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ARTICLE

Hyperglycaemia normalises insulin action on glucose metabolism but not the impaired activation of AKT and glycogen synthase in the skeletal muscle of patients with type 2 diabetes

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Abstract
Aims/hypothesis In type 2 diabetes, reduced insulin-stimulated glucose disposal, primarily glycogen synthesis, is associated with defective insulin activation of glycogen synthase (GS) in skeletal muscle. Hyperglycaemia may compensate for these defects, but to what extent it involves improved insulin signalling to glycogen synthesis remains to be clarified.
Methods Whole-body glucose metabolism was studied in 12 patients with type 2 diabetes, and 10 lean and 10 obese non-diabetic controls by means of indirect calorimetry and tracers during a euglycaemic-hyperinsulinaemic clamp. The diabetic patients underwent a second isoglycaemic-hyperinsulinaemic clamp maintaining fasting hyperglycaemia. Muscle biopsies from m. vastus lateralis were obtained before and after the clamp for examination of GS and relevant insulin signalling components.
Results During euglycaemia, insulin-stimulated glucose disposal, glucose oxidation and non-oxidative glucose metabolism were reduced in the diabetic group compared with both control groups ($p<0.05$). This was associated with impaired insulin-stimulated GS and AKT2 activity, deficient dephosphorylation at GS sites 2+2a, and reduced Thr308 and Ser473 phosphorylation of AKT. When studied under hyperglycaemia, all variables of insulin-stimulated glucose metabolism were normalised compared with the weight-matched controls. However, insulin activation and dephosphorylation (site 2+2a) of GS as well as activation of AKT2 and phosphorylation at Thr308 and Ser473 remained impaired ($p<0.05$).
Conclusions/interpretations These data confirm that hyperglycaemia compensates for decreased whole-body glucose disposal in type 2 diabetes. In contrast to previous less well-controlled studies, we provide evidence that the compensatory effect of hyperglycaemia in patients with type 2 diabetes does not involve normalisation of insulin action on GS or upstream signalling in skeletal muscle.

Keywords AKT · Glycogen synthase · Hyperglycaemia · Insulin signalling · Type 2 diabetes

Abbreviations

%FV	Per cent fractional velocity
G6P	Glucose 6-phosphate
GDR	Glucose disposal rates
GS	Glycogen synthase
GSK-3	Glycogen synthase kinase 3

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IR	Insulin receptor
IRS-1	Insulin receptor substrate 1
NOGM	Non-oxidative glucose metabolism
PCOS	Polycystic ovary syndrome
PI3K	Phosphatidylinositol 3-kinase
RER	Respiratory exchange ratio
TBC1D4	TBC1 domain family member 4
UDP	Uridine diphosphate

Introduction

Type 2 diabetes is characterised by insulin resistance defined as impaired insulin-stimulated glucose uptake in peripheral tissues. Skeletal muscle is the major site of glucose uptake during insulin stimulation, and correspondingly, the major site of insulin resistance in insulin-resistant conditions such as type 2 diabetes [1–10]. Reduced insulin-mediated glucose disposal is accounted for mainly by impaired stimulation of glycogen synthesis [1], but in obesity, polycystic ovary syndrome (PCOS) and type 2 diabetes also by reduced stimulation of glucose oxidation [3–7]. These abnormalities are associated with several defects at the molecular level in insulin-resistant skeletal muscle, including impaired signalling through proximal and distal components of the insulin signalling cascade such as the insulin receptor (IR), insulin receptor substrate-1 (IRS-1), phosphatidylinositol 3-kinase (PI3K), AKT, and TBC1 domain family, member 4 (TBC1D4), also known as AS160, in many [2, 6, 7, 11–13] although not all studies [3, 7, 8, 14–16], as well as impaired insulin activation of glycogen synthase (GS) in most studies [2–5, 7, 9, 10].

In everyday life, type 2 diabetic patients are characterised by different degrees of hyperglycaemia, and when studied at their prevailing fasting hyperglycaemia, the abnormalities in insulin-stimulated glucose metabolism disappear compared with weight-matched controls [17–21]. This effect has been explained by the mass action effect of glucose [21]. Most of the studies supporting this view were performed before the majority of components of the insulin signalling cascade were outlined [22, 23]. Nevertheless, based on the fact that impaired insulin action on glycogen synthesis was a major defect [1], a few studies investigated how insulin action on GS activity was affected by hyperglycaemia in type 2 diabetic patients [17–19, 21, 24]. In three studies using physiological levels of insulin [17, 18, 21], it was observed that hyperglycaemia normalised insulin-stimulated GS fractional velocity (FV), which reflects the sensitivity of GS to glucose 6-phosphate (G6P) and the degree of GS dephosphorylation [25]. In two other studies, in which hyperglycaemia was increased until insulin-stimulated glucose uptake matched that observed in controls, no effect on GS FV activity was seen during physiological hyperinsulinaemia [19, 24]. However, these reports all suffer from lack of sufficient control studies of type 2 diabetic patients during euglycaemic conditions [17–19, 21, 24]. If insulin regulation of GS activity is improved by hyperglycaemia, what are the molecular mechanisms involved? As GS activity is measured ex vivo, the changes have to be stable modifications, i.e. cannot simply be explained by allosteric mechanisms. One possibility is that an increase in the allosteric activator, G6P in vivo causes activation of a phosphatase or deactivation of a kinase affecting GS phosphorylation [21, 26]. Another possibility is that hyperglycaemia improves insulin signalling through IR, IRS-1, PI3K, AKT or glycogen synthase kinase-3 (GSK-3). This remains to be determined.

