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The evolution of insulin resistance in muscle of the glucose infused rat

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ABSTRACT

Glucose infusion into rats causes skeletal muscle insulin resistance that initially occurs without changes in insulin signaling. The aim of the current study was to prolong glucose infusion and evaluate other events associated with the transition to muscle insulin resistance. Hyperglycemia was produced in rats by glucose infusion for 3, 5 and 8 h. The rate of infusion required to maintain hyperglycemia was reduced at 5 and 8 h. Glucose uptake into red quadriceps (RQ) and its incorporation into glycogen decreased between 3 and 5 h, further decreasing at 8 h. The earliest observed change in RQ was decreased AMPKα2 activity associated with large increases in muscle glycogen content at 3 h. Activation of the mTOR pathway occurred at 5 h. Akt phosphorylation (Ser^{473}) was decreased at 8 h compared to 3 and 5, although no decrease in phosphorylation of downstream GSK-3β (Ser^{9}) and AS160 (Thr^{642}) was observed. White quadriceps showed a similar but delayed pattern, with insulin resistance developing by 8 h and decreased AMPKα2 activity at 5 h. These results indicate that, in the presence of a nutrient overload, alterations in muscle insulin signaling occur, but after insulin resistance develops and appropriate changes in energy/nutrient sensing pathways occur.

Introduction

Hyperglycemia and insulin resistance are hallmarks of type 2 diabetes. It is also clear that hyperglycemia by itself can cause, and/or exacerbate, insulin resistance in multiple tissues [1]. Skeletal muscle is quantitatively the most important tissue target of glucose postprandially and accounts for 70–80% of insulin stimulated glucose uptake [2].

Early studies revealed that insulin resistance develops in skeletal muscle of rats infused with glucose at a high, but constant rate for 1–7 days [3–5] and that it occurs as early as day 1 of a glucose infusion [4]. Laybutt et al. [5] reported that skeletal muscle of these animals had an increased long chain acyl-CoA (LCACoA) content, as well as activation of PKCε, potentially implicating elevated diacylglycerol (DAG) levels as a pathogenic factor. They proposed that increased intramyocellular fatty acid metabolites may feed back onto the insulin signaling pathway to inhibit glucose uptake [5].

More recent studies in which variable rates of infusion were used to clamp blood glucose levels at ~11 mM revealed that insulin resistance develops at 5 h [6,7]. They also revealed that at this time point it was accompanied by increases in the concentrations of malonyl-CoA and DAG and decreased AMPK activity, providing further evidence of a link between fatty acid metabolites and the hyperglycemia-induced insulin resistant state [7]. However, a subsequent study revealed that the insulin resistance generated by an acute glucose infusion at 5 h is not associated with decreased phosphorylation of proteins in the insulin signaling pathway (insulin receptor, Akt and AS160). Furthermore, it occurred without changes in maximal insulin-stimulated glucose transport capacity [6]. These findings suggested that the insulin resistance caused by a hyperglycemic insult initially may be due to excess glycogen storage and metabolic feedback, rather than a defect in insulin signaling [6].

A lack of an insulin signaling defect in the setting of insulin resistance is not in keeping with current thinking, which is based on the concurrent presence of abnormalities in insulin signaling and insulin resistance in a variety of circumstances [8]. The present study has two aims. The first is to determine if insulin signaling

Abbreviations: ACC, acetyl CoA carboxylase; AMPK, 5′ adenosine monophosphate-activated protein kinase; AS160, Akt substrate of 160KDa; DAG, diacylglycerol; EDL, extensor digitorum longus, GSK-3β, glycogen synthase kinase 3 beta; IR, insulin receptor; IRS-1, insulin receptor substrate-1; LCACoA, long chain acyl CoA; mTOR, mammalian target of rapamycin; p70S6K, p70S6 kinase; R_d, whole body glucose disposal rate; R_g', glucose uptake; RQ, red quadriceps; WQ, white quadriceps.

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defects occur during a more prolonged exposure to hyperglycemia and if so whether they are associated with a further exacerbation of insulin resistance in skeletal muscle. The second is to identify other factors modified during a glucose infusion that could account for the decrease in muscle glucose uptake prior to changes in insulin signaling.

Materials and methods

Animals

All surgical and experimental procedures performed were approved by the Garvan Institute/St. Vincent's Hospital Animal Ethics Committee and were in accordance with the National Health and Medical Research Council of Australia's guidelines on animal experimentation.

Adult male Wistar rats (Animal Resources Centre, Perth, Australia) were communally housed in temperature controlled (22 ± 0.5 °C) 12 h light–dark cycle rooms. Rats were fed *ad libitum* a standard chow diet (Rat Maintenance Diet; Gordon Specialty Feeds, Sydney, Australia). After a 1 week acclimatization period, cannulae were inserted into both jugular veins [6]. Average body weight on the day of experiment was 319 ± 8 g.

Glucose infusion

Seven days after surgery, rats were randomly divided into treatment groups. After a basal blood sample was taken, a 50% (w/v) glucose infusion commenced. Rats were infused for either 0, 3, 5 or 8 h using a peristaltic roller pump (101U/R; Watson-Marlow, Falmouth, UK). Blood samples were taken every 30 min and the glucose infusion rate was altered to maintain blood glucose concentration at ~11 mM. Red blood cells from each sample were resuspended in heparinised saline and returned to the animal. In one cohort, 2-deoxy-D-[2,6-^{3}H]glucose and [U-^{14}C]glucose (Amersham Biosciences, Buckinghamshire, UK) were administered as an intravenous bolus in the last 30 min of the glucose infusion. Blood samples were taken 2, 5, 10, 15, 20 and 30 min after administration of the tracer bolus for estimation of tracer clearance and blood glucose. Animals were then euthanized with an overdose of pentobarbital sodium (Nembutal; Abbott Laboratories, Sydney, Australia) and tissues were rapidly removed, freeze-clamped, and stored at −80 °C for later analysis.

