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ARTICLE

Partial rescue of in vivo insulin signalling in skeletal muscle by impaired insulin clearance in heterozygous carriers of a mutation in the insulin receptor gene

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Abstract
Aims/hypothesis Recently we reported the coexistence of postprandial hypoglycaemia and moderate insulin resistance in heterozygous carriers of the Arg1174Gln mutation in the insulin receptor gene (*INSR*). Controlled studies of in vivo insulin signalling in humans with mutant *INSR* are unavailable, and therefore the cellular mechanisms underlying insulin resistance in Arg1174Gln carriers remain to be clarified.
Subjects, materials and methods We studied glucose metabolism and insulin signalling in skeletal muscle from six Arg1174Gln carriers and matched control subjects during a euglycaemic–hyperinsulinaemic clamp.
Results Impaired clearance of exogenous insulin caused four-fold higher clamp insulin levels in Arg1174Gln carriers compared with control subjects ($p<0.05$). In Arg1174Gln carriers insulin increased glucose disposal and non-oxidative glucose metabolism ($p<0.05$), but to a lower extent than in controls ($p<0.05$). Insulin increased Akt phosphorylation at Ser473 and Thr308, inhibited glycogen synthase kinase-3α activity, reduced phosphorylation of glycogen synthase at sites 3a+3b, and increased glycogen synthase activity in Arg1174Gln carriers (all $p<0.05$). In the insulin-stimulated state, Akt phosphorylation at Thr308 and glycogen synthase activity were reduced in Arg1174Gln carriers compared with controls ($p<0.05$), whereas glycogen synthase kinase-3α activity and phosphorylation of glycogen synthase at sites 3a+3b were similar in the two groups.
Conclusions/interpretation In vivo insulin signalling in skeletal muscle of patients harbouring the Arg1174Gln mutation is surprisingly intact, with modest impairments in insulin-stimulated activity of Akt and glycogen synthase explaining the moderate degree of insulin resistance. Our data suggest that impaired insulin clearance in part rescues in vivo insulin signalling in muscle in these carriers of a mutant *INSR*, probably by increasing insulin action on the non-mutated insulin receptors.

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Keywords Glucose metabolism · Glycogen synthase · Insulin clearance · Insulin receptor mutation · Insulin signalling

Abbreviations

CHO	Chinese hamster ovary
G6P	glucose-6-phosphate
GDR	glucose disposal rate
GSK-3	glycogen synthase kinase-3

HRP	horseradish peroxidase
HSP90	heat shock protein 90
mt	mutant
INSR	insulin receptor gene
IRS	insulin receptor substrate
IRTK	insulin receptor tyrosine kinase
NOGM	non-oxidative glucose metabolism
PI3K	phosphoinositide 3-kinase
SLC2A4	solute carrier family 2 (facilitated glucose transporter), member 4 (previously known as GLUT4)
TBST	Tris-buffered saline with Tween 20
wt	wild-type

Introduction

Mutations in the human insulin receptor gene (*INSR*) have been discovered almost exclusively by sequencing this gene in patients with syndromes of severe insulin resistance [1]. Studies of these naturally occurring mutations of *INSR* using cell lines have provided valuable insights into the relationship between the structure and function of the receptor and have contributed to the understanding of the molecular mechanism involved in insulin signalling and insulin resistance [1, 2]. Mutations in the insulin receptor tyrosine kinase (IRTK) domain are characterised by decreased IRTK activity despite normal binding and affinity of insulin for the receptor [1, 2]. These mutations appear to cause insulin resistance in a dominant fashion, unlike mutations in other domains of the *INSR*. The dominant negative effect may result from the heterotetrameric ($\alpha_2\beta_2$) structure of the insulin receptor. Thus, of the three forms of heterotetramer formed in patients heterozygous for a single mutant allele, only insulin receptors formed by the two wild-type (wt) alleles—the wt/wt receptors—are expected to be fully functional [1, 2].

We have recently reported a novel syndrome of autosomal dominant hyperinsulinaemic hypoglycaemia in three generations of a family [3]. All ten family members affected by hypoglycaemia carried a heterozygous mutation in the IRTK domain of *INSR* (Arg1174Gln). This mutation has previously been found in three females with the type A syndrome of insulin resistance, and in an apparently healthy male [4–7]. Complete cosegregation (logarithm of the odds score 3.21) with the disease phenotype supported the proposition that the Arg1174Gln mutation was the cause of hypoglycaemia [3]. In addition to hypoglycaemia in the postprandial state, the Arg1174Gln carriers were physiologically characterised by moderate insulin resistance and impaired clearance of insulin. However, none of these subjects had type 2 diabetes [3]. This suggests preserved ability to compensate for insulin resistance.