Since these earlier reports [17–21], it has been shown that insulin activation of GS is to a major extent mediated by dephosphorylation of the NH2-terminal sites 2 and 2a, and the COOH-terminal sites 3a and 3b [22, 25, 27]. We have shown that type 2 diabetic patients are characterised by dysregulation at both NH2- and COOH-terminal sites in skeletal muscle under euglycaemic conditions [3, 7]. Thus, hyperglycaemia could normalise insulin activation of GS by decreasing the activity of kinases, which phosphorylate these sites. Although insulin action on GSK-3 appears normal in type 2 diabetic patients [3, 7], hyperglycaemia could still enhance inhibition of GSK-3, the major kinase that phosphorylates GS at sites 3a, 3b, 3c and 4 [22, 25]. This, and which sites are affected by hyperglycaemia, if any, remain to be clarified.

The present study was carried out to examine the molecular mechanisms involved in normalisation of insulin-mediated glucose metabolism during physiological hyperinsulinaemia when performed under hyperglycaemic compared with euglycaemic conditions in type 2 diabetic patients. Skeletal muscle biopsies obtained under both conditions in patients with type 2 diabetes were pairwise compared, and also compared with biopsies obtained from lean healthy and obese non-diabetic individuals, who were metabolically characterised by euglycaemic clamps and indirect calorimetry.

Methods

Study participants Twelve obese type 2 diabetic patients carefully matched to 10 healthy, lean and 10 obese, non-diabetic volunteers participated in the study (Table 1). Medication details and eligibility criteria are given in the electronic supplementary material (ESM) Methods.

Study design Participants were instructed to refrain from strenuous physical activity for a period of 48 h before the experiment. After an overnight fast, the lean and obese

Table 1 Subject characteristics at study entry

Characteristic	Lean controls	Obese controls	Type 2 diabetes
n	10	10	12
Sex (male/female)	4/6	6/4	6/6
Age (years)	54.0±1.5	55.4±1.2	53.5±1.7
BMI (kg/m^2)	23.2±0.6	31.1±0.9†††	29.1±1.2†††
Fat free mass (kg)	51.1±4.1	63.0±4.2	55.3±3.2
Fat mass (kg)	17.8±1.1	30.3±1.9††	27.8±2.5††
Plasma glucose (mmol/l)	5.6±0.1	5.8±0.1	8.5±0.6***,†††
Serum insulin (pmol/l)	32±5	40±6	68±12†
Serum C-peptide (pmol/l)	520±33	633±45	1053±136**,††
HbA_{1c} (%)	5.4±0.1	5.3±0.1	6.7±0.3***,††
Plasma cholesterol (mmol/l)	5.6±0.3	5.1±0.3	4.9±0.2
Plasma LDL-cholesterol (mmol/l)	3.6±0.2	3.2±0.3	3.2±0.2
Plasma HDL-cholesterol (mmol/l)	1.7±0.1	1.6±0.1	1.1±0.1**,†††
Plasma triacylglycerols (mmol/l)	0.85±0.08	1.0±0.13	1.54±0.14*,††
Plasma NEFA (mmol/l)	0.45±0.05	0.41±0.03	0.53±0.06
Diabetes duration (years)	–	–	6.3±1.5

Data are mean±SEM

*p<0.05, **p<0.01, and ***p<0.001 vs obese controls; $^{\dagger}p$<0.05, $^{\dagger\dagger}p$<0.01, and $^{\dagger\dagger\dagger}p$<0.001 vs lean controls

controls underwent a euglycaemic-hyperinsulinaemic clamp (4 h of insulin infusion at 40 mU m^2 min^1) with tracer glucose and combined with indirect calorimetry (see ESM Methods) to assess total glucose disposal rates (GDR), respiratory exchange ratio (RER), and rates of glucose and lipid oxidation, and non-oxidative glucose metabolism (NOGM) as described [28–31]. The diabetic patients were clamped twice starting randomly with either a euglycaemic or an isoglycaemic-hyperinsulinaemic clamp separated by 4–6 weeks. Three of twelve diabetic patients could not participate in the second clamp, leaving paired data for nine patients. In diabetic patients, plasma glucose was allowed to decline to 5.5 mmol/l during the euglycaemic clamp before glucose infusion was initiated, whereas during the isoglycaemic clamp, glucose infusion was started simultaneously with insulin infusion in order to clamp plasma glucose at their prevailing levels of fasting hyperglycaemia. Plasma glucose and lactate and serum insulin, C-peptide, cholesterols, triacylglycerols and NEFA were measured as described previously [32]. Muscle biopsies were obtained from the vastus lateralis muscle before and after insulin, and homogenates and lysates were prepared as described previously [33] and in ESM Methods.

Muscle glycogen, glucose and glucose 6-phosphate Muscle glycogen content was measured in muscle homogenates (150 μg of protein) as glycosyl units after acid hydrolysis determined by a fluorometric method [34]. Muscle specimens were extracted with perchloric acid, neutralised and analysed for glucose, G6P and uridine diphosphate (UDP)-glucose [35] using standard enzymatic methods [34].

Glycogen synthase activity Muscle GS activity in the presence of 8, 0.17 or 0.02 mmol/l G6P was measured in triplicate using a 96-well plate assay (Unifilter 350 Plates, Whatman, Cambridge, UK) [7, 9]. GS activities are reported either as total GS activity (determined at 8 mmol/l G6P), the percentage of G6P-independent GS activity (%I-form) (100×activity in the presence of 0.02 mmol/l G6P divided by the activity at 8 mmol/l G6P [saturated]) or as the percentage of fractional velocity (%FV) (100×activity in the presence of 0.17 mmol/l G6P divided by the activity at 8 mmol/l G6P).

IRS-1 associated PI3K activity and AKT activities IRS-1 associated PI3K activity was measured on immunoprecipitates of IRS-1 from 300 μg of muscle lysate using an IRS-1 antibody as described (ESM Methods). AKT2 activity was measured on immunoprecipitates of AKT2 from 300 μg lysate protein using an anti-AKT2 antibody, and AKT1 activity was measured on 255 μg of the supernatant fraction from the initial AKT2 immunoprecipitates (ESM Methods).