In the second cohort, rats were euthanized after the glucose infusion period ended (no tracer period). The soleus and EDL muscle from each leg was carefully dissected into 3 longitudinal strips from tendon to tendon with the use of a 25G needle and two strips of a similar weight from each leg were used. The third strip was immediately freeze-clamped (without further incubation) and stored at −80 °C for later immunoblotting analysis. Glucose transport was assayed in isolated muscles from one leg in sealed vials containing pregassed (95% O_2–5% CO_2) Krebs-Henseleit bicarbonate buffer supplemented with 4 mM sodium pyruvate, 8 mM mannitol, and 0.1% wt/vol BSA at 30 °C. Muscles were preincubated for 30 min and were then incubated with or without insulin for 30 min. Insulin-stimulated glucose transport capacity was then assayed for 15 min using [^{3}H]2DG (1 mM, 0.128 μCi/ml) in the presence or absence of 300 μU/ml insulin, a dose similar to that reached *in vivo* (Table 1) [6].

After the preincubation phase, the remaining muscle strips were transferred to buffer containing 5 mM glucose and 2 μCi of [U-^{14}C]glucose to monitor glucose incorporation into glycogen. The resulting glycogen concentration and [^{14}C]glucose incorporation into glycogen were measured as described previously [6,9].

Analytic methods

Blood and plasma glucose levels (YSI2300; Yellow Springs Instruments, Yellow Springs, OH), and plasma insulin (Linco, St. Louis, MO) were measured.

Plasma and tissue levels of ^{3}H- and ^{14}C-labelled tracers were measured to calculate whole body glucose disposal rate (R_d) and to estimate red (RQ) and white (WQ) quadriceps glucose uptake (R_g'), [^{14}C]glucose incorporation into glycogen and glycogen concentration. Assays and calculations are as previously described [9].

Long chain acyl-CoA (LCACoA) levels were measured as described by Antinozzi et al. [10]. Malonyl-CoA was assayed radioenzymatically by a modification of the method of McGarry et al. [11]. The measurement of DAG and ceramide content was determined as previously described [12]. Glucose metabolites were measured spectrophotometrically by standard enzymatic assays [13].

Western blot analysis

Protein extraction and immunoblots from RQ and WQ muscle homogenates were performed as previously described [6]. Antibodies raised against insulin receptor β were obtained from BD Biosciences (San Jose, CA), phospho-Tyr$^{1162/63}$ insulin receptor from BioSource International (Camarillo, CA), phospho-Tyr^{612} IRS-1 from Sigma–Aldrich (St. Louis, MO), Akt, phospho-Ser^{473} Akt, GSK-3β, phospho-Ser^{9} GSK-3β, IRS-1, phospho-IRS-1^{307} IRS-1, anti

Table 1
Blood, whole body and muscle parameters of basal and glucose infused animals.

	Basal	Time of glucose infusion		
		3 h	5 h	8 h
Whole body				
Glucose (mM)	4.4 ± 0.2	11.5 ± 0.5[a]	11.5 ± 0.2[a]	11.8 ± 0.3[a]
Insulin (mU/L)	36 ± 8	261 ± 44[a]	335 ± 24[a]	345 ± 39[a]
GIR (mg/min/kg)	N/A	63 ± 3	53 ± 2[b]	48 ± 1[b]
R_d (mg/min/kg)	12 ± 1	58 ± 3[a,c,d]	52 ± 1[a,b]	48 ± 1[a,b]
Red quadriceps				
R_g' (μmol/min/100 g)	8 ± 3	40 ± 2[a,c,d]	30 ± 2[a,b,d]	23 ± 1[a,b,c]
Glycogen content (μmol/g)	36 ± 3	111 ± 8[a,d]	122 ± 6[a,d]	154 ± 5[a,b,c]
Glycogen synthesis rate (μmol/100 g/min)	2 ± 1	24 ± 3[a,c,d]	16 ± 2[a,b,d]	8 ± 1[b,c]
White quadriceps				
R_g' (μmol/min/100 g)	5 ± 1	39 ± 4[a,d]	35 ± 2[a,d]	26 ± 2[a,b,c]
Glycogen content (μmol/g)	38 ± 3	70 ± 5[a,d]	71 ± 3[a,d]	94 ± 5[a,b,c]
Glycogen synthesis rate (μmol/100 g/min)	0.3 ± 0.1	4.6 ± 0.4[a]	4.2 ± 0.5[a]	3.1 ± 0.3[a]

Data are means ± SEM. [a]$P < 0.05$ vs basal, [b]$P < 0.05$ vs 3 h, [c]$P < 0.05$ vs 5 h, [d]$P < 0.05$ vs 8 h glucose infusion. $n = 4$–13. GIR, glucose infusion rate; R_d, whole body glucose disposal; R_g', glucose uptake into red or white quadriceps.

phospho-Ser^{2448} mTOR, anti phosphor-Thr^{389} p70S6K, total mTOR, p70S6K, phospho-Ser^{79} ACC and total ACC from Cell Signaling Technology (Danvers, MA), AS160 from Upstate Biotechnology (Lake Placid, NY), and phospho-Thr^{642} AS160, antibody was a gift from Symansis (Auckland, New Zealand).