Insulin stimulation of glycogen synthesis is believed to involve insulin receptor autophosphorylation, activation of insulin receptor substrates (IRSs), phosphoinositide 3-kinase (PI3K) and protein kinase Akt, which in turn leads to inhibition of glycogen synthase kinase-3 (GSK-3), and hence activation of glycogen synthase by decreasing phosphorylation at regulatory sites, of which the COOH-terminal residues Ser640 (site 3a) and Ser644 (site 3b) are probably the most important [8, 9]. In patients with type 2 diabetes, failure of insulin to activate glycogen synthase is a hallmark feature of skeletal muscle insulin resistance [10–15]. However, despite reports of impaired activation of PI3K by insulin [14–16], most studies have failed to demonstrate impaired insulin signalling downstream of PI3K, including activation of Akt, inhibition of GSK-3 and dephosphorylation of glycogen synthase at sites 3a+3b in muscle from subjects with type 2 diabetes both in vivo and in vitro [10, 15, 17, 18]. Recently, impaired activation of glycogen synthase in type 2 diabetes was linked to increased phosphorylation at NH_2-terminal residues Ser7 (site 2) and Ser10 (site 2a), suggesting that other pathways downstream of the insulin receptor could be involved [10]. In cell lines transfected with the Arg1174Gln mutation and thus expressing the mutant *INSR* in a homozygous state, insulin in physiological concentrations (1,000 pmol/l) showed impaired ability to increase tyrosine phosphorylation of IRS-1, PI3K activity, solute carrier family 2 (facilitated glucose transporter), member 4 (SLC2A4, previously known as GLUT4) translocation and glycogen synthesis [7, 19]. However, controlled studies of the effects of the Arg1174Gln mutation or other mutations in *INSR* on downstream insulin signalling in skeletal muscle in humans in vivo are unavailable.

The present study was undertaken to elucidate the molecular mechanisms underlying insulin resistance in heterozygous carriers of the Arg1174Gln mutant *INSR*. Such carriers are an in vivo model of moderate insulin resistance apparently without type 2 diabetes, and we investigated the effects of insulin on glucose metabolism, downstream insulin signalling and phosphorylation of glycogen synthase in their skeletal muscle, compared with matched healthy control subjects.

Subjects, materials and methods

Study subjects

Clinical and genetic data have been reported previously [3]. Six family members carrying the Arg1174Gln mutation and six healthy control subjects, matched for age, sex and BMI, participated in the study (Table 1). The Arg1174Gln carriers all suffered from episodes of hypoglycaemia in the

Table 1 Clinical and biochemical characteristics

	Control group	Arg1174Gln group	*p* value
Male/female	5/1	5/1	ns
Age (years)	44.0±2.3	41.7±5.6	ns
BMI (kg/m^2)	24.5±0.8	25.4±1.0	ns
HbA_{1c} (%)	4.8±0.1	5.7±0.2	<0.01
Total cholesterol (mmol/l)	5.2±0.3	5.7±0.4	ns
Plasma triglycerides (mmol/l)	1.2±0.4	1.3±0.2	ns
Basal plasma glucose (mmol/l)	5.3±0.1	5.6±0.1	ns
Clamp plasma glucose (mmol/l)	5.1±0.1	5.3±0.1	ns
Basal serum insulin (pmol/l)	18±2	177±31	<0.01
Clamp serum insulin (pmol/l)	328±17	1313±127	<0.01
Basal serum C-peptide (pmol/l)	403±46	553±64	ns
Clamp serum C-peptide (pmol/l)	465±83	527±101	ns
Insulin clearance (ml/min per m^2)	885±132	192±45	<0.01

Data are mean±SEM
ns Not significant

postprandial state and all were without diabetes and showed no clinical features of severe insulin resistance. The control subjects had normal glucose tolerance and no family history of diabetes. All subjects had normal results of screening blood tests of hepatic and renal function. All subjects were instructed to refrain from strenuous physical activity for 48 h before the experiment. Informed consent was obtained from all subjects before participation. The study was approved by the Local Ethics Committee and performed in accordance with the Declaration of Helsinki-II.

Study design

All study subjects were admitted to the Diabetes Research Centre at Odense University Hospital, Denmark. After a 10-h overnight fast, the subjects underwent a euglycaemic–hyperinsulinaemic clamp (2-h equilibrium period followed by 3 h of insulin infusion, 40 mU/m^2 per min) combined with indirect calorimetry and muscle biopsies. Refer to our previous report [3] for further details about calculation of glucose disposal rate (GDR), glucose oxidation, non-oxidative glucose metabolism (NOGM) and insulin clearance. Plasma glucose, serum insulin, C-peptide and NEFA were measured as described previously [20]. Muscle biopsies were obtained from the vastus lateralis muscle before and after the insulin infusion period using a modified Bergström needle with suction under local anaesthesia (10 ml lidocaine 2%). Muscle samples were immediately blotted free of blood, fat and connective tissue and frozen in liquid nitrogen within 20–30 s.

Muscle lysate

Muscle tissue was freeze-dried and dissected free of visual blood, fat and connective tissues. Muscle lysate was prepared by homogenisation of muscle tissue (1:80, weight:volume) in a buffer containing 50 mmol/l HEPES (pH 7.5), 150 mmol/l NaCl, 20 mmol/l Na-pyrophosphate, 20 mmol/l β-glycerophosphate, 10 mmol/l NaF, 2 mmol/l Na-orthovanadate, 2 mmol/l EDTA, 1% Nonidet P-40, 10% glycerol, 2 mmol/l phenylmethylsulfonyl fluoride, 1 mmol/l $MgCl_2$, 1 mmol/l $CaCl_2$, 10 μg/ml leupeptin, 10 μg/ml aprotinin and 3 mmol/l benzamidine. Homogenates were rotated end over end for 1 h at 4°C, and then cleared by centrifugation at 17,500 *g* at 4°C for 1 h. Protein content in the supernatant was measured by the bicinchoninic acid method (Pierce Chemical Company, Rockford, IL, USA).

