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ARTICLE

Differential aetiology and impact of phosphoinositide 3-kinase (PI3K) and Akt signalling in skeletal muscle on in vivo insulin action

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Abstract
Aims/hypothesis Insulin resistance in skeletal muscle is a key factor in the development of type 2 diabetes and although some studies indicate that this could be partly attributed to reduced content and activity of various proximal and distal insulin signalling molecules, consensus is lacking. We therefore aimed to investigate the regulation of proximal insulin signalling in skeletal muscle and its effect on glucose metabolism in a large non-diabetic population.

Methods We examined 184 non-diabetic twins with gold-standard techniques including the euglycaemic–hyperinsulinaemic clamp. Insulin signalling was evaluated at three key levels, i.e. the insulin receptor, IRS-1 and V-akt murine thymoma viral oncogene (Akt) levels, employing kinase assays and phospho-specific western blotting.
Results Proximal insulin signalling was not associated with obesity, age or sex. However, birthweight was positively associated with IRS-1-associated phosphoinositide 3-kinase (PI3K; IRS-1-PI3K) activity (p=0.04); maximal aerobic capacity ($\dot{V}O_{2max}$), paradoxically, was negatively associated with IRS-1-PI3K (p=0.02) and Akt2 activity (p=0.01). Additionally, we found low heritability estimates for most measures of insulin signalling activity. Glucose disposal was positively associated with Akt-308 phosphorylation (p<0.001) and Akt2 activity (p=0.05), but not with insulin receptor tyrosine kinase or IRS-1-PI3K activity.
Conclusions/interpretation With the exception of birthweight, 'classical' modifiers of insulin action, including genetics, age, sex, obesity and $\dot{V}O_{2max}$, do not seem to mediate their most central effects on whole-body insulin sensitivity through modulation of proximal insulin signalling in skeletal muscle. We also demonstrated an association between Akt activity and in vivo insulin sensitivity, suggesting a role of Akt in control of in vivo insulin resistance and potentially in type 2 diabetes.

Keywords Assay methods · Hormone receptors · Human · Insulin action · Insulin resistance · Insulin sensitivity

Abbreviations

Akt	V-akt murine thymoma viral oncogene
Akt1	Akt homologue 1
Akt2	Akt homologue 2

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Akt-308phos	Phosphorylation of Akt-308
Akt-473phos	Phosphorylation of Akt-473
DXA	Dual-energy X-ray absorptiometry
IGT	Impaired glucose tolerance
IRS-1-PI3K	IRS-1-associated phosphoinositide 3-kinase
IRTK	Insulin receptor tyrosine kinase
NGT	Normal glucose tolerance
PI3K	Phosphoinositide 3-kinase
R_d clamp	Rate of glucose disappearance during insulin stimulation
$\dot{V}O_{2max}$	Maximal aerobic capacity

Introduction

Insulin resistance in skeletal muscle is a key factor in the development of type 2 diabetes [1]. Defective insulin-stimulated glucose uptake in muscle is associated with impaired muscle membrane glucose transport [2, 3] and reduced muscle glycogen synthase activity [2, 4–7], which in turn may both result from impaired insulin signalling.

Upon binding to the insulin receptor, insulin promotes receptor autophosphorylation, thus activating the intrinsic tyrosine kinase activity of the insulin receptor. The receptor phosphorylates tyrosine residues on IRS-1 and -2 [8], the first of which is a major post-receptor component involved in glucose metabolism in skeletal muscle [9]. IRS-1 binds and thus activates phosphoinositide 3-kinase (PI3K). Through several kinases, PI3K facilitates the activating phosphorylation of threonine 308 and serine 473 residues on V-akt murine thymoma viral oncogene (Akt) homologue 1 (Akt1) and 2 (Akt2), which are proposed to be necessary for insulin-stimulated glucose uptake and glycogen synthesis [10].

Multiple defects at several levels in insulin signalling have been described in patients with overt type 2 diabetes [5, 11–14] and strongly associated diseases [15], but also in conditions predisposing to type 2 diabetes. These conditions include an adverse intrauterine environment as evidenced by low birthweight, which has previously been linked to reduced levels [16] and function [17] of key insulin signalling proteins. Obesity has also been associated with decreased insulin signalling capacity in some [5, 11, 12, 18], but not all studies [13, 14]. The nature of the reduced insulin signalling activity has not been fully elucidated, but the involvement of circulating mediators has been proposed. For instance, TNF-α and NEFA have been associated with increased inhibitory phosphorylation of serine residues on IRS-1 and lower activity of Akt in humans [19–21].