AMPK activity assay

RQ and WQ muscle were homogenized in lysis buffer (50 mM Hepes (pH 7.4) 50 mM NaF, 5 mM NaPP, 10% Glycerol, 1 mM EDTA (pH 8.0)) and incubated for 2 h at 4 °C. Samples were then centrifuged at 13,000 rpm for 10 min at 4 °C. Protein concentrations were determined by a Bradford assay (Bio-Rad Regents Park, NSW, Australia). 100 μg of the cleared lysate was incubated at 4 °C with gentle mixing with an antibody to either the α1- or α2-subunit of AMPK (supplied by D. Carling, MRC, London) and with Protein G Sepharose beads (GE Healthcare BioSciences, Uppsala, Sweden). After 2 h, samples was spun at 13,000 rpm, the supernatant was removed and washed in lysis buffer and then in HGE buffer (50 mM Hepes pH 7.4, 10% glycerol, 0.1 mM EDTA). Beads were then incubated at 30 °C with constant mixing in reaction buffer containing 40 mM Hepes pH 7.4, 80 mM NaCl, 5 mM $MgCl_2$, 8% glycerol, 800 μM EDTA, 200 μM AMP, 200 μM AMARA peptide, 800 μM dithiothreitol, 200 μM ATP (0.148 MBq [γ-^{32}P]ATP). After 20 min, the beads were pelleted and duplicate aliquots were spotted onto P81 Whatman photo-cellulose paper (Whatman Bioscience, Clifton, NJ), and washed three times in 1% phosphoric acid before scintillation counting.

Statistical analysis

Data are expressed as means ± SEM. Differences between groups were determined via ANOVA and regression analysis using GraphPad Prism (Version 5.00 for Windows, GraphPad Software, San Diego, California, USA). When the ANOVA reached significance, a Newman-Keuls multiple comparison post hoc test was conducted. Significance was set at $P \leqslant 0.05$.

Results

Blood and plasma parameters

Blood glucose and plasma insulin levels were similar in all 4 groups in the basal state. Blood glucose was maintained at ~11 mM with a variable glucose infusion which also caused a 7- to 9-fold increase in plasma insulin levels (Table 1).

Whole body

The glucose infusion rate (GIR) required to maintain hyperglycemia was decreased significantly at 5 h ($P < 0.05$; Table 1) and tended to decrease further by 8 h. Whole body glucose disposal (R_d) showed a similar pattern of change ($P < 0.05$; Table 1).

Skeletal muscle

Glucose uptake (R_g') into red quadriceps (RQ) muscle was significantly decreased after 5 h of glucose infusion compared to 3 h and was decreased further at 8 h (−22% and −40% vs 3 h, respectively, $P < 0.05$; Table 1). RQ glycogen content was increased ~3-fold after 3 and 5 h of hyperglycemia with a small further increase at 8 h (Table 1). Accordingly, the rate of glycogen synthesis was significantly decreased at 5 h and was further diminished at 8 h (−32% and −67% vs 3 h respectively, $P < 0.05$). When the data from the insulin stimulated groups were pooled, a negative relationship between glycogen concentration and glucose uptake in the RQ was evident ($r^2 = 0.245$, $P = 0.004$; Fig. 1).

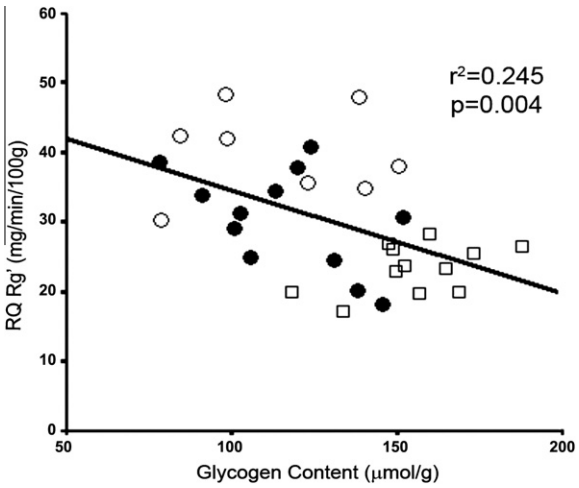

Fig. 1. Negative correlation between glucose uptake (RQ R_g') and glycogen content in the red quadriceps muscle from insulin stimulated animals. Data expressed as individual points. Open circles 3 h, closed circles 5 h, open square 8 h.

Glucose uptake (R_g') into white quadriceps (WQ) muscle was decreased at 8 h compared to 3 and 5 h glucose infusion (−33% and −27%, respectively, $P \leqslant 0.05$, Table 1) suggesting a delay in the development of insulin resistance in WQ compared to RQ. Glycogen content was increased at 3 and 5 h compared to basal with a further increase at 8 h, while the glycogen synthesis rate showed only a small, non-significant decrease at 8 h (Table 1). As in the RQ, when the data from the insulin stimulated groups were pooled, there was a negative relationship between glycogen content and glucose uptake, albeit weaker ($r^2 = 0.121$, $P = 0.05$).

During the entire 8 h glucose infusion, in the RQ, no defects in either the tyrosine phosphorylation of the insulin receptor (Fig. 2A), nor insulin receptor substrate-1 (IRS-1; Fig. 2B) were observed, although serine phosphorylation of IRS-1 at Ser^{307} was increased (Fig. 2C), compared to basal at 3, 5, and 8 h. Phosphorylation of IRS-1 at Ser$^{636/639}$ showed a similar pattern (Fig. 2D). An alteration in the insulin signaling cascade did occur at the level of Akt, with its phosphorylation at Ser^{473} decreased at 8 h of glucose infusion compared to 3 and 5 h (−50%, $P < 0.05$; Fig. 2E). Phosphorylation of Akt at Thr^{308} showed a similar trend to be decreased at 8 h; however, it did not reach significance (Fig. 2F). Two well described targets of Akt are GSK-3β and AS160. As shown in Fig. 2G and H, the phosphorylation state of these proteins was increased compared to basal values at 3, 5 and 8 h of glucose infusion ($P < 0.05$).

mTOR, a nutrient sensor and target of Akt involved in protein synthesis and many other events, showed an increased phosphorylation at 5 h glucose infusion when compared to basal and 3 h but was decreased slightly from 5 h levels at 8 h (Fig. 3A). p70S6K, a target of mTOR, showed similar changes to that of mTOR (Fig. 3B).