Protein content and protein phosphorylation

Muscle lysate proteins were boiled in Laemmli buffer, separated using 7.5% or 10% Bis-Tris gels (Invitrogen, Taastrup, Denmark) (HCl-Tris gels; Biorad, Copenhagen, Denmark), and transferred (semidry) to PVDF membranes (Immobilon Transfer Membrane; Millipore, Glostrup, Denmark). After blocking (Tris-buffered saline with Tween 20 [TBST]+1% skimmed milk overnight at 4°C), the membranes were incubated with primary antibodies (TBST+1% skimmed milk, 2 h at room temperature) followed by incubation in horseradish peroxidase (HRP)-conjugated secondary antibody (TBST+1% skimmed milk, 1 h at room temperature). Following detection (ECl+; Amersham Biosciences, Hillerød, Denmark) and quantification using a CCD image sensor and 1D software (Kodak Image Station, E440CF; Kodak, Glostrup, Denmark), the protein content was expressed in arbitrary units relative to a human skeletal muscle standard. The primary antibodies used were anti-IRS-1 (no. 06-248), anti-p85 (no. 06-497) and anti-Akt-1/PKB (no. 06-558) (Upstate Biotechnology, Lake Placid, MA, USA). The rabbit anti-glycogen synthase antibody

[21] was provided by O. Pedersen (Steno Diabetes Center, Gentofte, Denmark). The insulin receptor monoclonal antibody CT3 was raised against the COOH-terminal of the β-subunit and was a gift from K. Siddle (Cambridge University, Cambridge, UK). The phospho-specific antibodies used were anti-pAkt Ser473 (New England Biolabs, USA, no. 9271) and anti-pAkt Thr308 (06-801; Upstate Biotechnology). The sheep antibodies to anti-pGS site 2, anti-pGS site 3a+3b and anti-pGS site 2+2a were generated as described previously [10]. The secondary antibodies used were goat anti-rabbit HRP (P0448), goat-anti-mouse HRP (P0447) and rabbit-anti-sheep HRP (P0163) (Dako, Glostrup, Denmark).

IRTK and PI3K activity

IRTK activity was measured in muscle lysate after immunopurification of insulin receptor (anti-IR) (AB3; Oncogene Science, Cambridge, MA, USA) using a microtitre assay as described previously [22]. IRS-1-associated PI3K activity was measured in muscle lysate after immunopurification of IRS-1 (anti-IRS-1) (catalogue no. 06-248; Upstate Biotechnology) as described previously [10].

Akt1 and Akt2 activity

Sequential immunopurification of first Akt2 (anti-Akt2) (catalogue no. 06-606; Upstate Biotechnology) and then Akt1 (anti-Akt1) (catalogue no. 06-558; Upstate Biotechnology) was performed using 400 μg of muscle lysate protein before measuring Akt kinase activity. The prior depletion of Akt2 protein was necessary to avoid co-immunopurification of both Akt1 and Akt 2 using the anti-Akt1 from Upstate Biotechnology. The immunopurification (overnight at 4°C) was performed using G-Sepharose beads suspended in lysate buffer. The pellet was washed three times in a buffer containing 20 mmol/l Tris (pH 7.4), 5 mmol/l EDTA, 10 mmol/l $Na_4P_2O_7$, 100 mmol/l NaF, 1% NP-40 and 3 mmol/l Na_3VO_4 and twice in a buffer containing 20 mmol/l Tris (pH 7.4), 10 mmol/l $MgCl_2$ and 1 mmol/l dithiothreitol. The kinase activity assay was run in a buffer containing 50 mmol/l Tris (pH 7.4), 10 mmol/l $MgCl_2$, 1 mmol/l dithiothreitol and 1 μmol/l protein kinase inhibitor (catalogue no. P-0300; Sigma-Aldrich, St Louis, MO, USA) using 30 μmol/l Akt/SGK substrate peptide (catalogue no. 12-340; Upstate Biotechnology) and (111 kBq per sample) ^{32}P γ-ATP (Perkin Elmer, Boston, MA, USA) as substrates. The kinase assay was run for 30 min at 30°C before the reaction was stopped by addition of a buffer containing 0.6% HCl, 1 mmol/l ATP and 1% BSA. Aliquots of reaction mixture were spotted on p81 filter paper, washed in 75 mmol/l phosphoric acid and dried using acetone before determination of ^{32}P content by liquid scintillation counting.

GSK-3α and GSK-3β activity

GSK-3α was immunoprecipitated from muscle lysate using an anti-GSK-3α antibody (Upstate Biotechnology) bound to protein G-Sepharose. The supernatant from this immunoprecipitation was then added to new protein G-Sepharose and anti-GSK-3β antibody (Transduction Laboratories, BD Biosciences, San Jose, CA, USA). Kinase activity was measured in both immunoprecipitates in a buffer containing 8 mmol/l MOPS (pH 7.0), 10 nmol/l microcystin, 200 μmol/l EDTA, 0.5 mmol/l Na_3VO_4, 10 mmol/l Mg acetate and 125 μmol/l ATP using 20 μmol/l phospho-GS2-peptide (YRRAAV PPSPSLSRHSSPHQSpEDEEE [Sp=phosphoserine]; Schafer-N, Copenhagen, Denmark) as substrate and (55.5 kBq per sample) ^{32}P γ-ATP (Perkin Elmer). The kinase assay was run for 60 min at 30°C before the reaction was stopped by spotting 30 μl onto p81 filter paper. The preparation was washed in 75 mmol/l phosphoric acid and dried with acetone before determination of ^{32}P content by liquid scintillation counting.

Glycogen synthase activity

Activity was measured in homogenates by a method described previously [23]. Glycogen synthase activity was determined in the presence of 0.02 and 8 mmol/l glucose-6-phosphate (G6P) and expressed either as the percentage of G6P-independent glycogen synthase activity (I-form activity) (100 times the activity in the presence of 0.02 mmol/l G6P divided by the activity at 8 mmol/l G6P [saturated]) or as total glycogen synthase activity.