In a large well-characterised Danish twin population, we recently reported that insulin-stimulated glucose disposal is controlled to an equal degree by genetic and non-genetic factors [22]. Homozygous single gene defects (e.g. in the insulin receptor gene) are rare, but represent the most severe forms of insulin resistance [8]. Interestingly, combined heterozygous knockout of genes encoding insulin receptor and IRS-1 results in diabetes, despite the fact that heterozygous knockout of each gene on its own does not [23]. These findings support the notion that insulin resistance has a polygenic origin. Studies of monozygotic and dizygotic twins can disclose the relative importance of genetic and environmental influences on particular phenotypes [24], e.g. the activity of proteins involved in proximal insulin signalling.

In this study of a large twin population without known type 2 diabetes, we examined the regulation of proximal insulin signalling in skeletal muscle (by genes, age, sex, intrauterine environment, obesity and aerobic capacity) at the levels of the insulin receptor, IRS-1-associated PI3K (IRS-1-PI3K) and Akt. We also investigated the relationship between proximal insulin signalling and in vivo glucose metabolism.

Methods

Participants We identified participants through the population-based Danish Twin Registry as previously described [22, 25]. A total of 98 young and old twin pairs without known diabetes were enrolled in the clinical examination (Table 1). Of the old monozygotic twins, 76.2% (n=32) had normal glucose tolerance (NGT), 19.0% (n=8) had impaired glucose tolerance (IGT) and 4.8% (n=2) had previously unknown type 2 diabetes. Of the old dizygotic twins, 72.7% (n=32) had NGT, 25.0% (n=11) IGT and 2.3% (n=1) type 2 diabetes. All young dizygotic twins had NGT (n=44). In the young monozygotic twins group, 97.0% (n=64) had NGT and 3.0% had IGT (n=2). There was no significant difference in glucose tolerance status between monozygotic and dizygotic twins within each age group. Zygosity was determined by polymorphic genetic markers [25]. Glucose tolerance status was defined according to the WHO 1999 criteria [26]. The study was approved by the regional Ethical Committees and conducted according to the principles of the Helsinki Declaration. Informed consent was obtained from the study participants.

Clinical examination The clinical examination has been described in detail [22, 25]. In brief, it included a standard 75 g OGTT, anthropometric measurements including weight, height and both waist and hip circumferences, determination

Table 1 Clinical and metabolic characteristics of the young and old twins

Characteristics	Young	Old	*p* value
Participants			
All (*n*)	101	83	
Men (*n*)	56	37	
Women (*n*)	45	46	
Age (years)	28.0±1.8	62.0±2.3	<0.001
Birthweight (kg)	2.6±0.5	2.7±0.5	0.22
Body weight (kg)	73.9±15.1	75.0±13.5	0.60
BMI (kg/m^2)	24.2±3.2	26.3±4.5	<0.001
Total fat percentage	22.2±7.0	28.1±9.6	<0.001
$\dot{V}O_{2max}$ (ml min^{-1} kg^{-1})	39.6±7.9	26.5±7.0	<0.001
Lean body mass (kg)	55.0±11.2	50.8±11.8	0.01
Fasting plasma glucose (mmol/l)	5.4±0.5	5.9±0.8	<0.001
R_d clamp (mg kg^{-1} min^{-1})	11.8±3.2	9.9±3.4	<0.001

Unless specified otherwise, data are mean ± SD

of body composition by dual energy X-ray absorptiometry (DXA) scanning and estimation of maximal aerobic capacity ($\dot{V}O_{2max}$) by bicycle testing. Insulin sensitivity was examined by a 2 h euglycaemic–hyperinsulinaemic clamp (40 mU m^{-2} min^{-1}). A primed constant continuous infusion of [3-^{3}H]glucose (bolus 814 kBq, 8.14 kBq/min) was initiated at 0 min and continued throughout the clinical investigation (basal period 120 min, clamp period 120 min). Steady state (defined as the last 30 min of the basal and insulin-stimulated periods) was achieved for both young and elderly twin groups [27]. The rate of glucose disappearance during insulin stimulation (R_d clamp) was subsequently calculated using the non-steady-state equations proposed by Steele [28]. Muscle biopsies (available from 184 out of a total of 196 twins) were obtained during the basal and insulin-stimulated steady-state periods from the vastus lateralis muscle using a Bergström needle with suction applied. Specimens were quickly blotted on filter paper, frozen in liquid nitrogen and stored at −80°C until processed. All assays for this study were performed within 3 years (the duration of the elaborate clinical investigations of 200 individuals) of the clinical examination.