In the WQ, consistent with the delay in the development of insulin resistance, there was no decrease in the phosphorylation status of IR (Fig. 4A), AS160 (Fig. 4C) and more importantly, Akt (Fig. 4B). While GSK3β looked as if it increased at all time points over basal, this did not reach statistical significance (Fig. 4D).

Incubated muscles

We next conducted *ex vivo* experiments to assess glucose transport in isolated soleus muscles, obtained from rats infused with glucose. This would allow us to determine if the first step in glucose uptake is affected by hyperglycaemia (i.e. glucose transport

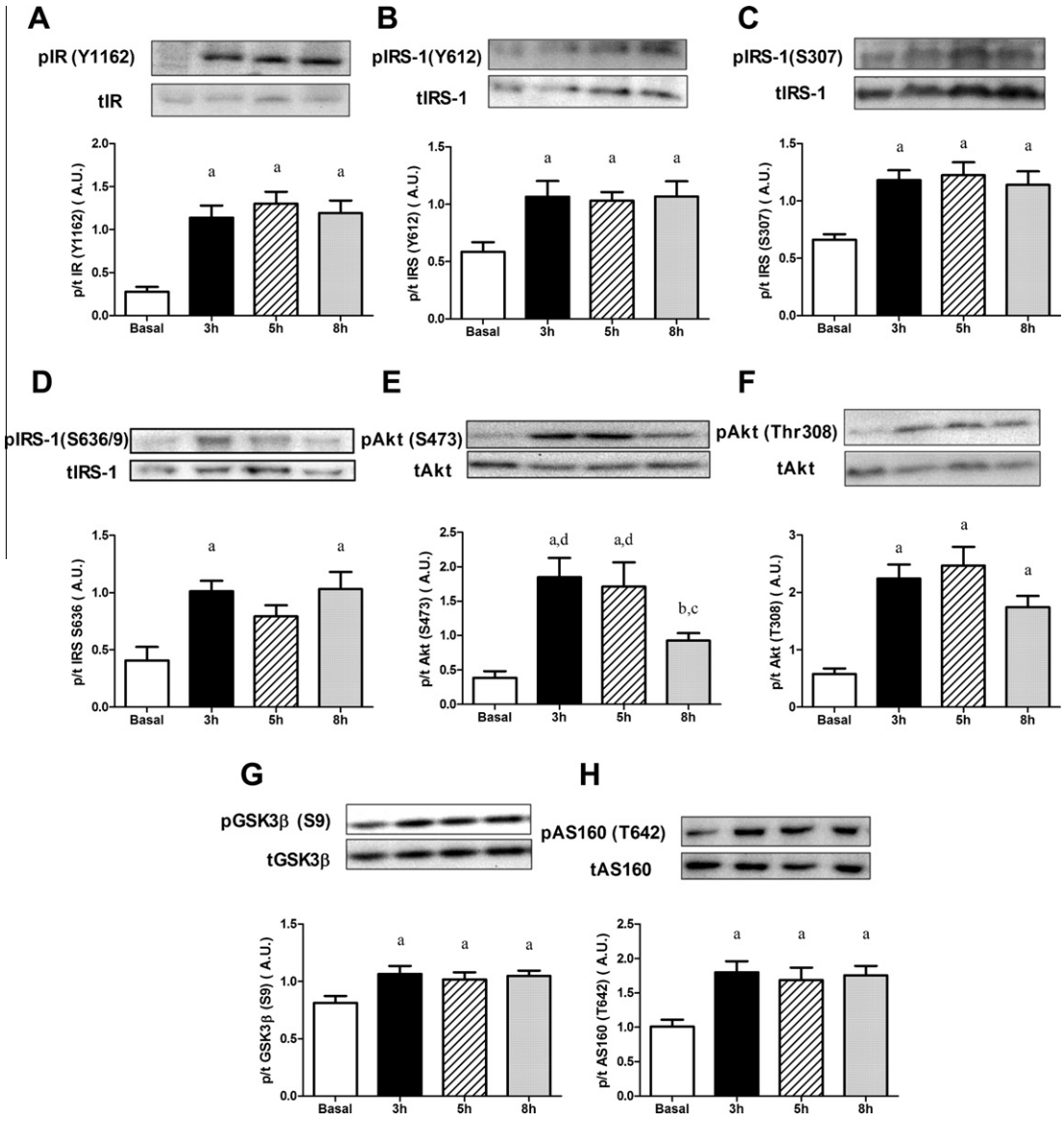

Fig. 2. Effects of glucose infusion on phosphorylation of insulin signaling intermediates in red quadriceps muscle. (A) Insulin receptor (IR Tyr^{1162}); (B) insulin receptor substrate-1 (IRS-1 Tyr^{612}); (C) IRS-1 (Ser^{307}); (D) IRS-1 ($Ser^{636/9}$); (E) Akt (Ser^{473}); (F) Akt (Thr^{308}); (G) glycogen synthase kinase-3β (GSK-3β; Ser^{9}); (H) Akt substrate of 160 kDa (AS160; Ser^{642}). Data expressed as means ± SEM. n = 6–15 rats per group $^{a}P < 0.05$ vs basal, $^{b}P < 0.05$ vs 3 h, $^{c}P < 0.05$ vs 5 h, $^{d}P < 0.05$ vs 8 h.

across the membrane). These studies revealed that insulin stimulated glucose transport was similar at all time points (Fig. 5A). Likewise, as was observed in the red quadriceps *in vivo*, the isolated soleus showed decreased phosphorylation of Akt Ser^{473} at 8 h, and increased glycogen content and decreased glycogen synthesis at 3, 5, 8 h (Fig. 5B, C and D).