SDS-PAGE and western blotting Muscle proteins were separated using 7–10% Tris–HCl gels and transferred (semi-dry) to PVDF-membranes (Immobilion Transfer Membrane, Millipore A/S, Denmark) as described previously [33]. Isoform (AKT1 and AKT2)-specific phosphorylation (Thr308 and Ser473) was determined by prior isoform-specific immunoprecipitation from 200 μg of protein following western blotting. Tyrosine phosphorylation of the IR was also determined by prior immunoprecipitation of the IR from 300 μg of lysate protein. A list of the antibodies used is given in ESM Methods. By loading a control sample in different amounts, it

was ensured that the quantification was within the linear response range for each particular protein probed for. An example of the whole gel set-up is given in ESM (Fig. 1).

Statistics Statistical analyses were performed using SigmaStat version 3.5 (Systat Software, San Jose, CA, USA). Data are presented as means±SEM. Differences between the groups were analysed by two-way ANOVA for one-way repeated measures, and significant main effects were further analysed by the Tukey post hoc test. p values below 0.05 were considered significant.

Results

Clinical and metabolic characteristics At study entry, fasting levels of plasma glucose, glycated haemoglobin (HbA_{1c}), plasma triacylglycerols and serum C-peptide were elevated and HDL-cholesterol reduced in the diabetic group compared with both control groups, whereas fasting serum insulin was higher in the diabetic group compared with the lean controls (Table 1).

During the insulin-stimulated steady-state period of the euglycaemic clamp, physiological hyperinsulinaemia, at a serum insulin concentration of ~400 pmol/l, was observed in all groups (Table 2). GDR, glucose oxidation and NOGM were similar among groups in the basal steady-state period. GDR and glucose oxidation were increased by insulin in all three groups ($p<0.05$), whereas NOGM was increased only in the two control groups ($p<0.05$). In the diabetic group, insulin-stimulated GDR, glucose oxidation and NOGM were reduced compared with both control groups (Table 2). Insulin-stimulated GDR and NOGM in obese controls were also lower than in lean controls. The ability of insulin to suppress lipid oxidation and to increase RER was reduced in diabetic patients compared with both lean and obese controls.

In the diabetic group clamped at prevailing levels of fasting hyperglycaemia (isoglycaemic clamp), serum insulin rose to 525 pmol/l during the insulin-stimulated steady-state period. Pairwise comparison of the diabetic patients who underwent both isoglycaemic and euglycaemic clamp studies showed that hyperglycaemia significantly increased insulin-mediated GDR, glucose oxidation and NOGM (Table 2). The average increase in insulin action on NOGM observed in these diabetic patients accounted for 76% of the increase in GDR. Compared with the matched obese controls, insulin-stimulated RER, GDR, glucose oxidation and NOGM were normalised in the diabetic group clamped at hyperglycaemia. However, insulin-stimulated GDR and NOGM were still slightly reduced in obese diabetic patients compared with lean controls. To account for the additional increase in serum insulin during the isoglycaemic clamp compared with the euglycaemic clamp, we calculated

Table 2 Metabolic characteristics during clamp

Characteristic	Lean (n=10)	Obese (n=10)	T2D-Eu (n=10)	T2D-Iso (n=11)
Plasma glucose, basal (mmol/l)	5.6±0.1	5.8±0.1	8.9±0.7***,†††	9.2±0.7***,†††
Plasma glucose, clamp (mmol/l)	5.5±0.1	5.4±0.1	5.4±0.1	9.3±0.7***,†††,‡‡‡
Serum insulin, basal (pmol/l)	19±2	33±7	69±14†	74±20
Serum insulin, clamp (pmol/l)	417±18	397±15	428±14	525±49*,††,‡‡
GDR, basal (mg min^{-1} m^{-2})	77±3	74±6	73±3	83±3
GDR, clamp (mg min^{-1} m^{-2})	378±28	309±24†	160±26***,†††	298±37†,‡‡‡
GDR per insulin, clamp (mg min^{-1} m^{-2}nmol/l^{-1})	928±91	804±81	385±70**,†††	653±113‡
Glucose oxidation, basal (mg min^{-1} m^{-2})	52±6	48±4	48±9	75±8
Glucose oxidation, clamp (mg min^{-1} m^{-2})	142±11	131±12	91±7**,†††	119±11‡
NOGM, basal (mg min^{-1} m^{-2})	24±4	26±6	25±7	8 ±7
NOGM, clamp (mg min^{-1} m^{-2})	236±21	178±25†	69±21***,†††	160±30¶,‡‡‡
Lipid oxidation, basal (mg min^{-1} m^{-2})	26±2	31±2	36±4	26±3‡
Lipid oxidation, clamp (mg min^{-1} m^{-2})	−4±4	1±4	16±4*,†††	11±4
RER, basal	0.82±0.01	0.81±0.01	0.80±0.01	0.84±0.01
RER, clamp	0.99±0.02	0.96±0.02	0.90±0.02*,†††	0.96±0.02‡
Lactate, basal (mmol/l)	0.70±0.06	0.74±0.06	1.14±0.11**,†††	1.23±0.11***,†††
Lactate, clamp (mmol/l)	1.30±0.08	1.19±0.09	1.01±0.07	1.24±0.11
NEFA, basal (mmol/l)	0.45±0.05	0.41±0.03	0.54±0.06	0.59±0.1¶
NEFA, clamp (mmol/l)	0.01±0.01	0.02±0.01	0.08±0.02	0.05±0.01††

Data are mean±SEM

n=9 for diabetic patients when isoglycaemic (T2D-Iso) and euglycaemic (T2D-Eu) clamp data are compared

All variables changed significantly in response to insulin ($p<0.05$), except for lactate in all groups, and NOGM in T2D-Eu

*$p<0.05$, **$p<0.01$, and ***$p<0.001$ vs obese controls; †$p<0.05$, ††$p<0.01$, and †††$p<0.001$ vs lean controls (¶$p<0.10$); ‡ $p<0.05$, ‡‡$p<0.01$, and ‡‡‡$p<0.001$ vs euglycaemic clamp

GDR per insulin unit in the insulin-stimulated periods. This showed that isoglycaemic clamp in type 2 diabetic patients still significantly increased insulin action on GDR, and actually abolished any significant difference when compared with both lean and obese controls (Table 2). The analyses gave similar results when calculated as mg min^{-1} (kg fat free mass)$^{-1}$ rather than mg min^{-1} m^{-2} (ESM Table 1).