Muscle protein levels Protein lysate was prepared and protein phosphorylation determined by SDS-PAGE and western blotting as previously described [29]. Protein content was expressed in arbitrary units relative to a skeletal muscle standard. The antibodies used were: phospho-specific anti-Akt-308 (06-678; Upstate Biotechnology, Lake Placid, NY, USA) and anti-Akt-473 (9271; Cell Signaling, Beverly, MA, USA), both of which recognise Akt1 and Akt2 isoforms, and goat-anti-rabbit horseradish peroxidase (P0448; Dako, Glostrup, Denmark).

Insulin receptor tyrosine kinase, PI3K and Akt activity Insulin receptor tyrosine kinase (IRTK) activity was measured after immunopurification using a microtitre assay as previously described [30]. All samples were loaded in duplicate. The intra-assay variation (calculated as the difference between duplicates divided by their mean) was 24%. To test whether this variability could explain the largely negative findings with regard to IRTK, we made a new dataset excluding the samples located in the upper tenth percentile of the variation. Using this dataset we reached the same conclusions as in the total dataset, thus demonstrating the robustness of our results. IRS-1-PI3K activity was measured after immunopurification of IRS-1 as previously described [4]. Isoform-specific Akt1 and Akt2 activity was determined after sequential immunopurification of Akt2 followed by Akt1 as previously described [31]. The human samples were normalised to a rodent muscle sample to account for assay variation.

Statistical methods All statistical tests were performed in SAS (version 9.1; SAS Institute, Cary, NC, USA). Data are presented as means ± SD. A p value of $p<0.05$ was considered significant.

For univariate analyses, a paired two-tailed t test was used to compare basal and insulin-stimulated insulin signalling activity. Spearman's Rho was calculated to evaluate the correlation between two continuous variables. The heritability coefficient (h^2) expresses the proportion of the total variation of a trait attributable to genetic variation. Heritability is expressed as twice the difference of the intra-class correlation coefficients of monozygotic and dizygotic twins $\left(h^2 = 2[r_{MZ} - r_{DZ}]\right)$, where MZ is monozygotic and DZ dizygotic [24].

For multivariate analyses, multiple regression analyses using the 'proc mixed' procedure in SAS allowed for adjustment of twin pair and zygosity status, and other contributing variables [24]. All explanatory variables had been associated with insulin signalling in the present or

previous studies. All response variables were transformed by the natural logarithm to avoid skewness of the residuals. This also resulted in estimates expressing percentage and not absolute changes of the response variable. For continuous variables the effect on the response variable was expressed per SD of the explanatory variable. Age, sex, zygosity, birthweight, $\dot{V}O_{2max}$ and total fat percentage were included as explanatory variables when investigating the association with measures of proximal insulin signalling. Age, sex, $\dot{V}O_{2max}$, total fat percentage and a measure of insulin-stimulated insulin signalling activity were included when investigating the effect on R_d clamp.

Results

Clinical and metabolic characteristics The physiological data from this population have been described in detail [6, 22, 25, 27, 29, 32, 33]. In brief, the old twins had significantly higher BMI, total fat percentage and fasting plasma glucose than the young twins (Table 1). They also had lower lean body mass, $\dot{V}O_{2max}$ and R_d clamp.

Determinants of in vivo insulin sensitivity R_d clamp was positively associated with $\dot{V}O_{2max}$ (r=0.34, p<0.001) and negatively associated with total fat percentage (r=−0.21, p=0.004). Increased age (Table 1) and the male sex were associated with lower R_d clamp (men 10.4 vs women 11.4 mg kg^{-1} min^{-1}, p=0.04).

Association between IRTK, IRS-1-PI3K and Akt activity IRTK activity was not positively correlated to any downstream kinase in proximal insulin signalling, whereas IRS-1-PI3K activity was associated with Akt2 activity (r=0.31, p<0.001; Fig. 1a). Additionally, phosphorylation of Akt-473 (Akt-473phos) was associated with Akt2 activity (r=0.28, p<0.001) and phosphorylation of Akt-308 (Akt-308phos) was associated with Akt1 (r=0.16, p=0.03) and Akt2 activity (r=0.39, p<0.001; Fig. 1b).