Similar to the soleus, the EDL showed no alterations in glucose transport across the membrane in any of the time points (Fig. 5E). Although at 8 h there seemed to be a decrease, this was not significant and more importantly, the ability of insulin to stimulate transport across the membrane (i.e. the change from basal) was not different. As with the *in vivo* WQ, ex vivo insulin stimulation of EDL strips showed no decrease in Akt phosphorylation, an increase in the glycogen content and delayed decrease in glycogen synthesis (5F, G and H).

AMPK activity, lipid content and glycolytic intermediates in vivo

AMP-activated protein kinase (AMPK) is known to be an important nutrient sensor and has been linked to the regulation of glycogen synthesis [14]. In the RQ we found no change in AMPKα1 activity (Fig. 6A); however, AMPKα2 activity was decreased at all three time points compared to baseline values in glucose infused animals (Fig. 6B). In keeping with this finding, the phosphorylation of ACC was decreased at 3, 5 and 8 h (Fig. 6C) and the concentration of malonyl CoA was increased at 5 and 8 h of glucose infusion (Fig. 6D). When data were pooled from all groups, a negative relationship was found between AMPKα2 activity and glycogen content ($r^2 = 0.253$, $P = 0.012$; Fig. 6E). As with the RQ, AMPKα1 activity did not change in the WQ (Fig. 6F). Consistent with the delay in insulin resistance development in WQ, there was a decrease in AMPKα2 activity at 5 and 8 h (Fig. 6G). Unlike the RQ there was no relationship between AMPKα2 activity and glycogen content ($P = 0.973$, $r^2 < 0.001$).

To investigate if changes in lipid intermediates might have played a role in the decreased phosphorylation of Akt in RQ at 8 h, LCACoA, DAG and ceramide levels were measured. Table 2 shows that the hyperglycemic insult reduced LCACoA levels to a similar extent at each time point ($P < 0.05$) and increased DAG levels at 5 and 8 h when compared to basal and 3 h ($P < 0.05$).

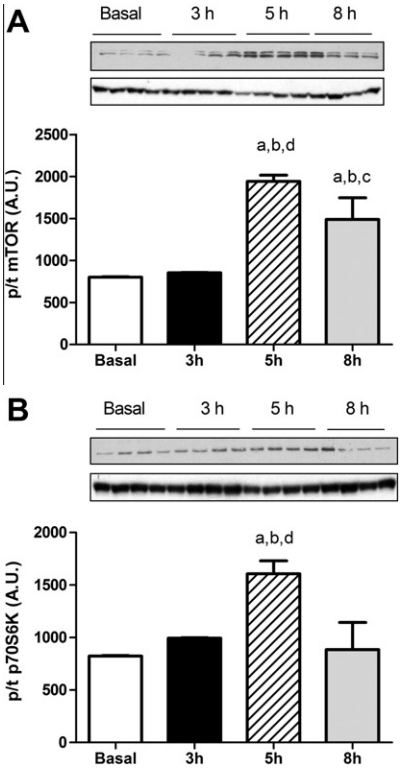

Fig. 3. Phosphorylation of (A) mTOR and (B) p70S6 K after glucose infusion. Data expressed as means ± SEM. $n = 4$ rats per group $^{a}P < 0.05$ vs basal, $^{b}P < 0.05$ vs 3 h, $^{c}P < 0.05$ vs 5 h, $^{d}P < 0.05$ vs 8 h.

Interestingly, DAG-Kinase activity was decreased after 5 and 8 h of glucose infusion which could account for the increased DAG levels observed. Since malonyl CoA is an inhibitor of CPT1 and therefore of mitochondrial fatty acid oxidation, the increase in malonyl CoA at 5 and 8 h infusion (Fig 6D) could lead to an increase in the cytosolic pool of lipids and therefore contribute to the increase in DAG content seen here. Ceramide levels were increased by 50% at 5 and 8 h; however the increases were not statistically significant (Table 2). There was no relationship between either DAG or ceramide content and Akt phosphorylation ($P = 0.535$, $r^2 = 0.04$ and $P = 0.457$, $r^2 = 0.05$, respectively). Glucose metabolites were also measured and there was a significant increase in cellular citrate levels after 8 h of glucose infusion and trends for increased G6P and decreased FDP at this time, neither of which were statistically significant (Table 2).

Discussion

The present work extends previous studies showing firstly that decreased glucose uptake in muscle in response to 5 h of hyperglycemia is not accompanied by defects in insulin signaling in skeletal muscle [6] and secondly that suppression in AMPK activity occurs in this model [7]. We confirm these findings but go onto make further novel findings that help to characterize the early onset of insulin resistance in skeletal muscle in response to excess glucose availability. We demonstrate that the AMPK activity suppression precedes the onset of insulin resistance and occurs in concert with activation of the mTOR pathway, also involved in nutrient signaling. By prolonging the hyperglycemia beyond 5 h, a further reduction in red quadriceps muscle glucose uptake occurs. This took place with a selective perturbation of one component of the insulin signaling pathway, namely a 50% reduction in the phosphorylation of Akt at Ser^{473} (a key site for maximal kinase activity). Interestingly, this reduction is not associated with decreases in either GSK3β or AS160 phosphorylation *in vivo* and the stimulation of glucose transport by insulin in isolated soleus muscle from these rats was not diminished. In addition, by extending the infusion