Plasma lactate levels in the basal state were increased in diabetic patients compared with controls. No differences between the groups were seen in the insulin-stimulated state, and no effect of the isoglycaemic clamp was observed in type 2 diabetic patients (Table 2).

Glycogen synthase To test whether the improved insulin-stimulated GDR and primarily NOGM was mediated by improved insulin activation of GS, we measured protein content, activity and phosphorylation of GS (Fig. 1). No differences in GS protein content or total GS activity were observed between the four groups. Moreover, in all four groups, insulin significantly increased GS activity measured as both %I-form and %FV. In the diabetic groups clamped at either euglycaemia or hyperglycaemia, insulin-stimulated GS activities were reduced compared with the control groups. Pairwise comparison of the diabetic patients who underwent both isoglycaemic and euglycaemic clamp studies showed no effect of the isoglycaemic clamp on the ability of insulin to activate GS. Thus, the normalised insulin action on GDR and NOGM during an isoglycaemic clamp in the diabetic group is not explained by improvements in insulin activation of GS as measured ex vivo.

In the control groups, physiological hyperinsulinaemia significantly reduced phosphorylation of GS at sites 2+2a and 3a+3b, whereas in diabetic patients, no effect of insulin on GS phosphorylation at sites 2+2a was seen during either the euglycaemic or the isoglycaemic clamp (Fig. 2). Although insulin significantly reduced GS phosphorylation at sites 3a+3b only during the isoglycaemic clamp in the diabetic patients, no enhancing effect of the isoglycaemic clamp on either sites 3a+3b, sites 2+2a, or site 1b compared with the euglycaemic clamp was demonstrated. Thus, these data correspond to the GS activities. Insulin-mediated phosphorylation of GSK-3α and -β, were not different between the groups, and in accordance with GS activity and phosphorylation, insulin-mediated phosphorylation of GSK-3α and -β were not affected by the isoglycaemic clamp versus the euglycaemic clamp in the diabetic group (Fig. 2).

Proximal insulin signalling To investigate whether the improved insulin action on GDR mediated by the isoglycaemic clamp was paralleled by improved insulin signalling to GS, we measured insulin action on IR tyrosine phosphorylation,

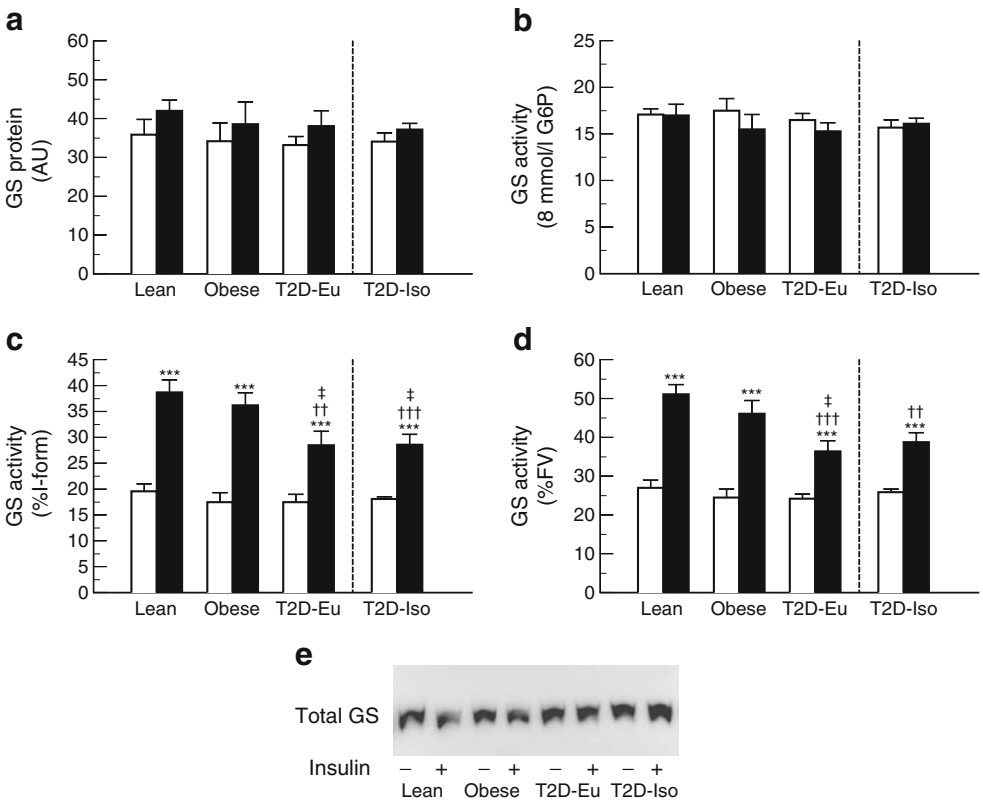

Fig. 1 Protein content (**a**), total activity (**b**), %I-form activity (**c**) and fractional velocity (%FV) (**d**) of GS. **e** Representative immunoblots. Measurements were performed in skeletal muscle biopsies obtained from lean, obese and type 2 diabetic (T2D-Eu) individuals during the basal (white bars) and insulin-stimulated (black bars) steady-state periods of a 4-h euglycaemic-hyperinsulinaemic clamp. T2D individuals (T2D-Iso) also underwent a isoglycaemic-hyperinsulinaemic clamp. Data are means±SEM. ***$p<0.001$ vs basal; $^{††}p<0.01$, and $^{†††}p<0.001$ vs lean; $^{‡}p<0.05$ vs obese