Impact of insulin stimulation and age Insulin stimulation increased IRTK and IRS-1-PI3K activity, as well as Akt-473phos significantly in both age groups (Table 2). The basal/insulin-stimulated ratio increased with increasing distance from the insulin receptor (Fig. 2). Young participants had significantly higher basal and insulin-stimulated IRTK activity than old ones but lower insulin-stimulated Akt-473phos, whereas IRS-1-PI3K activity and basal Akt-473phos were similar. However, after adjustment for sex, $\dot{V}O_{2max}$ and total fat percentage, we found no independent effect of age on insulin-stimulated IRTK, IRS-1-PI3K or Akt activity, or Akt phosphorylation (Table 3).

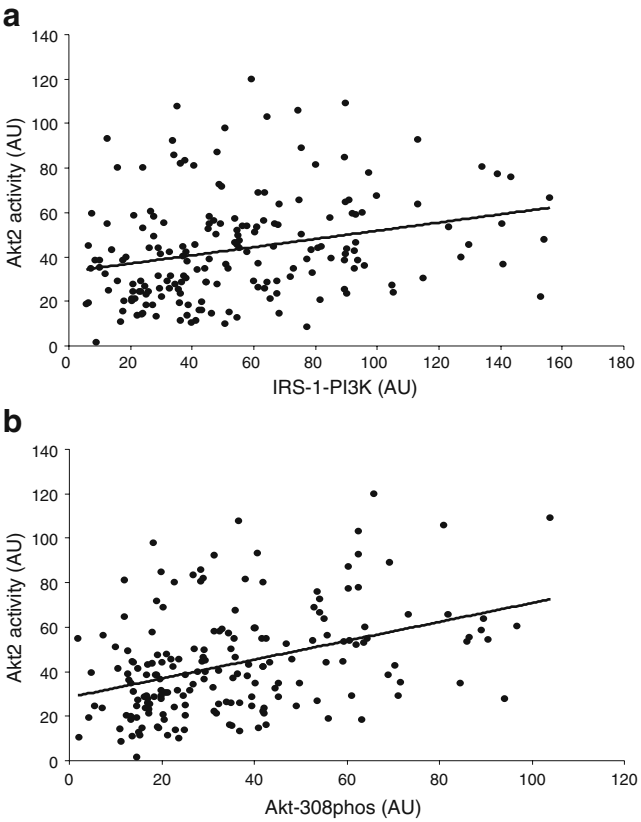

Fig. 1 Scatter plots of Akt2 activity as a function of (**a**) insulin-stimulated IRS-1-PI3K activity (r=0.31, p<0.001) and (**b**) Akt-308phos (r=0.39, p<0.001)

Explanatory variables of proximal insulin signalling Multiple regression analyses were performed with insulin-stimulated activity of IRTK, IRS-1-PI3K and Akt, and Akt phosphorylation (Table 3). IRTK and Akt1 activity and Akt-308phos were not associated with any of the explanatory variables including age, sex, zygosity, birthweight, $\dot{V}O_{2max}$ and total fat percentage. IRS-1-PI3K and Akt2 activity were negatively associated with $\dot{V}O_{2max}$. Akt-473phos was negatively associated with $\dot{V}O_{2max}$ (borderline significance) and Akt2 activity was negatively associated with total fat percentage (borderline significance).

Effect of intrauterine environment We found a positive association between IRS-1-PI3K activity and birthweight with a 1 SD increase of birthweight associated with 12% increased IRS-1-PI3K activity (Table 3). No impact of birthweight was seen on IRTK or Akt activity, or Akt phosphorylation.

Impact of inheritance High heritability estimates for insulin-stimulated IRTK and IRS-1-PI3K activity were seen in young but not in old twins (Table 4). In contrast, heritability estimates for any measure of Akt activity and phosphorylation in the young participants were low. In the old participants, heritability estimates for Akt-308phos, and

Table 2 Impact of insulin stimulation on proximal insulin signalling in the young and old twins

Variable	Basal		Insulin	
	Young	Old	Young	Old
IRTK activity	3.90±1.70	3.06±1.29[a]	4.82±2.18[b]	4.07±1.31[a,b]
IRS-1-PI3K activity	25.4±16.2	27.6±12.8	53.1±34.6[b]	57.3±33.1[b]
Akt-473phos	7.37±4.16	7.46±4.65	37.3±20.7[b]	45.7±24.4[a,b]

All activities are expressed in arbitrary units; data are means ± SD

As basal levels of Akt-308phos and Akt1 and Akt2 activity were below the detection limit of our assay, only values for IRTK and IRS-1-PI3K activity, and Akt-473phos were included. [a] $p<0.05$ vs young; [b] $p<0.05$ vs basal

Akt1 and Akt2 activity were low, whereas heritability for Akt-473phos was strong.