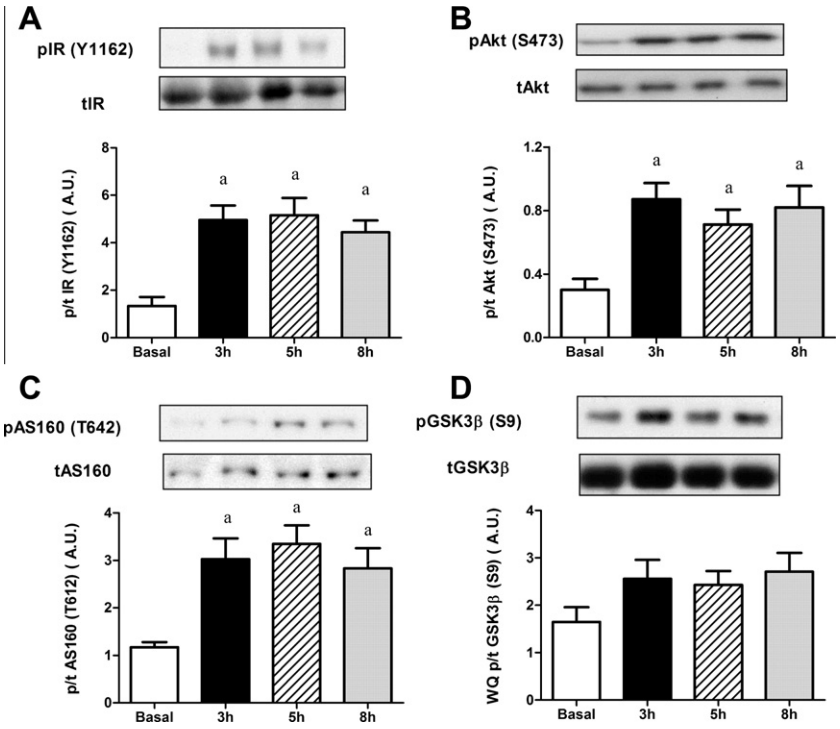

Fig. 4. Effects of glucose infusion on phosphorylation of insulin signaling intermediates in white quadriceps muscle. (A) Insulin receptor (IR Tyr^{1162}); (B) Akt (Ser^{473}); (C) glycogen synthase kinase-3β (GSK-3β; Ser^{9}); (D) Akt substrate of 160 kDa (AS160; Ser^{642}). Data expressed as means ± SEM. $n = 6$–13 rats per group $^{a}P < 0.05$ vs basal.

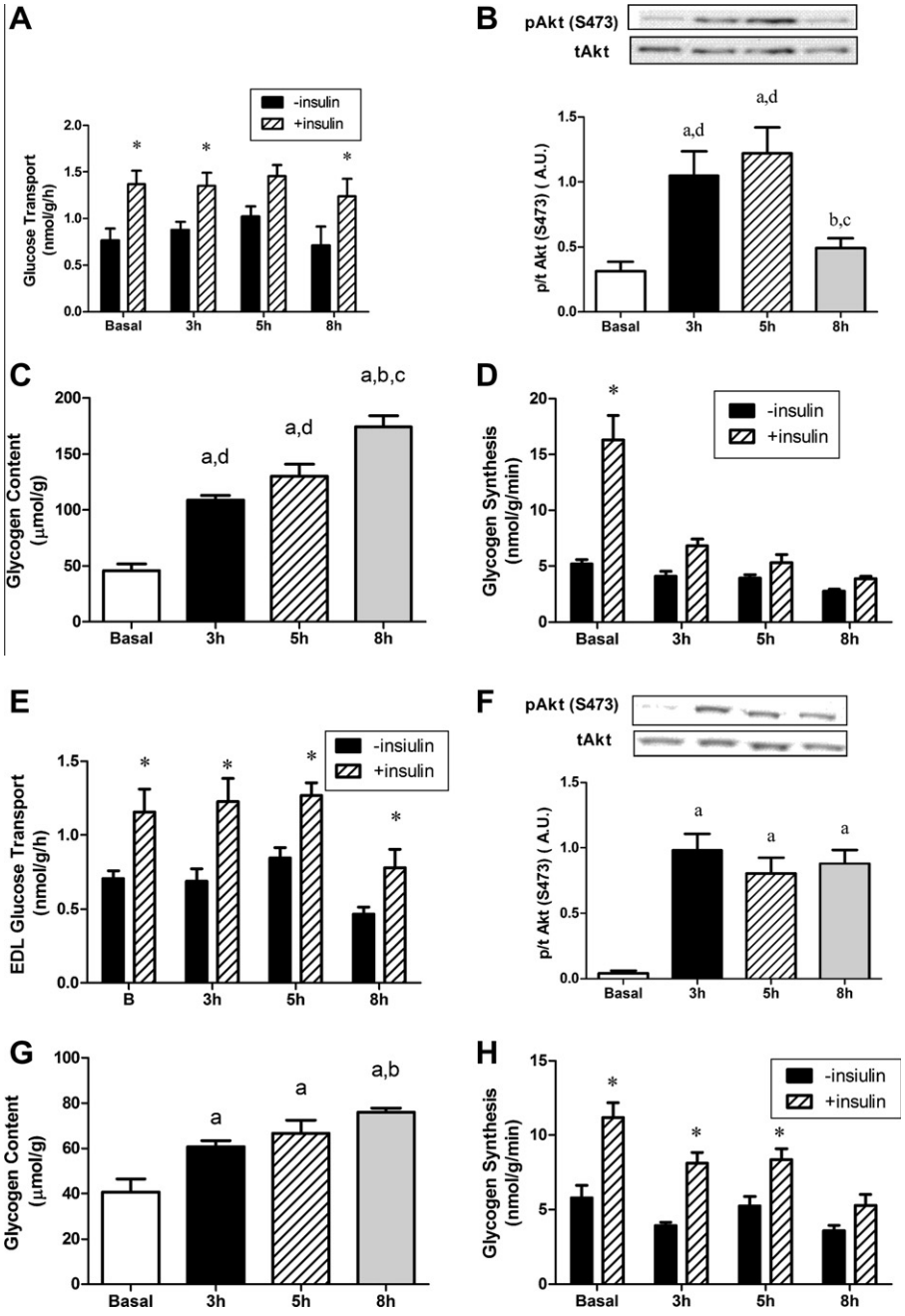

Fig. 5. Effects of glucose infusion on (A and E) glucose transport, (B and F) Akt (Ser^{473}) phosphorylation, (C and G) glycogen content, and (D and H) glycogen synthesis rate in response to insulin in isolated soleus (A–D) or EDL (E–H) muscles. Data expressed as means ± SEM. n = 4–6 rats per group $^{a}P < 0.05$ vs basal, $^{b}P < 0.05$ vs 3 h, $^{c}P < 0.05$ vs 5 h, $^{*}P < 0.05$ vs insulin.