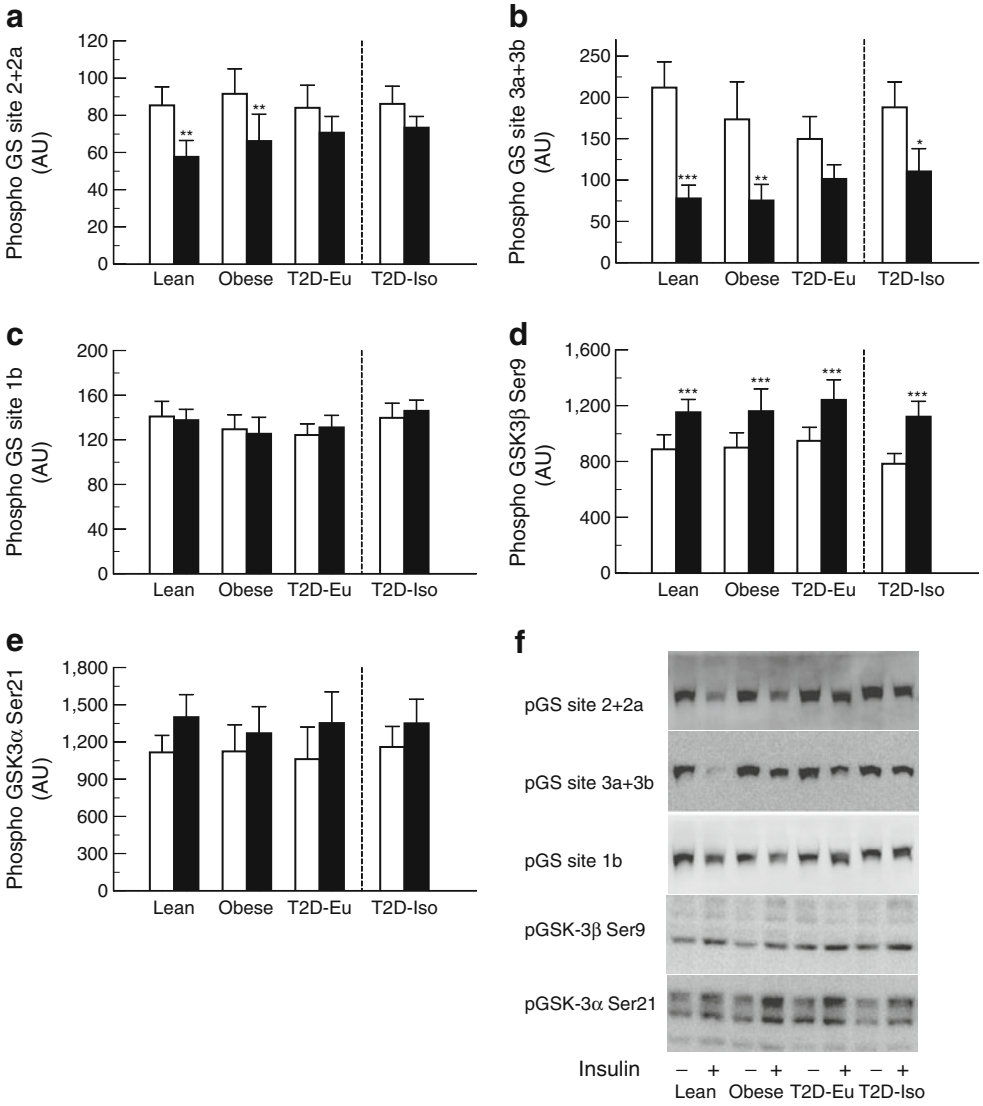

Fig. 2 Phosphorylation of GS at sites 2+2a (**a**), site 3a+3b (**b**) and site 1b (**c**), and phosphorylation of glycogen synthase kinase 3β (GSK3β) at Ser9 (**d**) and GSK3α at Ser21 (**e**). Findings are shown in representative immunoblots (**f**), quantified in bar graphs as arbitrary units (AU). Measurements were performed in skeletal muscle biopsies obtained from lean, obese and type 2 diabetic (T2D-Eu) individuals during the basal (white bars) and insulin-stimulated (black bars) steady-state periods of a 4-h euglycaemic-hyperinsulinaemic clamp. T2D individuals (T2D-Iso) also underwent a isoglycaemic-hyperinsulinaemic clamp. Data are means±SEM. *p<0.05, **p<0.01 and ***p<0.001 vs basal

IRS-1 associated PI3K activity and AKT activity/phosphorylation (Fig. 3, ESM Fig. 2). Physiological hyperinsulinaemia increased tyrosine phosphorylation of the IR in all groups (main effect p<0.001). Although a main effect of diabetes (p=0.042) was seen during the euglycaemic clamp, there was no interaction between groups and clamp level (basal vs insulin). Moreover, when corrected for IR protein content, no difference was evident between groups (ESM Fig. 2). Physiological hyperinsulinaemia increased IRS-1-associated-PI3K activity in all four groups. Although basal IRS-1-associated-PI3K activity appeared elevated leading to an apparently smaller increment in response to insulin in the diabetic group, there were no significant differences between the diabetic and control groups, and no effect of the isoglycaemic clamp in the diabetic group (Fig. 3). ANOVA showed a tendency for a difference in insulin-induced fold-changes in IRS-1-associated-PI3K activity between the lean (3.17±0.49), obese (2.76±0.48) and diabetic groups (1.74±0.27) during the euglycaemic clamp (main effect p=0.06), but not during the isoglycaemic clamps (diabetic group; 1.99±0.40, main effect p=0.18), and there was no effect of the isoglycaemic clamp in the diabetic group (p=0.46). In contrast, insulin-stimulated phosphorylation of AKT at Thr308 and Ser473, and activation and Thr308 phosphorylation of immunoprecipitated AKT2 were significantly impaired in the diabetic groups compared with the control groups (Fig. 3). This defect was unrelated to AKT2 protein content (similar between groups, ESM Figs 3 and 4). The isoglycaemic clamp did not abolish these defects in type 2 diabetic patients. It is noteworthy that in skeletal muscle, insulin activation of AKT was isoform-specific as no regulation of AKT1 activity was evident (ESM Fig. 5) and no AKT3 protein was detected (data not shown). Thus, no changes in insulin action on these proximal components of the insulin signalling cascade could explain the

