evidence from  
humans**

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**SUMMARY**

A generally accepted view posits that insulin resistant condition in type 2 diabetes is caused by defects at one or several levels of the insulin-signalling cascade in skeletal muscles, adipose tissue and liver, that quantitatively constitute the bulk of the insulin-responsive tissues. Hence, the gradual uncovering of the biochemical events defining the intracellular signalling of insulin has been quickly followed by clinical studies on humans attempting to define the molecular defect(s) responsible for the establishment of the insulin resistant state. While the existence of molecular defects within the insulin signal transduction machinery is undisputed, contrasting data exist on what is the principal molecular alteration leading to insulin resistance. Such discrepancies in the literature may depend on: 1) different subject characteristics, 2) methodological differences 3) small cohorts of subjects, and - not least - 4) intrinsic limitations in studying every detail of the insulin signalling cascade. Here, we review the studies on humans exploring the defects of the insulin signalling cascade generated by insulin resistance and type 2 diabetes, focusing on muscle and adipose tissue - which account for most of the glucose disposal capacity of the body - with focus on the unresolved discrepancies present in the literature.

**Abbreviations:**

aPKCs: atypical protein kinases C

GS: glycogen synthase

GSK-3: glycogen synthase kinase-3

IR: Insulin Receptor

JNK: Jun N-terminal kinase

MAPK: Mitogen-activated protein kinase

mTOR: mammalian Target of Rapamycin

PDK1: phosphoinositide-dependent protein kinase 1

PH domain: pleckstrin homology domain

PTB domain: phosphotyrosine binding domain

PI3K: phosphoinositide 3-kinase

PIP<sub>3</sub>: phosphatidylinositol (3,4,5) trisphosphate

PKB: protein kinase B

SHP2: Src homology 2 containing tyrosine phosphatase

## INTRODUCTION

The demonstration that insulin acts through the binding to a plasma membrane receptor, dating back from the early '70s, suggested that the altered sensitivity to insulin as observed in obesity, insulin resistance and type 2 diabetes could be dependent on a diminished receptor level or weak affinity towards its agonist [1]. Since then, the intracellular signalling machinery relaying the metabolic actions of insulin has been elucidated to a good extent, and several of the enzymes taking part in insulin action have been shown to undergo alterations of their physiological function in the insulin resistant state. The discovery of the intracellular steps of insulin signalling has paved the way to intensive investigation aiming at understanding the molecular mechanism responsible for the establishment of the insulin resistant state, both on rodent models and in humans. In this review, we survey the molecular events of the insulin signalling cascade that have been amenable to clinical investigation in human subjects. The focus will be on the current understanding of the molecular disturbances of the insulin signalling cascade in muscle and adipose tissue.

### **The insulin signalling cascade, an overview**

The action of insulin is initiated by binding to its cognate receptor and activation of the receptor's intrinsic protein tyrosine kinase activity, resulting in the phosphorylation of tyrosine residues located in the cytoplasmic face [2]. The activated receptor, in turn, recruits and phosphorylates a panel of substrate molecules. Among these, IRS1 and IRS2 appear to be the adapter molecules playing a major role in the coupling to the PI3K-PKB and MAPK downstream kinases [3]. Tyrosine phosphorylated IRS1/2 recruit the heterodimeric p85/p110 PI3K at the plasma membrane, where it produces the lipid second messenger PIP<sub>3</sub>, which in turn activates a serine/threonine phosphorylation cascade of PH-domain containing proteins

[4]. PIP<sub>3</sub> targets include PDK1, the serine/threonine protein kinase B (PKB)/Akt, and the atypical protein kinases C  $\zeta$  and  $\lambda$  isoforms [5, 6]. Mechanistically, PDK1, PKB and aPKCs, which all contain a PH-domain, are recruited at the plasma membrane by binding to PIP<sub>3</sub>; thereon, PDK1 phosphorylates PKB and aPKCs on a threonine residue located in the activation loop of the catalytic domain, causing their activation [7-9]. Major targets of activated PKB are GSK-3 [10] and AS160 [11]. Upon PKB-mediated phosphorylation on Ser9, GSK-3 is inactivated [10]. This inactivation, in parallel to protein phosphatase-1 (PP1) activation, relieves the inhibitory phosphorylation of GS, which becomes activated and promotes glycogen synthesis [12]. PKB also regulates the insulin-stimulated translocation of the glucose transporter GLUT-4 at the plasma membrane, resulting in increased glucose uptake. This pathway involves an inhibitory phosphorylation of the RabGTPase activating protein AS160. Inhibition of AS160 favours the GTP-loaded state of Rab and relieves an inhibitory effect towards GLUT-4 translocation from intracellular compartments to the plasma membrane [13]. In addition to the role of PKB in controlling GLUT-4 translocation, aPKCs act in parallel to - or can even be substitutive for - PKB. (Figure 1) [14].

On a parallel pathway, activated IRS1/2 recruit Grb2, which associates to SOS and activates the Erk1/2 MAPK pathway [15]. The p38 and JNK stress-activated kinases - whose activation is mainly dependent on stress signals and inflammatory cytokines [16, 17] - have also been shown to be phosphorylated/activated in response to insulin [18, 19]; although the pathway leading to their activation has not yet been fully elucidated. Overall, alterations of the activation status of the proximal insulin signalling enzymes (IR, IRS1/2, PI3K), and downstream targets (PDK, PKB and its targets GSK-3 and AS160, aPKCs, and MAPK-family protein kinases) have been studied in muscle and adipose tissue from insulin resistant, obese and type 2 diabetic subjects, and the underlying insulin resistance has been attributed to defects in one or more steps of the insulin signalling cascade.