Relation to in vivo glucose metabolism When analysed by simple correlation analyses R_d clamp was not associated with IRTK (Fig. 3a), IRS-1-PI3K (Fig. 3b), Akt1 or Akt2 activity, or Akt-473phos. However, Akt-308phos was positively correlated with R_d clamp (Fig. 3c). Using multiple regression analyses and adjusting for the effects of age, sex, $\dot{V}O_{2max}$ and total fat percentage, R_d clamp was not significantly associated with IRTK, IRS-1-PI3K or Akt1 activity, or with Akt-473phos. However, R_d clamp was positively associated with insulin-stimulated Akt-308phos and Akt2 activity, with an increase of 1 SD in Akt-308phos or Akt2 activity associated with a 9% ($p<0.001$) or 5% ($p=0.05$) increase in R_d clamp, respectively.

Discussion

In this study of 184 twins, we found that whole-body in vivo insulin sensitivity was positively associated with insulin-stimulated levels of Akt-308phos and Akt2 activity, but not with IRTK, IRS-1-PI3K or Akt1 activity, or with Akt-473phos. We found that IRS-1-PI3K activity was positively associated with birthweight, and IRS-1-PI3K and Akt2 activity were negatively associated with aerobic capacity. However, proximal insulin signalling was not significantly associated with age, sex or total fat percentage. Additionally, we saw a minor genetic component of proximal insulin signalling, except for IRTK and IRS-1-PI3K activity in the young twins and Akt-473phos in the old twin group, which were under more profound genetic influence.

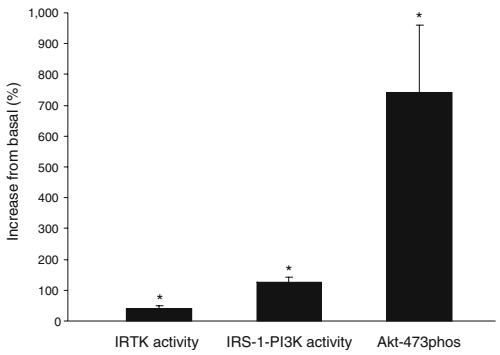

Fig. 2 Impact of insulin stimulation on three steps of proximal insulin signalling. The insulin-mediated mean fold increase in activity level (95% CI) is shown. Only fold changes for IRTK and IRS-1-PI3K activity, and Akt-473phos were calculated, because basal levels of Akt-308phos, and Akt1 and Akt2 activity were below the detection limit of our assay. $p<0.05$ for the difference between basal and insulin-stimulated states

This study was performed in a large twin population undergoing detailed clinical characterisation using gold-standard techniques including the euglycaemic–hyperinsulinaemic clamp and DXA scanning. No previous study of this magnitude has investigated signalling activities at three separate steps in proximal insulin signalling. Our population of predominantly non-diabetic participants covered a broad range of glucose disposal rates (4 to 20 mg glucose kg^{-1} min^{-1}) and BMI (17 to 40 kg/m^2), and included three participants with mild, previously unknown type 2 diabetes. This added to the heterogeneity of the population, but without altering the main conclusions of the study (the rationale for addition of these participants has been discussed previously [22]). Altogether, this setting allowed us to extensively evaluate the association between proximal insulin signalling and insulin sensitivity in a non-diabetic population of young and old participants. Furthermore, in line with previous studies investigating various aspects of glucose metabolism in this population [22, 24, 29, 33], the unique twin design of this study allowed us to estimate the relative importance of genetic vs environmental factors in the control of skeletal muscle insulin signalling.

The fold change of insulin-stimulated activity from basal increased with distance from the insulin receptor (Fig. 2) as previously demonstrated for IRTK to IRS-1-PI3K activity [11] and for IRS-1-PI3K activity to phosphorylation of Akt1 and Akt2 [34]. Of note, the fold changes demonstrated were of similar magnitudes to those in previous studies [11, 34, 35]. Our results are consistent with: (1) signal

Table 3 Regulation of insulin signalling activity

Response variable	Explanatory variable	Explanatory variable SD	Estimate	*p* value[a]
IRTK	–	–	–	–
IRS-1-PI3K activity	Birthweight	481 g	1.12	0.04
	$\dot{V}O_{2max}$	9.9 ml min^{-1} kg^{-1}	0.82	0.02
Akt-308phos	–	–	–	–
Akt-473phos	$\dot{V}O_{2max}$	9.9 ml min^{-1} kg^{-1}	0.88	0.07
Akt1 activity	–	–	–	–
Akt2 activity	Total fat percentage	8.6%	0.88	0.07
	$\dot{V}O_{2max}$	9.9 ml min^{-1} kg^{-1}	0.83	0.01