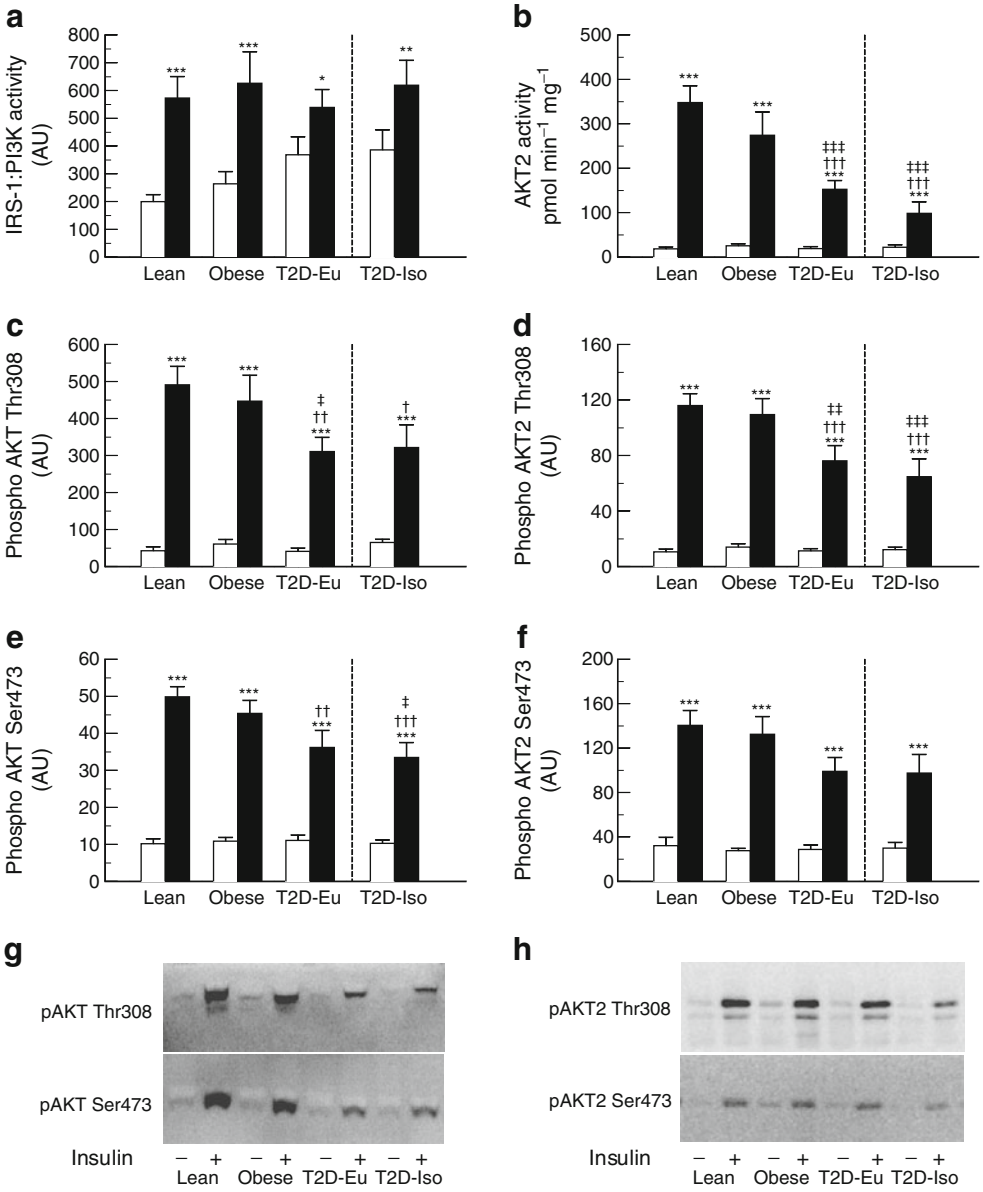

Fig. 3 IRS-1 associated PI3K activity (**a**), AKT2 activity (**b**), Thr308 phosphorylation of AKT (**c**) and AKT2 (**d**), and Ser473 phosphorylation of AKT (**e**) and AKT2 (**f**). Findings are shown in representative immunoblots (**g** and **h**), quantified in bar graphs as arbitrary units (AU) or as substrate phosphorylation per minute per mg lysate protein used in the IP of AKT2 (pmol min^{-1} mg^{-1}). Measurements were performed in skeletal muscle biopsies obtained from lean, obese and type 2 diabetic (T2D-Eu) individuals during the basal (white bars) and insulin-stimulated (black bars) steady-state periods of a 4-h euglycaemic-hyperinsulinaemic clamp. T2D individuals (T2D-Iso) also underwent a isoglycaemic-hyperinsulinaemic clamp. Data are means±SEM. *$p<0.05$, **$p<0.01$ and ***$p<0.001$ vs basal. $^{\dagger}p<0.05$, $^{\dagger\dagger}p<0.01$, and $^{\dagger\dagger\dagger}p<0.001$ vs lean; $^{\ddagger}p<0.05$, $^{\ddagger\ddagger}p<0.01$, $^{\ddagger\ddagger}p<0.001$ and $^{\ddagger\ddagger\ddagger}p<0.001$ vs obese

improvements in GDR caused by the isoglycaemic clamp in patients with type 2 diabetes.