## **Methodologies to study insulin signalling in human skeletal muscle and adipocytes**

Skeletal muscle accounts for a large percentage of the whole body glucose uptake [20] and is an important site for insulin resistance in type 2 diabetes, as proved in a pioneering study by DeFronzo et al. [21]. At the same time, interest was also drawn to the adipose tissue, as it was shown that adipose tissue in type 2 diabetic subjects was resistant to insulin with respect to glucose transport [22], and had a decreased insulin receptor kinase activity [23].

The standard method for assessing insulin sensitivity in human volunteers is the euglycaemic hyperinsulinaemic clamp. The clamp technique, with or without radioactive tracers, was developed almost 30 years ago [21, 24]. This method consists in the infusion of glucose and insulin and concomitant monitoring of insulin and glucose levels over several hours, allowing evaluation of peripheral insulin sensitivity, and hepatic glucose production if the infused glucose is labelled either with radioactive or stable isotopes. Additionally, this study design can be accompanied by the obtention of muscle/adipose tissue biopsies, taken before and after the clamp protocol, to study the biochemical/physiological effects of insulin and glucose in the peripheral tissues of the patients.

Investigations published so far on the alterations in insulin signalling in insulin resistant and / or type 2 diabetic subjects included clamp studies of 40 minutes [6], 2 hours [25-27], 3 hours [28-32], 4 hours [33] and 5 hours [34]. Furthermore, insulin infusion rates employed in these studies ranged from 40 to 300  $\text{mU} \cdot \text{m}^{-2} \cdot \text{min}^{-1}$ , a parameter that - together with the time of clamp - might explain some of the discrepancies in the literature. The importance of the insulin infusion rate used is controversial, since Nolan et al. [35] reported a dose-dependent difference in IR autophosphorylation between lean nondiabetic, obese nondiabetic and obese type 2 diabetic subjects, while Cusi et al. [27] reported no differences

in insulin-stimulated PI3K, PKB and GS activity using infusion rates between 120 and 300  $\text{mU} \cdot \text{m}^{-2} \cdot \text{min}^{-1}$  in patients with similar characteristics. Regarding the duration of the clamp, previous studies have shown that post-receptor signalling defects are observed as early as 2 h, 3 h, and even 30 minutes after insulin infusion [6, 25, 26] and it has been shown that defective insulin-stimulated ( $120 \text{ mU} \cdot \text{m}^{-2} \cdot \text{min}^{-1}$ ) tyrosine phosphorylation was detected after 30 minutes and sustained throughout 5 h [36].

Even if *in vivo* studies have been of great importance to determine the metabolic changes that occur during insulin resistance and diabetes, complementary *in vitro* techniques have likewise been developed, providing a system which allow investigation of the molecular mechanisms responsible for insulin resistance in muscle and adipose tissue.

Performing biopsies before and after a clamp is instrumental to pinpoint the alteration(s) of insulin signalling in patients with insulin resistance and type 2 diabetes, as compared to healthy control subjects. Yet, a number of differences regarding the origin of the tissue used may complicate the interpretations. These complications arise by the location of the biopsy (mainly needle biopsy from vastus lateralis obtained under local anaesthesia, or rectus abdominus strips obtained during a surgical procedure), and whether the biopsy is directly analysed or used to establish a biopsy-derived primary cell culture. Overall, studies on muscle include muscle biopsies, muscle-strips in culture and biopsy-derived primary muscle cells ( Figure 2).

In 1988 Dohm et al. elaborated an *in vitro* muscle preparation derived from the rectus abdominus muscle that is structurally intact, metabolically viable and responsive to insulin [37]. These muscle strips were obtained through surgery and directly incubated *ex-vivo*. This study model was used to study muscle fibre strips from obese patients with or without type 2 diabetes, showing a reduced insulin-stimulated glucose transport rate in muscle strips derived from type 2 diabetic patients versus control subjects [38-40]. A cell system of differentiated

human myotubes, derived from human satellite cells obtained from muscle biopsies, was subsequently developed that display several features of mature skeletal muscles [41] and is a model that has largely been used to study insulin resistance and diabetes since it retains the diabetic phenotype [42-50].

Studies regarding insulin resistance and type 2 diabetes in adipose tissue have also been performed, but to a lesser extent than in muscle tissue. The techniques used for the study of adipose tissue is based on isolated adipocytes from biopsy prepared by the collagenase method developed by Rodbell [51] in rats and modified for use in human adipocytes [23, 52-62]. Other techniques that have been used include whole tissue adipose explants [63].

An important issue regarding the adipose tissue is relative to the time-span between the obtention of the biopsy and the performance of the experimental treatment(s) [64]. Analysis of adipocytes from lean nondiabetic subjects four hours after biopsy and after an overnight incubation showed that ERK1/2 and p38 basal phosphorylations were higher soon after the explant, compared to an overnight incubation. Thus, when ERK1/2 was analysed after 4h it was strongly phosphorylated and did not further respond to insulin, while - following overnight dephosphorylation - it responded to insulin the following day. Therefore it might be difficult to compare studies in which adipose tissue biopsies have been incubated for different time periods.