SLC2A4 protein in total crude membranes

Freeze-dried muscle was homogenised (1:110, weight: volume) in a buffer containing 250 mmol/l sucrose, 30 mmol/l HEPES, 2 mmol/l EGTA, 40 mmol/l NaCl, 2 mmol/l phenylmethylsulfonyl fluoride and pH 7.4 using a Polytron PT 3100 (Kinematica, Littau-Luzern, Switzerland) and spun at 200,000 *g* for 60 min at 4°C using a TL-100 ultracentrifuge (Beckman Coulter, Fullerton, CA, USA). The resulting pellet (corresponding to the membrane fraction) was resuspended by short-term sonication (50 mmol/l Tris, 4% SDS, 1 mmol/l EDTA, pH 7.4) and cleared by centrifugation at 4,000 *g* for 1 min. SLC2A4 protein was determined by western blotting as described above (blocking: TBST+2% skim milk overnight at 4°C; primary antibody: anti-GLUT4 antibodies [no. AB1346; Chemicon, Temecula, CA, USA] in TBST+2% skim milk for 2 h at room temperature; HRP-conjugated secondary antibody: goat anti-rabbit HRP [P0448] in 2% skim milk for 1 h at room temperature).

Statistical analysis

Calculations and statistical analysis were performed using SSSP for Windows version 10.0. Data are presented as mean± SEM. Differences between and within groups were evaluated using the Mann–Whitney *U*-test for unpaired data and the Wilcoxon signed ranks test for paired data, respectively (two-sided). Significance was accepted at the $p<0.05$ level.

Results

Euglycaemic–hyperinsulinaemic clamp

Family members carrying the Arg1174Gln mutation (Arg1174Gln group) had higher HbA_{1c} levels than the controls, but all values were within the normal range (Table 1). Data obtained from the euglycaemic–hyperinsulinaemic clamp studies have been reported previously [3]. Basal levels of plasma glucose and serum C-peptide were similar, and C-peptide was unaffected by insulin infusion in both groups (Table 1). Basal serum insulin levels were higher in the Arg1174Gln group than in the controls. Insulin infusion at a rate of 40 mU/m^2 per min raised serum insulin to 4-fold higher levels in the Arg1174Gln group compared with control subjects. This was caused by markedly reduced clearance of insulin in the Arg1174Gln group.

Basal GDR, glucose oxidation and NOGM were similar in the two groups. In both groups, GDR, glucose oxidation and NOGM increased in response to insulin infusion (Table 2). However, during the insulin-stimulated state GDR and NOGM in the Arg1174Gln group were lower than in the control group. The lower GDR was almost exclusively (87% of the reduction) accounted for by impaired insulin stimulation of NOGM.

Proximal insulin signalling

In the total population ($n=12$), insulin infusion caused an increase in both IRTK (7.02±0.86 vs 9.63±0.73 arbitrary units; $p=0.04$) and IRS-1-associated PI3K activity (72.0± 11.4 vs 107.5±16.5 arbitrary units; $p=0.05$). Insulin infusion caused similar increases in IRTK activity in the control (42%) and Arg1174Gln (33%) groups, but in neither of the groups did this achieve statistical significance (Fig. 1a). In control subjects, insulin infusion increased IRS-1-associated PI3K activity, whereas in the Arg1174Gln group no significant response to insulin was observed ($p=0.46$) (Fig. 1b). However, there was no difference between the groups in IRS-1-associated PI3K activity in either the basal or the insulin-stimulated state.

In the control subjects, insulin infusion increased phosphorylation of Akt at Thr308 and Ser473 as well as Akt1 and Akt2 activity (Fig. 1c–f). In the Arg1174Gln group, insulin infusion also increased phosphorylation of Akt at Ser473 and Thr308, whereas Akt1 ($p=0.08$) and Akt2 ($p=0.08$) tended to be increased by insulin. However, insulin-stimulated Thr308 phosphorylation and Akt2 activity were reduced in the Arg1174Gln group compared with the control group. Insulin-stimulated Akt1 activity also tended to be reduced in the Arg1174Gln group ($p=0.09$), whereas no difference in Ser473 phosphorylation was observed ($p=0.59$).

Downstream insulin signalling

In the total population ($n=12$), insulin infusion inhibited GSK-3α activity (0.111±0.013 vs 0.071±0.007 pmol min^{-1} mg^{-1}; $p=0.01$) and tended to reduce GSK-3β activity (0.760±0.039 vs 0.654±0.042 pmol min^{-1} mg^{-1}; $p=0.07$). No differences in either GSK-3α or GSK-3β activity were observed between the groups (Fig. 2a,b). In the Arg1174Gln group, insulin infusion caused a reduction in GSK-3α activity, whereas in the control group insulin

Table 2 Euglycaemic–hyperinsulinaemic clamp data

	Control group	Arg1174Gln group	*p* value
GDR in basal condition	77±4	76±3	ns
GDR during clamp	346±33[a]	200±34[a]	0.03
Glucose oxidation in basal condition	45±6	48±6	ns
Glucose oxidation during clamp	117±5[a]	100±14[a]	ns
NOGM in basal condition	32±5	27±5	ns
NOGM during clamp	229±28[a]	100±24[a]	0.02

Metabolic rates are expressed as milligram per milligram per minute squared (mg mg^{-1} min^{-2})
Data are mean±SEM
[a] $p<0.05$ vs basal
GDR Glucose disposal rate, *NOGM* non-oxidative glucose metabolism, *ns* not significant