Intracellular glucose, G6P, UDP-glucose and glycogen We next examined whether the improved insulin action on GDR during the isoglycaemic clamp was accompanied by changes in intracellular levels of glucose, G6P, UDP-glucose or glycogen in muscle biopsies. We were not able to demonstrate any differences between the diabetic and control groups, or between the isoglycaemic and euglycaemic clamp in type 2 diabetic patients either in the basal or the insulin-stimulated steady-state periods (Table 3). As reported in rodent muscle [35], insulin significantly reduced intracellular levels of UDP-glucose ($p<0.001$, main effect) in the present study with no significant difference between the groups. In both control groups, insulin significantly increased glycogen levels ($p<0.05$), whereas no such effect was seen in the diabetic groups clamped at either euglycaemia or hyperglycaemia.

Discussion

Several studies have shown that insulin-stimulation of glucose disposal is normalised in type 2 diabetic patients if investigated at their prevailing fasting hyperglycaemia [17–21]. In agreement, we demonstrate by direct comparison, that hyperglycaemia normalises insulin-mediated GDR in type 2 diabetic patients compared with weight-matched non-diabetic individuals, and that normalisation of NOGM

Table 3 Intramyocellular concentrations of glycogen, G6P, UDP-glucose and glucose

	Lean (n=10)	Obese (n=10)	T2D-Eu (n=10)	T2D-Iso (n=11)
Glycogen, basal (mmol/kg dw)	393±37	382±52	394±17	436±39
Glycogen, clamp (mmol/kg dw)	479±30	471±36	415±31	460±32
G6P, basal (mmol/kg dw)	0.56±0.20	0.40±0.06	0.32±0.08	0.63±0.27
G6P, clamp (mmol/kg dw)	0.34±0.09	0.39±0.09	0.36±0.07	0.32±0.07
UDPG, basal (mmol/kg dw)	0.098±0.015	0.100±0.009	0.095±0.007	0.118±0.006
UDPG, clamp (mmol/kg dw)	0.073±0.006	0.073±0.009	0.083±0.009	0.066±0.008
Glucose, basal (mmol/kg dw)	6.35±1.17	6.18±0.73	8.09±0.82	9.20±1.30
Glucose, clamp (mmol/kg dw)	5.99±0.87	4.12±0.58	5.91±1.25	7.37±1.20

Data are mean±SEM

n=9 for diabetic patients when isoglycaemic (T2D-Iso) and euglycaemic (T2D-Eu) clamp data are compared

Insulin decreased concentrations of UDP-glucose in all groups (p<0.001, main effect), and increased glycogen levels in both control groups (p< 0.05), but not in the diabetic groups

dw, dry weight

accounts for ~75% of this improvement. The major findings of the present study were, however, that contrary to our working hypothesis, and previous less well-controlled studies [17, 18, 21], hyperglycaemia did not abolish defective insulin activation of GS, or related deficient dephosphorylation at sites 2+2a in the muscle of patients with type 2 diabetes [2, 3, 7]. Moreover, impaired insulin signalling to GS via AKT phosphorylation at Thr308 and Ser473 was not repaired by hyperglycaemia. Finally, we did not detect any changes in intramyocellular levels of glucose, G6P, UDP-glucose or glycogen in response to hyperglycaemia that could explain improved glucose metabolism. We, therefore, conclude that normalisation of whole-body insulin-mediated glucose metabolism does not involve improved insulin signalling to GS via AKT or dephosphorylation of GS. Thus, further studies are warranted to elucidate the molecular mechanism underlying this beneficial effect of hyperglycaemia during clamp conditions.

Our results are in line with an earlier study of the effect of hyperglycaemia in healthy individuals [36]. Thus, during a 4 h hyperglycaemic clamp using an insulin infusion rate of 40 mU min^{-1} m^{-2}, no effect of hyperglycaemia (10 mmol/l) was seen on insulin activation of GS, despite large increases in GDR and NOGM [36]. Moreover, in agreement with our data, no effect was seen on glycogen levels, probably because the expected increase due to improved glycogen synthesis is below 10%, and therefore difficult to detect [36]. Concerning GS, our findings are in contrast to most previous studies of type 2 diabetic patients during hyperglycaemia [17–19, 21]. In these studies, the diabetic patients were, however, examined only during hyperglycaemia, and not during euglycaemia [18, 19, 21], or only a few patients (n=4) were studied under euglycaemia [17]. In those studies that reported an apparently normalised GS activity during hyperglycaemia [17, 18, 21], the isoglycaemic clamp procedure was very similar to the one used in the present study. Thus, the lack of effect on GS activity in our study cannot be explained by differences in the insulin concentration used or duration of infusion. Although we and others have demonstrated impaired insulin activation of GS in type 2 diabetes [2, 3, 7], not all studies have been able to detect a significant difference between obese type 2 diabetic patients and matched non-diabetic individuals [7, 10]. Without sufficient control clamps performed at euglycaemia in type 2 diabetic patients, it remains a possibility that the apparently normal insulin action on GS activity observed during hyperglycaemia in previous studies does not represent a true improvement [17, 18, 21]. In two studies, which also reported a lack of effect on GS activity, the study design was quite different using 5-h insulin infusion and aiming for hyperglycaemia (20 mmol/l) that normalised glucose uptake [19, 24]. This caused a decrease in insulin stimulation of glucose oxidation whereas NOGM was markedly increased. The increase in NOGM was explained by increased non-oxidative glycolysis, which leads to exaggerated muscle lactate production, and was suggested to increase flow through the Cori cycle [19]. This was supported by the finding of a pronounced increase in plasma lactate in the insulin-stimulated state in response to hyperglycaemia [19]. In our study, the increase in NOGM cannot be explained by this mechanism. Thus, glucose oxidation was also increased, and plasma lactate levels in the insulin-stimulated state were not increased in type 2 diabetic patients clamped at hyperglycaemia compared with euglycaemia, or even compared with controls.