### **Alterations of insulin receptor functionality in insulin resistance**

The advent of tools to determine the activatory status of the insulin receptor allowed demonstration of its downregulation in rodent models of insulin resistance and streptozotocin-induced diabetes [65, 66]. The first attempt to evaluate the functionality of insulin receptor in obese and obese type 2 diabetic subjects was performed on partially purified insulin receptors - from abdominal muscle biopsies - stimulated ex-vivo with insulin.  $^{125}\text{I}$ -insulin binding to the

receptor was impaired in both obese and obese type 2 diabetic patients, as was IR tyrosine kinase activity towards an exogenous substrate; yet insulin-stimulated autophosphorylation was comparable between control subjects, obese and type 2 diabetic patients [67]. Other studies, performed on partially purified IR from isolated abdominal skeletal muscle strips from nonobese type 2 diabetic patients, have also shown decreased  $^{125}\text{I}$ -insulin binding and IR kinase activity as compared to control subjects [68]. A major advancement in the determination of the IR functionality was set by the study of muscle biopsies taken before and after a euglycaemic-hyperinsulinaemic clamp. Nolan et al. performed dose-escalation (insulin from 0 to  $1200 \text{ mU} \cdot \text{m}^{-2} \cdot \text{min}^{-1}$ ) euglycaemic clamps on lean control subjects, obese nondiabetic and obese type 2 diabetic patients and showed a modest impairment of autophosphorylation of the IR [35]. A time course study performed by the same group (with insulin infusions of  $120 \text{ mU} \cdot \text{m}^{-2} \cdot \text{min}^{-1}$ ) to evaluate kinetic defects of insulin action demonstrated only modest impairments in type 2 diabetes and obesity, thus suggesting the existence of defects downstream of the activated IR. In adipocytes, a reduction in  $^{125}\text{I}$ -insulin binding was observed in adipose tissue from type 2 diabetic patients - irrespective of their degree of obesity - and in insulin resistant subjects [52, 56], as well as a reduction of insulin-stimulated IR kinase activity in adipose tissue from type 2 diabetic patients [23, 56]. These observed defects resulted in a reduction in insulin-stimulated lipogenesis [22] and glucose transport in adipose tissue from type 2 diabetic patients [55, 57]. Importantly, kinetic defects in the activation of the IR that were observed in adipocytes from obese and obese type 2 diabetic patients [69], could be corrected by a 2-weeks intensive insulin treatment [70]. In conclusion, a comparison of the activation defects of the IR between muscle and adipocytes in insulin resistant conditions indicates that the IR of adipocytes is more subjected to dysregulation as compared to the muscle's receptor (Fig. 2 and 3). It should be noted, however, that studies on muscle IR activation kinetics compared biopsies collected before and

after a euglycaemic-insulinaemic clamp, while studies on adipose tissue were performed on explants of fat tissue treated ex-vivo with insulin which may explain the discrepancy in insulin responsiveness seen between skeletal muscle and fat.

Following these first studies mainly biochemical in nature, a most widely used approach to determine the degree of IR activation has consisted in selective immunoprecipitation of the receptor followed by determination of its tyrosine phosphorylation by western blotting. This analysis has in most cases [30, 31, 39, 71], but not all [72], demonstrated a mild to non-existing impairment of IR activation on samples ranging from biopsies and muscle strips to primary cultured muscle cells.

### **Dysfunctions of IRS1/2-PI3K signalling**

*Defects of IRS1/2:* The earlier observation that a phospho-protein with apparent molecular weight of 180 kDa is a major target of the activated insulin receptor led to the discovery of IRS1 and IRS2 [73]. Tyrosine phosphorylated IRS proteins provide docking sites for SH2 domain containing molecules, thus allowing the recruitment/activation of the PI3K p85/p55/p50 adapter subunits and Grb2 (mediating the transmission of the insulin signal) [73], and the tyrosine phosphatase SHP2 which may be implicated in the extinction of the signal via IR and IRS dephosphorylation [74]. To date, most of the studies on human samples have investigated the alterations occurring on IRS1, but recent research is also tackling the dysfunctions of IRS2 occurring during insulin resistance.

*IRS1:* Independent reports have shown that IRS1 tyrosine phosphorylation is diminished in skeletal muscle of obese and type 2 diabetic patients undergoing an hyperinsulinaemic clamp - leading to an almost blunted interaction with p85 [27]; and in mounted muscle strips from non-obese type 2 diabetic and morbidly obese non-diabetic patients [39, 72]. Similarly, in adipocytes derived from type 2 diabetic patients, reduced IRS1

tyrosine phosphorylation appeared to be the first upstream signalling step to be reduced [75]. A central aspect of IRS proteins regulation during insulin signalling resides in their serine/threonine phosphorylation, which can take place on more than 20 identified phosphorylation sites leading to a complex regulation of the IRS activatory state [76]. Initial studies in rodents demonstrated that JNK-mediated phosphorylation of IRS1 Ser307 (i.e. Ser312 in human IRS1), a residue located close to the PTB domain, promoted insulin resistance by impairing IRS1 binding to the activated IR [77, 78]. Other kinases have subsequently been shown to target Ser312, including IKK $\beta$  [79] and mTOR [80]. Ser312 phosphorylation by JNK and IKK $\beta$  established a plausible link between the instalment of insulin resistance and the activation of pro-inflammatory pathways [81]. Likewise, the targeting of Ser312 by mTOR demonstrated the existence of a feedback controlling mechanism within the insulin signalling cascade. Besides Ser312, other serine/threonine phosphorylation sites endowed with potential regulatory roles are those located in proximity to the SH2 domain binding motifs YxxM. Among those residues is Ser636, that, once phosphorylated by MAPK or mTOR, negatively modulates p85 PI3K binding to IRS1 [82, 83]. In primary culture of myotubes from type 2 diabetic patients, basal and insulin-stimulated levels of IRS1 phospho-Ser636 were increased [47]. Similarly, IRS1 Ser312 phosphorylation was increased in muscle biopsies from obese and type 2 diabetic patients [34]. Interestingly, while in muscle biopsies from control subjects IRS1 Ser312 phosphorylation was modulated by insulin infusion - increasing  $\approx$  3 fold - the increased basal phosphorylation in obese and type 2 diabetic patients did not allow for a further insulin-stimulated increase [34], suggesting that either i) in the insulin resistant condition IRS1 Ser312 is stoichiometrically phosphorylated or ii) insulin signalling leading to IRS1 Ser312 phosphorylation is impaired. In keep with this second idea, Danielsson et al. have demonstrated that - in cultured adipocytes from type 2 diabetic patients - the ability of insulin to induce IRS1 Ser312