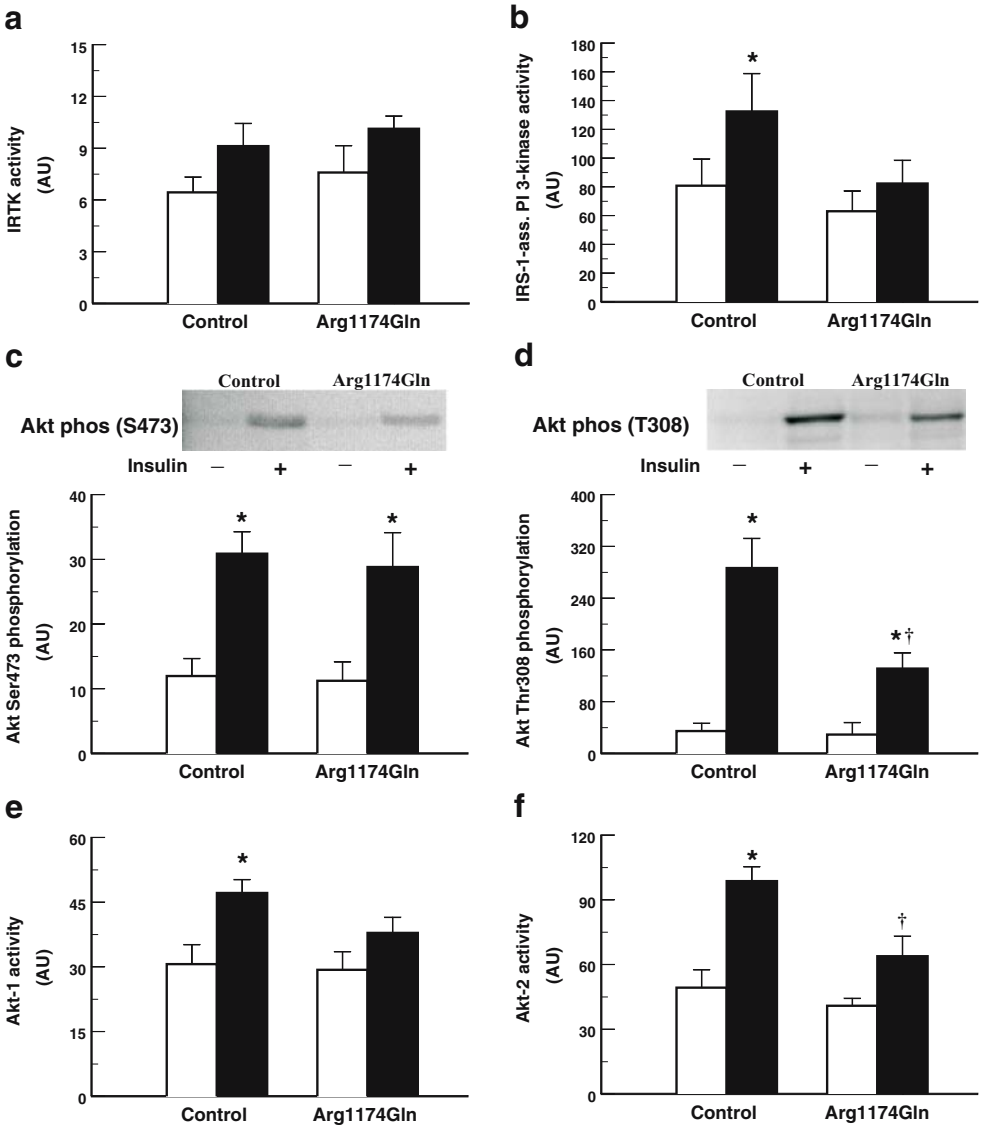

Fig. 1 Effect of insulin on insulin receptor tyrosine kinase activity (IRTK) (**a**), IRS-1-associated phosphoinositide 3-kinase (PI 3-kinase) activity (**b**), phosphorylation of Akt at Ser473 (**c**) and Thr308 (**d**), and Akt-1 (**e**) and Akt-2 (**f**) activities in skeletal muscle of six family members carrying the Arg1174Gln mutation in *INSR* and six control subjects. Representative immunoblots (**c, d**) for a control subject and an Arg1174Gln carrier are also shown. Measurements were performed in muscle biopsies obtained from m. vastus lateralis during the basal (*open bars*) and insulin-stimulated (*filled bars*) steady-state periods of a 3-h euglycaemic–hyperinsulinaemic clamp at an insulin infusion rate of 40 mU/m^2 per min. Data are mean±SEM. *p<0.05 for clamp vs basal values; †p<0.05 vs control subjects. *AU* Arbitrary units

tended to decrease GSK-3α activity (p=0.11). Insulin infusion had no significant effect on GSK-3β activity in either of the groups.

Total glycogen synthase activity was similar in the two groups in the basal and the insulin-stimulated state (Fig. 2c). Insulin increased the I-form activity of glycogen synthase in both groups, but insulin-stimulated I-form activity of glycogen synthase was lower in the Arg1174Gln group compared with the control group (Fig. 2d).

Phosphorylation of glycogen synthase at regulatory sites

Insulin infusion reduced phosphorylation of glycogen synthase at sites 3a+3b by ~50% in both groups (Fig. 3a), and there were no significant differences in phosphorylation at sites 3a+3b under basal or hyperinsulinaemic conditions. The phosphorylation of glycogen synthase either at site 2 alone or at sites 2+2a was unaffected by insulin infusion in the total population as well as in both the individual groups, and no differences between the groups were observed in the basal or the insulin-stimulated state (Fig. 3b,c).