to 8 h we show that while similar metabolic changes occur in white muscle (quadriceps) to that in red muscle, these changes are delayed (significant after 8 h but not 5 h infusion). It is noteworthy that this occurs without significantly altered Akt phosphorylation that may occur later. Our studies do not establish causality among the early muscle AMPK and mTOR changes and later insulin resistance. However, based on the time course data obtained here, taken with a recent in vitro study where insulin resistance is prevented by maintenance of AMPK activity in the presence of glucose elevation [15], we hypothesize that AMPK plays an important regulatory role influencing insulin potency in response to increased nutrient availability.

Under conditions of glucose oversupply, in the presence of insulin (e.g. during a glucose infusion *in vivo),* one mediator of decreased muscle glucose metabolism may be the degree of accumulation of muscle glycogen. Here we confirm previous reports demonstrating that muscle glycogen levels correlate negatively with glucose uptake (Fig. 1) [16,17]. While the precise mechanism for this remains uncertain, a number of reports attribute this to either inhibition of glucose transport, hexokinase and/or glycogen synthase activity [18–20]. Hyperglycemia has been shown to lead to a shift of the rate limiting step from glucose transport to post-transport steps [21].

AMPKα2 but not AMPKα1 activity was decreased in both red and white muscle prior to any decrease in glucose uptake and any changes to the insulin signaling pathway (Fig. 4). It recently has been hypothesized that when the pool of muscle glycogen is enlarged, more AMPK is bound via its glycogen binding domain

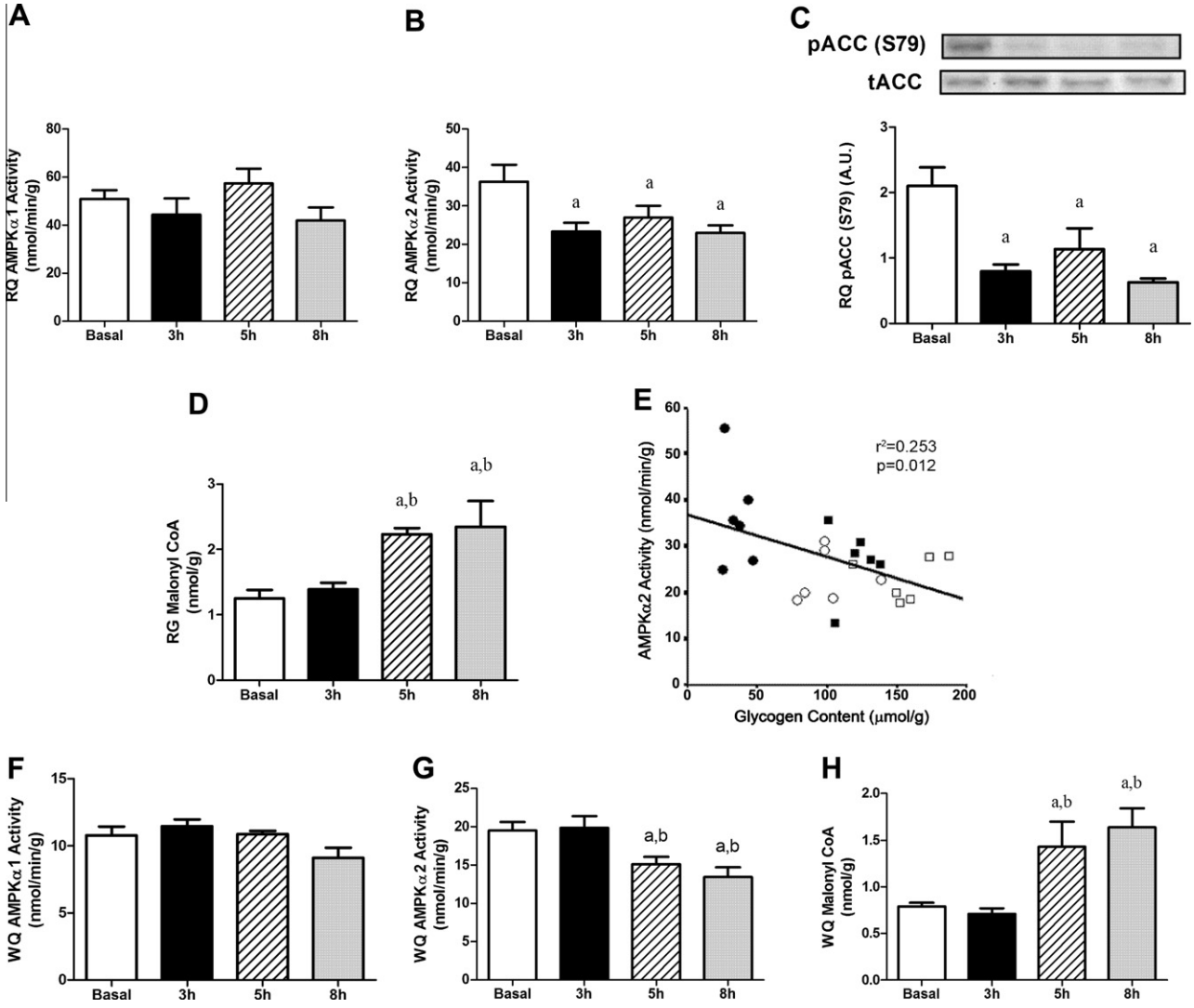

Fig. 6. AMPKα1 (A) or α2 (B) activities, malonyl CoA content (C) phosphorylation of ACC (D) and (E) negative correlation between AMPKα2 activity and glycogen content in red quadriceps following glucose infusion. AMPKα1 (F) or α2 (G) activities, malonyl CoA content (H) in white quadriceps muscle. Data expressed as means ± SEM, except (D) individual points. n = 4–6 rats per group $^{a}P < 0.05$ vs basal, $^{b}P < 0.05$ vs 3 h. Closed circles basal, open circles 3 h, open square 5 h, closed square 8 h.