phosphorylation is attenuated, implying that augmented IRS1 Ser312 following insulin stimulation is a physiologically normal response, possibly implicated in the determination of the correct amplitude and length of insulin action [75].

Further research in cell culture systems has allowed the determination of a large number of Ser/Thr phosphorylation sites on IRS1, the responsible kinases and the impact of such phosphorylations on the insulin signalling capacity [76]. Several new Ser/Thr phosphorylation sites modulating IRS1 function have recently been reported [84-95], as summarised in figure 4. While IRS1 Ser/Thr phosphorylation was initially viewed solely as a negative regulatory mechanism, it is now clear that both positive and negative modulation of IRS1 function arise from different phosphorylation patterns. Thus, the current understanding on the modulation of IRS1 function by Ser/Thr phosphorylation in human samples (either from control subjects or type 2 diabetic patients) is rather limited inasmuch: i) only Ser312 and Ser636 phosphorylations have been studied in detail in muscle and adipose tissue and ii) the standard methodological approach implies the use of phosphospecific antibodies, with the associated specificity problems and the difficulty to test a panel of antibodies directed to all the known IRS1 phosphorylation sites within a single biological sample. These experimental limitations will likely be overcome by the use of more sensitive technologies. Recently, several groups have started to apply mass spectrometry-based techniques to identify new aspects of IRS1 regulation through Ser/Thr phosphorylation. The advantages of mass spectrometry-based techniques reside in the fact that the need for isotopic/chemical labelling or phosphospecific antibodies is overcome and low-abundance proteins, such as IRS1, are amenable to full characterization, since the overall Ser/Thr phosphorylation pattern can be analysed within the same sample after tryptic digestion, with the unphosphorylated peptides serving as endogenous internal standards [96]. By this approach, Thr446 has been identified as a TNF $\alpha$ -induced phosphorylation site, likely relaying a negative regulation [96], and

Ser570 has been identified as a PKC- $\zeta$  target involved in the negative modulation of the insulin signal [92]. In addition, after overexpression of human IRS1, Luo et al. simultaneously identified 12 phosphorylation sites - among whom 10 were newly identified sites - and assigned to Ser1223 a positive regulatory role [95]. While these studies dealt primarily with the identification of new phosphorylation sites on IRS1 in cell culture studies, a major advance has recently been provided by a study by the group of Mandarino, in which insulin-induced IRS1 Ser and Thr phosphorylations have been analysed in human percutaneous biopsies from vastus lateralis muscle obtained before and after a 2-hours euglycemic-hyperinsulinemic clamp [97]. This study allowed the simultaneous identification of 22 Ser/Thr phosphorylation sites and the quantitative evaluation of Ser/Thr phosphorylation levels of 15 sites [97]. This study has provided, for the first time, an overall picture of the dynamic changes of IRS1 phosphorylation status after insulin stimulation in healthy humans and set the technological standard for the study of phosphorylation-dependent IRS protein dysfunctions in insulin resistant subjects and type 2 diabetic patients.

*IRS2*: While studies on IRS1 regulation through Ser/Thr phosphorylation mechanism flourished, and studies on humans have started to define the physiological response of IRS-1 to insulin, much less is known on the modulatory processes regulating IRS2, although IRS1 and IRS2 appear to equally contribute to peripheral glucose metabolism, at least in mice [98]. In the vast majority of the studies, PI3K activity associated to IRS proteins has been monitored in IRS1 immunoprecipitates only (figure 2). In the few studies in which PI3K activity associated to IRS1 or IRS2 have been performed side by side [32, 47, 71], a decrease has been observed in both IRS1- and IRS2-associated insulin-stimulated PI3K activity in muscle biopsies from obese type 2 diabetic patients [32], whereas a selective alteration of insulin-induced IRS1-associated PI3K activity - after a 10-min stimulation with 100 nmol/l insulin - is observed in myotubes derived from obese type 2 diabetic patients [47]. Another

group has showed IRS2-associated defect, yet in a sustained way from 3 to 60 min stimulation with 17 nmol/l insulin in myotubes from insulin resistant obese individuals [71]. Whether alterations of Ser/Thr phosphorylation pattern on IRS2 play a role in its modulation is largely undetermined and should provide a fruitful field of investigation, as IRS2 is known to be subjected to extensive phosphorylation events that modulate its function both positively and negatively [99]. Although the IRS1 key regulatory residue Ser312 appears to be absent in IRS2, sequence analysis revealed that IRS2 Thr348 could be the homologous residue, being located within a potential JNK phosphorylation site, and biochemical studies demonstrated that IRS2 Thr348 can be phosphorylated by JNK *in vitro* [100]. This biochemical observation underscores a common theme in the negative regulation of IRS1 and IRS2. Conversely, very recent data indicate that there is a divergence in the regulation of IRS proteins through PKC- $\zeta$ -mediated phosphorylation, with the IRS1 (and IRS3/4) being PKC- $\zeta$  targets, but not IRS2 [101].