Protein expression

The average insulin receptor protein content tended to be lower (by 36%) in the Arg1174Gln group compared with control subjects (p=0.09). We found no differences in the protein content of IRS-1, p85, Akt, GSK-3α, GSK-3β, glycogen synthase or SLC2A4 protein between the groups (Fig. 4).

Discussion

The signalling properties of the naturally occurring Arg1174Gln mutation in *INSR* have been studied previous-

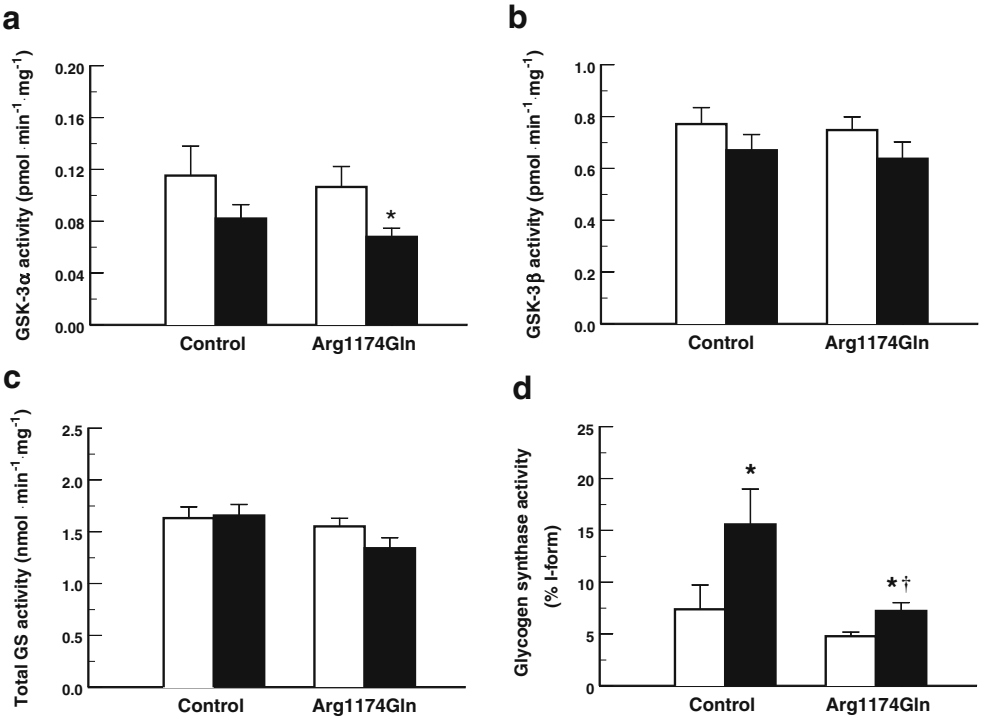

Fig. 2 Effect of insulin on activity of glycogen synthase kinase-3α (GSK-3α) (**a**), GSK-3β activity (**b**), total glycogen synthase (GS) (**c**), and glycogen synthase as percentage of I-form activity (**d**) in skeletal muscle of six family members carrying the Arg1174Gln mutation in *INSR* and skeletal muscle of six control subjects. Measurements were performed in muscle biopsies obtained from m. vastus lateralis during the basal (*open bars*) and insulin-stimulated (*filled bars*) steady-state periods of a 3-h euglycaemic–hyperinsulinaemic clamp at an insulin infusion rate of 40 mU/m^2 per min. Data are mean±SEM. *p<0.05 for clamp vs basal values; †p<0.05 vs control subjects

ly in Chinese hamster ovary (CHO) cells transfected with mutant *INSR* [7, 19] and in patient lymphocytes transformed by Epstein–Barr virus [5]. In transfected cells, only receptors formed by two mutant (mt) alleles—mt/mt receptors—are synthesised, and the mutation is present in a homozygous state, which facilitates the interpretation of receptor function studies. In contrast, patient cells transformed by Epstein–Barr virus provide the possibility of studying the mutation in a heterozygous state similar to the situation in vivo, in which all three possible receptor species—wt/wt, wt/mt hybrid and mt/mt receptors—are formed in an expected ratio of 1:2:1 [1, 5]. In the present case–control study, we had the opportunity to investigate the functional consequences of a heterozygous mutation in *INSR* in skeletal muscle in vivo. We report functional impairment but also partial preservation of insulin action on insulin signalling in six family members harbouring the naturally occurring Arg1174Gln mutation of *INSR*.

Studies of the functional properties of the Arg1174Gln mutation have shown normal insulin binding and affinity to the receptor, but a 70–75% reduction in insulin-stimulated IRTK activity [5–7]. Furthermore, in CHO cell lines homozygous for the mutant *INSR*, stimulation with insulin in the physiological range (1,000 pmol/l) showed a pronounced inability to increase tyrosine phosphorylation of IRS-1, PI3K activity, SLC2A4 translocation and glycogen synthesis [7, 19]. This suggests that homozygosity for the Arg1174Gln mutation in vivo would cause either extreme insulin resistance or be incompatible with life. In contrast, we report here that the presence of this mutant *INSR* in its heterozygous form in vivo is associated with a surprisingly intact response to insulin administered in physiological concentrations. Although the ability of insulin to stimulate IRTK and PI3K activity was low and inconclusive in both groups, we found no significant differences in the activities of these kinases in the insulin-stimulated state, and pooling of data from the Arg1174Gln carriers and controls in fact rendered the response to insulin significant for both kinases. More convincingly, we show that infusion of insulin at physiological rates in Arg1174Gln carriers was enough to increase Akt phosphorylation and activity, inhibit GSK-3α activity, reduce phosphorylation of glycogen synthase at sites 3a+3b, and stimulate G6P-independent glycogen synthase activity in vivo. Thus, in Arg1174Gln carriers the four-fold higher clamp insulin levels apparently compensate for the pronounced signalling defects found in vitro. This partial rescue of the action of insulin on glucose storage and in vivo insulin signalling by impaired insulin clearance is likely to involve an effect of higher insulin levels on the fraction of fully functional (wt/wt) insulin receptors. Additionally, insulin levels of ~1,300 pmol/l may be enough to overcome the ~10-fold lower affinity of insulin for IGF-I and insulin/IGF-I hybrid receptors and evoke signalling through these receptors [24]. Reports of an increased number of insulin/IGF-I hybrid receptors in muscle of patients with chronic hyperinsulinaemia [25, 26] provide further support for a potential compensatory action of insulin through the IGF-I receptors in patients with mutant *INSR*.