The mathematical model can be written as: ln(insulin-stimulated measure of activity) = age+sex+zygosity+birthweight+total fat percentage+$\dot{V}O_{2max}$

[a] Only significant (and borderline significant) effects are included in the table

amplification due to activation of several downstream enzymes by each upstream enzyme; or (2) convergence of different pathways. Our study suggests involvement of both mechanisms. Thus IRS-1-PI3K activity was positively correlated to Akt2 activity, indicating activation of Akt2 by IRS-1-PI3K, and only measures of Akt activity and phosphorylation were associated with glucose disposal, suggesting convergence of separate pathways at the level of Akt. The amplification of the signal might in part explain why only the distal parts of proximal insulin signalling could be clearly linked to whole-body insulin sensitivity. Only the fold change of Akt-473phos was calculated, because basal levels of Akt-308phos, and Akt1 and Akt2 activity were below the detection limit.

We demonstrated that IRS-1-PI3K activity was more strongly associated with Akt2 than with Akt1 activity, a finding in agreement with a study of human myoblasts [9].

Table 4 Heritability of proximal insulin signalling activity

Variable	r_{MZ}	r_{DZ}	h^2
Young			
IRTK	0.59	0.26	0.67
IRS-1-PI3K activity	0.59	0.24	0.70
Akt-308phos	0.22	0.49	[a]
Akt-473phos	0.36	0.27	0.18
Akt1 activity	0.18	0.66	[a]
Akt2 activity	0.36	0.60	[a]
Old			
IRTK	0.63	0.43	0.41
IRS-1-PI3K activity	0.42	0.66	[a]
Akt-308phos	0.45	0.50	[a]
Akt-473phos	0.80	0.00	~1.00
Akt1 activity	0.00	0.76	[a]
Akt2 activity	0.64	0.38	0.51

The heritability of insulin signalling activity is expressed as h^2 $\left(h^2 = 2[r_{MZ}^* - r_{DZ}^*]\right)$, where MZ is monozygotic and DZ is dizygotic, and * represents intra-class correlations within mono- or dizygotic twin groups

[a] Could not be calculated due to higher correlation among dizygotic than among monozygotic twins