Using an insulin infusion rate of 40 mU min^{-1} m^{-2}, we observed a 20% higher increase in plasma insulin levels during the isoglycaemic clamp compared with the euglycaemic clamp. However, even after adjustment for this additional increase in insulin, insulin-mediated GDR were normalised in type 2 diabetic patients compared with

controls. Higher levels of insulin were also observed in a previous study reporting a positive effect of hyperglycaemia on glucose metabolism and GS in patients with type 2 diabetes [21]. Despite this extra increase in insulin, we could not demonstrate any beneficial effect of the isoglycaemic clamp on insulin action on GS FV, dephosphorylation of GS or signalling from the IR through PI3K and AKT to GSK-3. This indicates that skeletal muscle was unaffected by this minor increase in insulin. This is supported by the study of Kelley et al. [17], in which the ability of hyperglycaemia to normalise insulin action on glucose metabolism and GS was observed at comparable levels of hyperinsulinaemia in controls and diabetic patients.

Of interest, we could not reproduce the finding of increased phosphorylation of muscle GS at site 1b in type 2 diabetic patients [7]. Similar to the inconsistent finding of impaired insulin action on AKT phosphorylation (6,7,11–13), this probably reflects the large heterogeneity in both type 2 diabetic and obese non-diabetic individuals combined with the relatively small sample sizes often used in such studies. However, the impaired insulin activation of GS and the lack of dephosphorylation of GS at sites 2+2a appear to be consistent findings in type 2 diabetic patients [3, 7], as well as in other insulin-resistant conditions such as PCOS [9] and HIV lipodystrophy [37]. Consistently, we have demonstrated that insulin-mediated dephosphorylation of GS at sites 2+2a correlates significantly with insulin-stimulated NOGM in a large cohort of twins [38]. Currently, it appears that insulin resistance in muscle of type 2 diabetes is associated with defect insulin activation of GS [2–4, 7, 10], deficient dephosphorylation of GS at sites 2+2a [3, 7] and impaired activation of AKT2 and phosphorylation of AKT at Thr308 and Ser473 [7, 11]. Whether dephosphorylation of GS sites 3a+3b is also impaired in type 2 diabetes remains a possibility. In this and two previous studies [3, 7], we have, however, been unable to demonstrate a significant reduction in the insulin-mediated dephosphorylation of sites 3a+3b in type 2 diabetic patients, although the effect has seemed lower in all three studies. Moreover, these sites are to a major extent regulated by GSK-3 during insulin stimulation [22, 25], and in none of our studies was any evidence found that the ability of insulin to inhibit GSK-3 is compromised in skeletal muscle of type 2 diabetic patients.

Importantly, none of the signalling defects observed during euglycaemia in muscle of type 2 diabetic patients were restored by hyperglycaemia during a hyperinsulinaemic clamp, and there was no improvement in signalling through either IR, PI3K or AKT. This lack of effect of hyperglycaemia on insulin signalling has to our knowledge not been demonstrated in human skeletal muscle before. Although these findings lend support to the hypothesis [20] that the effect of hyperglycaemia is mediated by a mass action, there is accumulating experimental evidence that glucose transport via GLUT4 is regulated by several distinct mechanisms including exocytosis, endocytosis and intrinsic activity of GLUT4 [23]. Hyperglycaemia during a hyperinsulinaemic clamp may regulate one or more of the enzymes involved in these processes in skeletal muscle of both healthy and type 2 diabetic patients. Moreover, we cannot exclude the possibility that hyperglycaemia repairs an abnormality in insulin signalling to glucose transport further downstream of the AKT→TBC1D4/TBC1D1→Rab axis, via selective actin remodelling through the Rac→actin→α-actinin-4 axis, changes in binding of GLUT4 interacting proteins such as GAPDH and hexokinase II, or a defect in enzymes mediating fusion of GLUT4 vesicles with the plasma membrane such as synaptosomal-associated protein 23 (SNAP23), mammalian homologue of Unc-18c (MUNC18C), syntaxin4 and vesicle associated membrane protein 2 (VAMP2) [23, 39]. Thus, studies of the several processes that regulate GLUT4 vesicle movement, and content and activity in the plasma membrane in response to hyperglycaemia may help to reveal the mechanisms involved in improved insulin-mediated glucose metabolism during a hyperglycaemic hyperinsulinaemic clamp.

In contrast to previous studies measuring G6P in human muscle using magnetic resonance spectroscopy [40], we could not demonstrate an increase in muscle G6P in response to physiological hyperinsulinaemia, or in response to hyperglycaemia during insulin stimulation. Thus, in our hands, changes in muscle G6P concentrations do not seem to explain increased insulin-mediated glycogen synthesis during hyperglycaemia. Although the lack of response to insulin has been reported before using the same biochemical assay [21], the cause of these discrepancies remains to be defined. We therefore measured muscle levels of UDP-glucose. As reported in mouse muscle [35], we observed that insulin caused a significant reduction in muscle UDP-glucose. However, there was no difference between diabetic and non-diabetic groups, and, most importantly, no effect of hyperglycaemia on UDP-glucose that could explain improved insulin-mediated glycogen synthesis. Our data do not exclude an increased flux of G6P or UDP-glucose in response to hyperglycaemia, and this may push the control of GS activity toward a more G6P-dependent activation in vivo.

In summary, we confirm that hyperglycaemia normalises insulin-mediated GDR, primarily due to improved NOGM in type 2 diabetic patients. The novel finding is that this effect is achieved without improved insulin signalling through IR, PI3K, AKT and GS, or changes in the intramyocellular levels of glucose metabolites. These results support the view that hyperglycaemia works by the mass action effect of glucose. However, we cannot exclude the possibility that hyperglycaemia could augment insulin-mediated glucose uptake by molecular mechanisms yet to be defined, and that this augmentation could bypass the

defects in insulin action on key enzymes regulating glucose transport and glycogen synthesis. If such mechanisms could be identified, they may represent novel targets for treatment. That is, if a similar beneficial effect could be achieved without a sustained increase in glucose levels.

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Contribution statement BFV, JBB, HB-N, JFPW and KH were responsible for the conception and design, analysis and interpretation of data, drafting the article, revising it critically for important intellectual content and final approval of the version to be published. SGV and BA were responsible for analysis and interpretation of data, revising the article critically for important intellectual content and final approval of the version to be published.

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