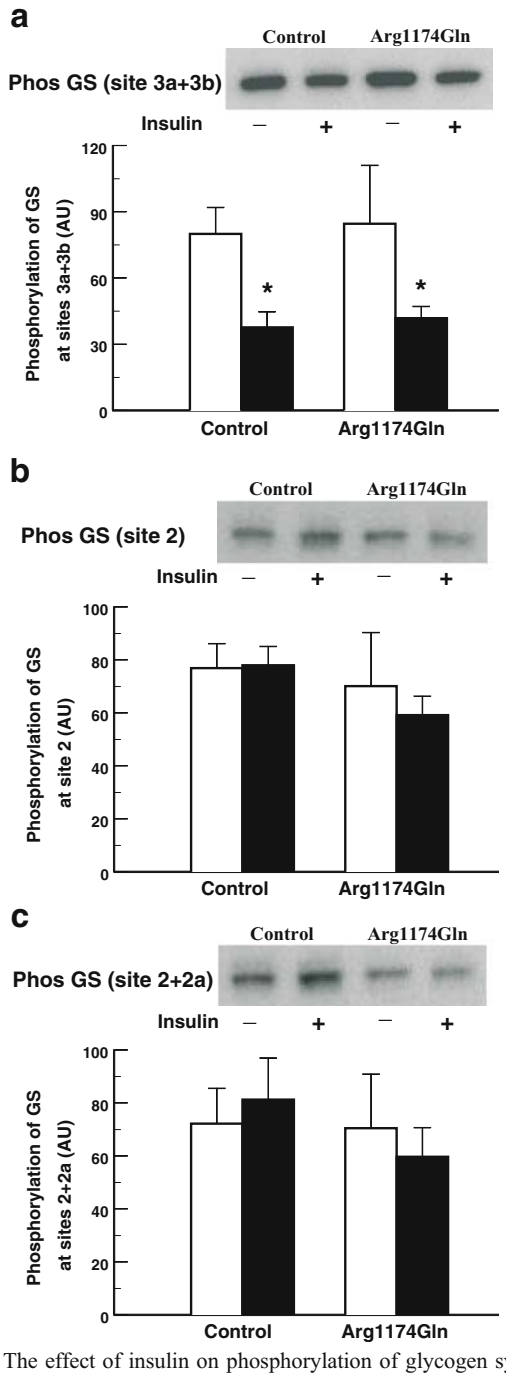

Fig. 3 The effect of insulin on phosphorylation of glycogen synthase (GS) at sites 3a+3b (**a**), site 2 alone (**b**) and sites 2+2a (**c**) was determined in skeletal muscle of six family members carrying the Arg1174Gln mutation in *INSR* and skeletal muscle of six control subjects. Representative immunoblots (**a–c**) for a control subject and an Arg1174Gln carrier are also shown. Measurements were performed in muscle biopsies obtained from m. vastus lateralis during the basal (*open bars*) and insulin-stimulated (*filled bars*) steady-state periods of a 3-h euglycaemic–hyperinsulinaemic clamp at an insulin infusion rate of 40 mU/m^2 per min. Data are mean±SEM. *p<0.05 for clamp vs basal values. *AU* Arbitrary units

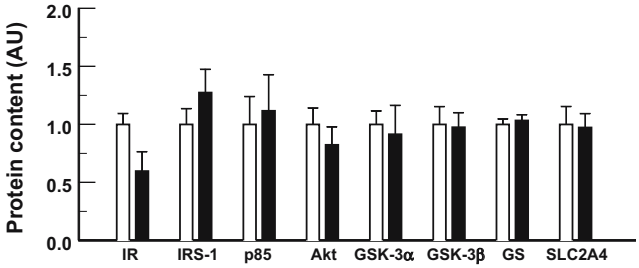

Fig. 4 Protein content of insulin signalling components in skeletal muscle of six family members carrying the Arg1174Gln mutation in *INSR* and in skeletal muscle of six control subjects. The protein levels were assessed by western blotting using muscle lysates of biopsies obtained from m. vastus lateralis during basal conditions. The protein level in Arg1174Gln carriers was normalised to the level observed in the control subjects. Data are mean±SEM. *AU* Arbitrary units, *IR* insulin receptor

Despite preservation of a significant effect of exogenous insulin on glucose uptake, glucose storage and most insulin signalling components in vivo, activation of PI3K and insulin-stimulated activities of Akt and glycogen synthase were modestly impaired in Arg1174Gln carriers compared with controls. This probably explains the moderate degree of insulin resistance. Thus, blunted activation of PI3K has been observed in several insulin-resistant states [14–16, 27]. Reduced Thr308 phosphorylation of Akt has been reported in obese non-diabetic subjects [28] and in non-obese patients with type 2 diabetes [29], but contrasts with findings in skeletal muscle of obese patients with type 2 diabetes and cultured muscle cells established from such patients [10, 15, 17]. The lower Thr308 phosphorylation of Akt may cause lower glucose transport in Arg1174Gln carriers, but was not responsible for the impaired insulin activation of glycogen synthase, because insulin-mediated inhibition of GSK-3 and subsequent dephosphorylation of glycogen synthase at sites 3a+3b was normal.