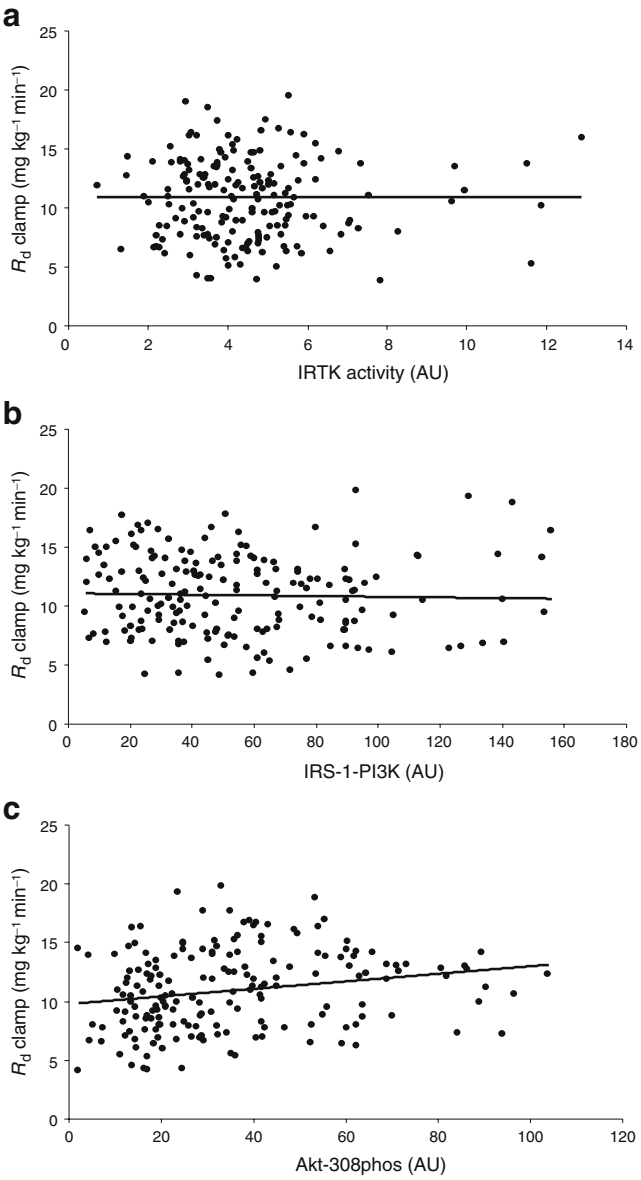

Fig. 3 Scatter plots of R_d clamp as a function of (**a**) insulin-stimulated IRTK activity (r=−0.02, p=0.81), (**b**) IRS-1-PI3K activity (r=−0.10, p=0.20) and (**c**) Akt-308phos (r=0.25, p<0.001)

Interestingly, the same study demonstrated that Akt1 was phosphorylated on threonine 308 in response to insulin, whereas Akt2 was phosphorylated on serine 473 and to some extent on threonine 308. This suggests that Akt1 activation involves Akt-308phos, whereas Akt2 activation primarily involves Akt-473phos and to some degree Akt-308phos. In contrast, we found no or only weak association between Akt1 activity and Akt-308phos or Akt-473phos, and although we also demonstrated an association between Akt2 activity and both Akt-308phos and Akt-473phos, Akt-308phos was the best predictor of Akt2 activity in our study. The discrepancy between the studies could largely be explained by the indirect evaluation of Akt activity in the former study (phospho-specific western blotting only) and by the transition from in vitro to in vivo studies.

After adjustment for confounding variables, no independent effects of sex or age were seen on proximal insulin signalling. This is surprising since increasing age (Table 1) and male sex were associated with lower insulin sensitivity in this and other studies [36, 37]. Thus, in agreement with recent studies of the effects of sex [38] and age [29] on insulin signalling, our data indicate that the effects are not mediated through altered proximal insulin signalling.

Based on the positive association between $\dot{V}O_{2max}$ and glucose metabolism seen in this and other studies [34, 39], a similarly positive association with insulin signalling was expected. However, contradictory to one [40] and in accordance with other studies [18, 34, 41], no positive effect of increased $\dot{V}O_{2max}$ was observed on proximal insulin signalling. This suggests that exercise-induced increases in glucose disposal are mainly mediated by other insulin-dependent mechanisms, e.g. activation of insulin signalling proteins downstream of Akt, including Akt substrate of 160 kDa, or improved haemodynamic effect of insulin [41], or alternatively by predominantly insulin-independent mechanisms, e.g. activation of the 5′AMP-activated protein kinase pathway [42]. Although paradoxical, the negative association between $\dot{V}O_{2max}$ and IRS-1-PI3K activity is in line with other studies [18, 41]. This, along with the negative association between $\dot{V}O_{2max}$ and Akt2 activity, suggests a more complex association between physical activity and insulin signalling than previously thought. In contrast to some previous studies [11, 12] but in line with others [43], no effect of obesity as such was found on IRTK or IRS-1-PI3K activity. Possible explanations of this discrepancy include problems with the matching of lean vs obese participants in previous studies. In the present study, adjustments for many confounding factors were performed in an attempt to reduce such problems. We found a borderline significant negative association between total fat percentage and Akt2 activity, which in part could account for the negative association between obesity and insulin sensitivity seen in this study.

We have previously demonstrated an association between low birthweight and reduced muscle insulin signalling, including reduced protein levels of PI3K and protein kinase Cζ [16], and reduced insulin-stimulated Akt-473phos [17]. As Akt and protein kinase Cζ are both involved in glucose uptake [44], these results indicated a mechanistic link between an adverse intrauterine environment and skeletal muscle insulin resistance. This is partly supported by the present results, which demonstrate a positive association between birthweight and IRS-1-PI3K activity. Interestingly, we found no effect of birthweight downstream of IRS-1-PI3K, indicating a limited effect on Akt-dependent insulin signalling. Our results thus suggest that the effects of an adverse intrauterine environment are primarily mediated through Akt-independent pathways, possibly involving the protein kinase Cζ pathway, as our previous studies suggest [16]. High birthweight (>4 kg) has also been associated with type 2 diabetes [45] and might thus also be associated with decreased insulin signalling. However, all participants in the current study population had birthweights below 4 kg. Future studies including participants with high birthweight are needed to further investigate the association between high birthweight and insulin signalling.