*PI3K*: Insulin-induced PI3K action - leading to the production of PIP<sub>3</sub> at the plasma membrane catalysed by class I p110 $\alpha$ / $\beta$  PI3K catalytic subunits - is the result of a complex interplay of many biochemical events including:

- recruitment of PI3K at tyrosine phosphorylated IRS1/2
- relative contribution of the p110 $\alpha$  and p110 $\beta$  catalytic isoforms, which are combinatorially associated to the p85/55/50 $\alpha$  and p85 $\beta$  and p55 $\gamma$  adapter subunits
- modulation of the enzyme's action through interaction with RAS
- availability of the lipid substrate PtdIns(4,5)P<sub>2</sub> at the plasma membrane
- modulation of the resulting PIP<sub>3</sub> levels by the PTEN and SHIP2 phosphoinositide phosphatases.

Although all these molecular events have been extensively investigated (reviewed in [102-106]) and both PI3K and the PIP<sub>3</sub> phosphatases are promising targets for therapeutic

intervention to improve insulin action [107, 108], the current understanding of the alterations of PI3K in insulin resistance and type 2 diabetes rests essentially on in vitro PI3K assays performed on immunoprecipitates to IRS1/2 or phosphotyrosines. While this procedure mirrors the extent of interaction of PI3K to its upstream partners, it does not provide information on all the other mechanisms implicated in the regulation of intracellular PIP<sub>3</sub> levels (such as the contribution of RAS to PI3K activation and the activities of the phosphoinositol phosphatases PTEN/SHIP2), thus leaving us in an uncertainty as to what extent PI3K signalling is actually dysregulated in insulin resistance and type 2 diabetes. Furthermore, the activities of class II and class III PI3Ks should be taken into account to have an overall picture of the contribution of PI3Ks in insulin signalling. Indeed, the involvement of class II PI3Ks in insulin signalling, in cultured cells, was demonstrated by the group of Shepherd [109] and the exact mechanism of action has been elucidated more recently [110]. These reports demonstrate the importance of PI3K-C2 $\alpha$ , and its unique in vivo lipid product, PtdIns3P as a lipid second messenger in insulin action, in cell culture models, these findings suggest that dysregulation of class II and class III PI3Ks may also occur in the insulin-resistant state. A more reliable way to determine the extent of PI3K-dependent defects would thus be the direct monitoring of the PI3K lipid product PtdIns3P, PtdIns3,4P<sub>2</sub> and PIP<sub>3</sub>.

While in vivo imaging techniques to visualise and quantify PIP<sub>3</sub> in cell culture have been developed and used in L6 cells [111], to the best of our knowledge such approaches have not yet been used on human-derived skeletal muscle cells or adipocytes. Furthermore, direct visualisation of PIP<sub>3</sub> within an organism is as yet technically out of reach. These intrinsic limitations in the study of PI3K action in biopsy-derived samples can help explaining the current discrepancies existing in the literature as to whether PI3K is impaired or not in insulin resistance and type 2 diabetes. As an example, several groups have reported defective PI3K activation (measured in vitro as IRS1-associated activity) but unaltered PKB activation in

obese diabetic patients [6, 30, 32]. To fully understand this seemingly paradoxical observation we would need to know whether phosphoinositide phosphatase activities of PTEN and SHIP2 are altered in those subjects, as decreased activities could result in increased PIP<sub>3</sub> levels in vivo, which may explain the retained PKB activation. Furthermore, the contributions of class II and class III PI3Ks - that are known to actively control insulin signalling [110, 112] - to the insulin resistant state have not yet been studied in humans.

### **Akt/PKB**

The PI3K downstream target PKB, a serine/threonine kinase of which  $\alpha$ ,  $\beta$  and  $\gamma$  isoforms have been reported, is a key enzyme mediating the metabolic actions of insulin [113-115]. PKB activation occurs through phosphorylation of Thr308 and Ser473, mediated by PDK1 and the rictor-mTor complex respectively [9, 116]

While PKB $\alpha$  is involved in the regulation of lipid metabolism [117], the activation of glycogen synthesis in skeletal muscle [118] and in insulin action in adipose cells [119]; it is PKB $\beta$  (the major isoform expressed in skeletal muscle [120]) that is considered to be the key isoform involved in insulin metabolic actions [118]. Finally, although PKB $\gamma$  is preferentially expressed in non-insulin dependent tissues [121, 122], reduced PKB $\gamma$  activity in skeletal muscle of obese and insulin resistant subjects has been reported [123].