The decrease of ~50% in the phosphorylation of glycogen synthase at sites 3a+3b and the unchanged phosphorylation at sites 2+2a in response to insulin in both Arg1174Gln carriers and controls correspond to what has been reported previously in healthy subjects [10, 30] and provide further support for the notion that insulin activates glycogen synthase mainly by dephosphorylating sites 3a, 3b and 3c in vivo [9]. In patients with type 2 diabetes, impaired activation of glycogen synthase was explained by hyperphosphorylation at sites 2+2a despite normal insulin-mediated dephosphorylation at sites 3a+3b [10]. In the Arg1174Gln carriers, blunted activation of G6P-independent glycogen synthase activity was not, however, associated with increased phosphorylation of sites 2+2a. This suggests the possibility of increased phosphorylation at site 3a alone or at sites believed to be less important for glycogen synthase activity, such as sites 1a, 1b, 3c, 4 and 5 [8, 9]. Alternatively, other post-translational modifications could be involved. Recently, it was reported that *O*-linked glycosylation of glycogen synthase induced by exposure to high concentrations of glucose or hexosamines could

decrease glycogen synthase activity without changes in the phosphorylation state [31]. However, in the light of fasting plasma glucose and HbA_{1c} levels within the normal range, it seems unlikely that *O*-linked glycosylation plays a role in the observed blunted activation of glycogen synthase in Arg1174Gln carriers.

In agreement with a study of the Arg1174Gln mutation in patient lymphocytes transformed by Epstein–Barr virus [5], the number of insulin receptors in muscle tended to be lower (36%) in Arg1174Gln carriers compared with control subjects. Studies of CHO cells cotransfected with wild-type and mutant *INSR* cDNA have shown that the Arg1174Gln mutation is more susceptible to proteasomal degradation and impairs the formation of mt/wt hybrids, causing an increased fraction (~55%) of fully functional (wt/wt) receptors at the cell surface [32]. If this reflects the situation in vivo, this would explain the preserved insulin action mediated by impaired insulin clearance in spite of a small reduction in the total number of insulin receptors. In cell lines transfected with other kinase-deficient *INSR* mutations, accelerated receptor degradation has been reported to involve heat shock protein 90 (HSP90) [33], and recently the inhibition of HSP90 was shown to reduce the levels of functional non-mutated insulin receptors on the cell surface [34]. These studies suggest that HSP90 may serve to increase the amount of non-mutated relative to mutated insulin receptors. Interestingly, the level of HSP90 protein was recently reported to be increased in muscle of patients with type 2 diabetes [35]. Such an increase in HSP90 might antagonise the down-regulation of functional insulin receptors mediated by hyperinsulinaemia, and, as suggested by recent studies, preserve insulin signalling through Akt [36, 37]. Thus, increased levels of HSP90 in Arg1174Gln carriers could contribute to an increase in the relative amount of fully functional (wt/wt) insulin receptors, and hence the preservation of insulin action. This warrants further studies.

Using a euglycaemic–hyperinsulinaemic clamp, we provide evidence that the increased levels of insulin in Arg1174Gln carriers are caused by impaired insulin clearance. This effect, which is a known consequence of mutations in the IRTK domain [1], appears to play a major role in the partial preservation of the metabolic and signalling actions of insulin in muscle of patients harbouring the Arg1174Gln mutation. The data presented here provide no direct explanation for the coexistence of postprandial hypoglycaemia, but indirectly suggest the possibility that partial rescue of in vivo insulin signalling in other tissues, such as liver, beta cells and brain, may play a role. These findings may contribute to our understanding of the wide range of phenotypes observed in patients heterozygous for kinase-deficient *INSR* mutations, whereby some individuals develop syndromes of severe insulin resistance and type 2 diabetes whereas others, even in the same family, do not [2, 38]. Whether the coexistence of moderate insulin resistance and postprandial hypoglycaemia rather than type 2 diabetes is specific to the Arg1174Gln mutation itself or is a consequence of this mutation only in the family reported here, due to other background factors, remains to be elucidated. However, *INSR* mutations are in fact uncommon as the cause of type 2 diabetes [2, 38], and mice with a heterozygous *INSR* mutation develop diabetes with a frequency varying between 5 and 10% [39, 40]. This suggests that heterozygous *INSR* mutations exert only a predisposing role in susceptibility to type 2 diabetes and that additional susceptibility genes and environmental factors are needed to give manifest diabetes.

In summary, we have demonstrated that insulin resistance in skeletal muscle of patients harbouring the Arg1174Gln mutation in the IRTK domain of the *INSR* gene is associated with some defects in insulin signalling, which could explain impaired insulin-stimulated glucose uptake and NOGM. However, these responses to exogenous insulin were to a large extent preserved, indicating the existence of compensatory mechanisms. Impaired insulin clearance, a built-in consequence of the Arg1174Gln mutation, seems to play a major role in these compensatory mechanisms by increasing insulin levels. Further exploration of insulin signalling events in muscle and other insulin target tissues are warranted in order to understand the coexistence of postprandial hypoglycaemia and insulin resistance in subjects harbouring this *INSR* mutation.

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