To our knowledge, no previous twin study has addressed the heritability of proximal insulin signalling. For IRTK and IRS-1-PI3K activity, a stronger genetic impact was seen among young than among old participants, suggesting that with increasing age the environmental influence exerts a larger effect. Conversely, Akt-473phos was largely genetically controlled in the old, but not in the young twins, suggesting that ageing might be essential for penetrance of the heritable effects on Akt-473phos. Altogether, the activity levels of only a few proteins displayed high heritability coefficients (Table 4); proximal insulin signalling taken together thus appears to have a minor genetic component. Insulin sensitivity was related to Akt2 activity and Akt-308phos, and since the genetic impact on these was low, the previously shown genetic component in glycogen synthesis [29] does not seem to be mediated by modulation of Akt-dependent insulin signalling. A recent study including the present twin population demonstrated significant effects of a frequently occurring single nucleotide polymorphism on IRS-1 protein levels and IRS-1-PI3K activity [46]. However, using heritability estimates in this study, we were unable to document a genetic impact on IRS-1 protein levels (data not shown) or on IRS-1-PI3K activity in the old participants. Most likely, this is because heritability estimates only represent crude measures of heritability, allowing only the detection of major genetic components of a given phenotypic trait [47].

In contrast to some [11, 12, 14, 48] and in line with other studies [15, 30, 43], we saw no association between

IRTK as well as IRS-1-PI3K activity and glucose disposal (Fig. 3a, b). This discrepancy might be explained by (1) difficulties with the matching of insulin-sensitive vs insulin-resistant participants in previous studies and (2) by the fact that some studies included type 2 diabetic participants [14, 48] compared with our predominantly non-diabetic participants. Additionally, our results could be explained by attenuation of IRTK and IRS-1-PI3K activity after 2 h of insulin stimulation. However, this is unlikely, since we and others have demonstrated that IRTK, IRS-1-PI3K and Akt activity was not attenuated after 2 to 3 h of insulin stimulation in humans [30, 41, 43]. Finally, variability of the IRTK assay (see Methods) might have contributed to the lack of association between IRTK activity and glucose disposal. Nonetheless, the large number of participants included in this study would have enabled us to detect any major association. Although IRTK and IRS-1-PI3K activity was not significantly associated with whole-body glucose disposal, a local effect cannot be excluded, since the present clamp technique evaluated whole-body insulin sensitivity and not glucose disposal in distinct muscles. Accordingly, we previously demonstrated that young males with low compared with normal birthweight had normal whole-body in vivo insulin sensitivity, but reduced local insulin sensitivity in the forearm [49]. In contrast, Akt-308phos and Akt2 activity were positively associated with whole-body glucose disposal. Generally, this is in accordance with previous studies demonstrating that Akt2 is the most important Akt isoform in skeletal muscle glucose metabolism [9, 50, 51]. However, in contrast to several studies [9, 15, 52], we found no association between Akt-473phos and glucose metabolism, which in turn is in agreement with studies demonstrating that only Akt-308phos was significantly decreased in insulin-resistant participants or type 2 diabetic patients [5, 13]. It is also in line with our results demonstrating that Akt2 activity is more tightly associated with Akt-308phos than Akt-473phos. Altogether, our results demonstrate an association between reduced proximal insulin signalling and insulin resistance in a non-diabetic population. Due to the cross-sectional nature of the study and the inclusion of only very few diabetic participants, this study could not directly address the role of proximal insulin signalling in the pathogenesis of type 2 diabetes. Nonetheless, it seems plausible that the reduced insulin sensitivity associated with decreased Akt signalling could contribute to the development of overt insulin resistance and ultimately type 2 diabetes. Studies of a larger diabetic population are needed to further investigate the role of reduced proximal insulin signalling in the pathogenesis of insulin resistance and type 2 diabetes.

In conclusion, our results suggest that the ‘classical’ modifiers of insulin action, including genes, age, sex, obesity and aerobic capacity, mediate their most central effects on insulin sensitivity independently of modifications of proximal insulin signalling in skeletal muscle. In line with previous findings, the present study indicates that the intrauterine environment may influence PI3K-dependent insulin signalling involving Akt-independent pathways. Finally, we demonstrated an association between reduced insulin sensitivity and reduced skeletal muscle proximal insulin signalling at the level of Akt, thus demonstrating an early defect in insulin signalling that might later contribute to the development of type 2 diabetes. Future studies will investigate the molecular mechanisms linking reduced Akt activity and reduced insulin sensitivity in non-diabetic participants.

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Duality of interest B. F. Hansen is employed at Novo Nordisk. The remaining authors declare that there is no duality of interest associated with this manuscript.

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