Whether insulin-induced activation of PKB in insulin resistance and type 2 diabetes is impaired is currently a debated matter. Some studies reported significant reductions of insulin-stimulated Ser473 or Thr308 phosphorylation in skeletal muscle of type 2 diabetic patients [29, 31, 40], while others showed no alterations of phosphorylation or enzymatic activity of PKB between control subjects and type 2 diabetic patients [27, 31, 32, 124]. As previously mentioned, Kim et al. [32] did not observe, in type 2 diabetic subjects, defects in PKB $\alpha$  and  $\beta$  activities and phosphorylation, in spite of decreased insulin-induced PI3K

activation. This observation was subsequently confirmed by the observation of unaltered PKB phosphorylation in muscle cultures and biopsies from control subjects and type 2 diabetic patients by employing phospho-specific antibodies [31, 124]. On the other hand, Krook et al. [40] reported defective insulin-induced activation of PKB $\alpha$  in isolated muscle strips from moderately obese diabetic patients compared to BMI-matched control subjects. A more recent and comprehensive study reported on the specific activity of each PKB isoform, showing that PKB $\beta$  and  $\gamma$  (but not PKB $\alpha$ ) activities are diminished in skeletal muscle biopsies from morbidly obese insulin resistant patients [123]. Additionally, our laboratory recently demonstrated that isoform-specific alterations of the PKB phosphorylation pattern occur in cultured primary myotubes from type 2 diabetic patients in response to insulin, with defective Ser473 phosphorylation on PKB $\beta$  and defective Thr308 phosphorylation on PKB $\alpha$  [29]. Furthermore, it was shown that PHLPP1, which is able to dephosphorylate the Ser473 residue of PKB $\beta$ , is upregulated in the muscle of type 2 diabetic patients compared to control subjects [29].

Overall, the variability of PKB activation status must be viewed in the light of the different patient cohorts characteristics used in each study. As an example, Cozzone et al. [29] included overweight type 2 diabetic patients while Brozinick et al. included morbidly obese insulin resistant patients [123]. In most studies biopsies are from vastus lateralis [29, 32, 40, 124] but in some instances strips from the rectus abdominal muscle have been used [123].

Thus, unresolved issues on our current understanding of PKB defects in insulin resistance derive from the fact that i) global PKB phosphorylation is most often measured, disregarding isoform-specificities; ii) samples are of different origin, i.e. biopsies, biopsy-derived muscle strips or biopsy derived primary muscle cell culture and, finally iii) cohort characteristics are rarely fully comparable among different studies.

At present, a few studies have also interrogated PKB impairment in adipocytes from type 2 diabetic subjects. With respect to this issue, there is more consensus on the fact that PKB activation is impaired in adipocytes derived from adipose tissue of type 2 diabetic patients [125, 126].

### **Atypical PKCs**

The implication of aPKCs in insulin-stimulated GLUT4 translocation and glucose transport in muscle and adipose tissue has mainly been shown by studies in animals (Reviewed in [127]). In human isolated adipocytes, expression of PKC- $\zeta$  or PKC- $\lambda/\iota$  increases glucose transport, while expression of kinase-inactive forms inhibited glucose transport [128]. Numerous studies on animals report abnormalities in the activation of aPKC in muscle and adipose tissue in insulin resistance and type 2 diabetes (Reviewed in [127], and these abnormalities are also found in humans.

In lean, nondiabetic humans subjects, insulin stimulation leads to a twofold increase in aPKC activity in muscle [6, 14, 30, 34, 71, 129] and adipose tissue [129], and a positive relationship was seen between insulin-stimulated PKC-  $\zeta/\lambda$  activity and insulin-stimulated glucose disposal rate [30]. The insulin stimulated activation was diminished in myotubes from obese and/or obese type 2 diabetic patients [6, 14, 30, 34, 71, 129] and adipocytes from obese glucose intolerant subjects [129]. A further study using weight- and age-matched nondiabetic and diabetic subjects, showing that insulin-stimulated activation of aPKC was still diminished in the diabetic group, allowed excluding obesity as the only reason behind the abnormalities [6].

Also, PIP<sub>3</sub> directly activates aPKCs in vitro, to a similar extent as does insulin, in muscle and adipose tissue from nondiabetic patients [6, 128], while this activation is diminished in muscle samples from glucose intolerant and type 2 diabetic patients [6] and

myotubes and adipocytes derived from obese glucose intolerant women [129]. This abnormality in aPKC activity is sometimes accompanied by decreased protein levels of PKC- $\zeta$  and/or PKC- $\lambda/\iota$  [6, 30]. Even if there is no evidence of direct interaction in vivo of PIP<sub>3</sub> with the PH domain of aPKCs, there are several studies showing that direct addition of PIP<sub>3</sub> to aPKC immunoprecipitates provokes insulin-like increases in aPKC activities [6, 14, 129-131]. Alternatively, aPKCs activation may be subsequent to PIP<sub>3</sub>-induced PLC $\gamma$  activation [132, 133].

Generally, all studies in humans, independently from the tissue under study and the methodology employed, have shown impairment of aPKC activity in obesity, insulin resistance and type 2 diabetes, as summarised in figure 2 and figure 3.

#### **MAPK signalling - ERK, JNK, p38**

Besides the PI3K-PKB, insulin activation leads, through Grb2/Sos and RAS, to the phosphorylation and activation of members of the MAP kinase family, specifically ERK1/2, JNK and p38 MAPK, which phosphorylate and control the activity of other downstream protein kinases and transcription factors. However, not much is known about whether the MAPK pathway is equally affected by insulin resistance as the PI3K-PKB pathway.

The first reports regarding MAP kinases showed that ERK1/2 phosphorylation in muscle biopsies was similar between control subjects and type 2 diabetic patients [39] as was ERK1/2 phosphorylation and activity and MEK1 activity [27]. These observations were followed by the study of muscle biopsy-derived myotubes from control subjects and type 2 diabetic patients, showing that the phosphorylation levels of ERK1/2 were similar, both in the absence and presence of insulin [124]. However, other studies have reported a high basal phosphorylation state of ERK1/2 in muscle [34, 47] and adipose tissue [58, 64] from type 2 diabetic patients compared to control subjects. Some of the studies reported that the high

levels of ERK1/2 basal phosphorylation is accompanied by a lack of further insulin stimulated phosphorylation in the type 2 diabetic patients [34, 58, 64].

A few studies have also explored the dysregulations of other members of the MAP kinase family. The level of JNK1 phosphorylation has been reported to be elevated in the basal state in type 2 diabetic patients compared to control subjects, both in muscle [34] and adipose tissue [58], with the high basal state possibly causing the lack of an insulin-stimulated JNK activity. p38 MAPK phosphorylation was also shown to be increased in basal state in muscle tissue from obese and type 2 diabetic patients compared to control subjects [34, 134], with similar results observed in adipose tissue [58]. Upon insulin stimulation, p38 phosphorylation increased in control subjects, while it decreased in muscle tissue of type 2 diabetic patients [34, 134], and remained unchanged in adipose tissue from type 2 diabetic patients [58].

The few studies available on the MAPK pathways in insulin resistance indicate that this pathway is possibly altered, even if discrepancies exist regarding this pathway. Similar effort needs to be done regarding this pathway as for the PI3K-PKB pathway to elucidate the alterations present during insulin resistance and type 2 diabetes.

## CONCLUSIONS

During the past three decades, studies on the molecular alterations of the insulin signalling cascade have allowed to establish a link between the alterations of glucose metabolism observed in insulin resistant and type 2 diabetic patients and the underlying molecular defects in muscle and adipose tissue. While initial studies on the IR suggested that its dysfunctionality could account for the insulin-resistant phenotype, analysis of the intracellular components of the insulin signalling pathway suggested that the defects mainly reside intracellularly. As reviewed in this paper, and clearly pointed out in Figure 2 and 3, the

only consensus reached by all studies to date is the presence of alterations in aPKCs as well as in metabolic responses such as glucose disposal and transport. On the contrary, the occurrence of defects of upstream signalling components is more controversial, with studies showing alterations in insulin resistance while others find no differences with respect to control subjects. This is mainly apparent relatively to the defects observed at the levels of IRS proteins, PI3K and PKB, as we discussed in detail in the relevant paragraphs.

The complexity of the insulin signalling pathway does not cease to grow as new regulatory phosphorylation sites, proteins, protein isoforms and interactions are reported. So far, the analysis of the insulin signalling pathway in insulin resistance has been mainly focused on IRS1/2, PI3K and PKB. Further investigations on the phosphoinositide phosphatases PTEN and SHIP2 may explain some of the discrepancies reported between the PI3K and PKB activities. Therefore, the study of PTEN and SHIP2 dysregulation in insulin resistance is warranted. Other proteins participating in the signalling cascade might also need to be taken into account, e.g. the newly discovered PKB isoform-specific phosphatase PHLPP [135], of which some isoforms seem to be altered in the muscle of type 2 diabetic patients [29]. Insulin also generates a pool of the lipid PtdIns3P at the plasma membrane, a lipid messenger that has been shown to be implicated in GLUT4 translocation [136]. While the crucial role of PIP<sub>3</sub> in many signalling events and intracellular functions is well established, less is known about the role of other PI3K products, even if it has been shown that PtdIns3P may play a dynamic role as a second messenger, being synthesized de novo to transduce specific signals downstream to cellular stimulation. Therefore, studies on the implication of class II and class III PI3Ks, and their lipid products, in insulin resistance are warranted.

The use of a mass spectrometry-based techniques for quantification of phosphorylation of IRS1 described by Yi et al. [96, 97] is a technique that allow the quantification of several phosphorylation sites simultaneously using the same sample, thereby reducing the problem of

sample availability. To date, this technique has only been used to study phosphorylation of IRS1 protein in muscle samples from healthy human muscle samples. Yet, with the availability of the technology, it is likely that studies evaluating IRS1 and IRS2 phosphorylation in obese, insulin resistant and type 2 diabetic patients will be performed in the near future. Similarly, it is likely that mass spectrometry-based techniques will also be used to study the phosphorylation patterns of other proteins of the signalling cascade (e.g. PKB isoforms, aPKCs).

Since the clinical characteristics of lean and obese subjects as well as insulin resistant and type 2 diabetic patients are different, the subjects enrolled in the studies need to be well characterised and study groups clearly defined. As discussed earlier, differences in experimental procedures (e.g. biopsies versus primary cell culture) and duration and/or rate of insulin infusion needs to be considered when trying to reach a consensus on defects in insulin signalling. Furthermore, all studies reviewed in this article are based on studies in small cohorts of insulin resistant, obese or type 2 diabetic patients, with cohort sizes around 10 patients per group. The lack of bigger cohort studies might also be one of the reasons for the reported discrepancies. In future studies, to overcome the problem of patient recruitment, which is not an easy task, one solution would be multicentric studies, either national or international.

Our understanding of the defects of the insulin signalling cascade in insulin resistance and type 2 diabetes will probably be fully attained in the next few years as novel technologies are employed and more consideration is given to the experimental approaches and subject characteristics.

**Current limitations and future prospects in the study of insulin signalling in human muscle/adipose tissue*****Limitations:***

- Only a few IRS1 Ser/Thr phosphorylation regulatory sites are studied within each independent investigation
- The knowledge of IRS2 regulation by Ser/Thr phosphorylation is still very limited
- The extent of PI3K activation is only calculated on the basis of in vitro PI3K activity associated to IRS1 and IRS2
- Only a few studies have addressed the isoform-specific deregulation of PKB

***Future prospects:***

- Use of mass-spectrometry techniques to simultaneously study multiple Ser/Thr phosphorylation sites in IRS1 and IRS2
- Evaluation of PI3K lipid-kinase activity by measuring the actual PIP<sub>3</sub> production, in vivo
- Defects of class II and III PI3Ks need to be studied
- Study of PKB isoform specific deregulations
- More extensive study of MAPK pathway

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## Figure Legends

**Figure 1:** Schematic depiction of the insulin regulated intracellular signal transduction cascade, reporting the signalling steps whose alterations have been studied in insulin resistant and type 2 diabetic subjects.

**Figure 2:** Schematic diagram of signalling and metabolic events studies in human muscle from control subjects versus insulin resistant, obese or type 2 diabetic patients. The diagram summarises studies from i) biopsy-derived primary human skeletal muscle cells, ii) needle biopsies, iii) muscle strips from abdominal surgeries. Red dots indicate the observation of impairments versus control subjects, green dots indicate the absence of alteration in the biochemical/metabolic events studied, and red/green dots indicate either unchanged or impaired versus control subjects.

**Figure 3:** Schematic diagram of signalling and metabolic event studies in human adipose tissue ex-vivo from control subjects versus glucose intolerant, obese, insulin resistant or type 2 diabetic patients. Red dots indicate the observation of impairments versus control subjects, green dots indicate the absence of alteration in the biochemical/metabolic events studied, and red/green spots indicate either unchanged or impaired versus control subjects.

**Figure 4:** Schematic diagram of IRS1, reporting newly discovered phosphorylation sites, the kinases mediating the site's phosphorylation, and the resulting effect caused on IRS1 functionality (red, inhibitory phosphorylation; green, activatory phosphorylation). On the bottom, previously identified phosphorylation sites, Ser312, Ser612 and Ser636, are reported (reviewed in [76]). IRAK: IL-1 receptor associated kinase.

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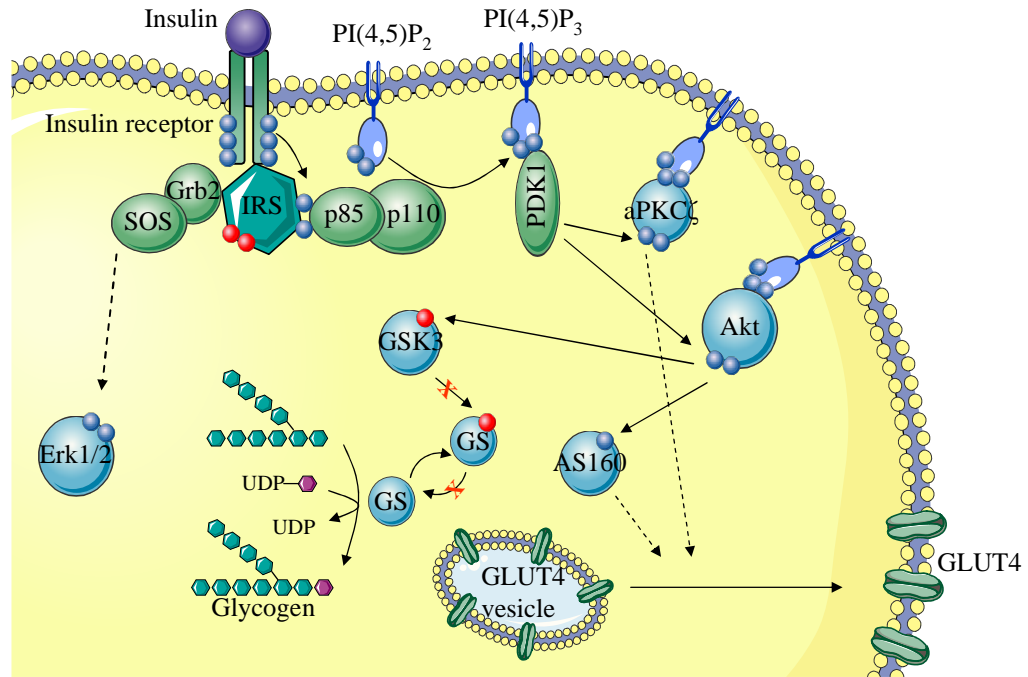


Figure 2

Controls versus	IR activity or autophosphorylation	pY associated PI3K	IRS-1 associated PI3K	IRS-2 associated PI3K	PDK-1	PKB activation	PKC $\zeta$ activation	ERK1/2 activation	GS activity	Glucose transport/disposal	Reference
Obese insulin resistant subjects											
Biopsies											(6, 27, 30, 32, 34)
Muscle strips											(72, 123)
Primary muscle cells											(71, 124, 129)
Type 2 diabetic patients											
Biopsies											(6, 27, 30, 31, 32, 33, 34)
Muscle strips											(39, 40)
Primary muscle cells											(29, 47, 48, 49, 50, 124)

Unchanged versus controls    Impaired versus controls    Either unchanged or impaired versus control

Figure 3

Controls versus	Insulin binding	IR activity or autophosphorylation	IRS-1/2 associated PI3K	PKB activation	PKC activation	ERK1/2 activation	GLUT4 protein level	Glucose transport/disposal	Reference
Insulin resistant subjects									
Obese	●	●	●	●	●		●	●	(23, 52, 60, 61, 69, 129)
Morbidly obese	●							●	(57)
Type 2 diabetic patients									
Nonobese	●							●	(52)
Obese	●	●	●	●		●	●	●	(23, 52, 55, 58, 60, 62, 64, 69, 125, 126)
Morbidly obese	●	●						●	(56, 57)

● Unchanged versus controls    ● Impaired versus controls    ● Unchanged/Impaired versus control

Figure 4