to glycogen where it phosphorylates glycogen synthase on site 2, decreasing its activity [14]. Since glucose incorporation into muscle glycogen is perhaps its major fate in a rat infused with glucose, a decrease in glycogen synthase activity could have been a major contributor to the decreased glucose uptake seen here.

A test of the role of suppression of AMPK activity on subsequent insulin resistance could conceivably be made by interventional studies such as concomitant AICAR infusion. This was investigated in pilot studies (not shown) but these confirmed that due to the inhibitory effects of AMPK activation on the pancreatic β-cell to release insulin [22,23], systemic infusion of AICAR cannot easily be interpreted in the current context. However, studies in isolated muscle strips have shown co-incubation in high glucose (+/- insulin) and AICAR can reverse the decreased AMPK activity, Akt phosphorylation [15] and the decreased glucose uptake [24] associated with hyperglycaemia. These studies, combined with our own data, suggest a role for AMPK in the development of insulin resistance due to hyperglycemia.

The present study indicates that decreased Akt phosphorylation is a relatively late marker of the decrease in glucose uptake by the red quadriceps muscle since it developed between 5 and 8 h of glucose infusion. Akt needs to be phosphorylated on two sites for full activation [25]. Thus, the decrease in its phosphorylation at Ser^{473} should theoretically have been sufficient to decrease the kinase activity of the enzyme. However, there was no change in the phosphorylation state of two well described targets of Akt, GSK-3β or AS160 (Fig. 2E and F). Furthermore, insulin stimulated glucose transport in isolated soleus muscle strips was unaltered at 8 h glucose infusion (Fig. 3D), indicating that the glucose transport step is unaltered. Thus decreased Akt phosphorylation of the magnitude observed may not be responsible for the further decline in glucose uptake during the 5–8 h time period. The non-linearity of Akt phosphorylation and downstream responses has support in the literature both from cell and animal studies. Whitehead et al. [26] showed that in cultured adipocytes, only 20% of the total Akt pool needs to be phosphorylated for maximal glucose uptake (as measured by 2DG uptake) to occur. More recently, Hoehn and colleagues [27] showed that in L6 myotubes, GLUT4 translocation is maximal despite as little as 5% of the Akt pool being phosphorylated. In rats, 3 days of high fat feeding caused adiponectin resistance in skeletal muscle and this occurred with a decrease in Akt Ser^{473} phosphorylation but without changes in glucose uptake [28]. An important point from the current study is that although we observed a defect in Akt phosphorylation, the level was still a

Table 2
Lipid intermediates, DAG-Kinase activity and glucose metabolites in red muscle of basal and glucose infused animals.

	Basal	Time of glucose infusion		
		3 h	5 h	8 h
Lipid intermediates				
LCACoA (nmol/g)	21 ± 2	5 ± 1[a]	5 ± 1[a]	8 ± 1[a]
Ceramide (nmol/g)	25 ± 7	27 ± 8	43 ± 8	40 ± 9
DAG (nmol/g)	100 ± 24	112 ± 43	290 ± 18[a,b]	310 ± 18[a,b]
DAG-kinase (a.u.)	2434 ± 66	2240 ± 149	1461 ± 64[a,b]	1407 ± 22[a,b]
Glucose metabolites				
Citrate (μmol/g)	0.19 ± 0.01	0.20 ± 0.01	0.20 ± 0.01	0.27 ± 0.02[a,b,c]
G6P (μmol/g)	1.54 ± 0.06	1.87 ± 0.15	1.53 ± 0.05	1.96 ± 0.30
F6P (μmol/g)	0.20 ± 0.01	0.22 ± 0.02	0.22 ± 0.01	0.19 ± 0.03
FDP (μmol/g)	0.40 ± 0.06	0.29 ± 0.03	0.36 ± 0.05	0.35 ± 0.02

Data are means ± SEM. [a]$P < 0.05$ vs basal; [b]$P < 0.05$ vs 3 h; [c]$P < 0.05$ vs 5 h glucose infusion. $n = 4–13$. LCACoA, long chain acyl-CoA; DAG, diacylglycerol; G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; FDP, fructose-1,6-bisphosphate. LCACoA measured in RQ, rest measured in RG.

2-fold increase over basal and appeared sufficient to maintain the ability of insulin to stimulate glucose transport.

In the red quadriceps we have shown an increased Tyr^{612}, Ser^{307} and $Ser^{636/639}$ phosphorylation of IRS-1 from 3 h of glucose infusion (Fig. 4). We also show an increase in phosphorylation of mTOR and p70S6 K at 5 h glucose infusion when the insulin resistance is occurring (Fig. 6). mTOR, and its downstream target p70S6 K, are nutrient sensors that are known to negatively feedback on the insulin signaling cascade by phosphorylating IRS-1 on Ser^{307} and $Ser^{636/639}$ [29–31]. This is interesting as multiple studies have shown that serine phosphorylation can interfere with subsequent tyrosine phosphorylation, disrupting signal transduction [32,33]. However, a recent study by Paul et al. [34] showed a dissociation between tyrosine and serine phosphorylation. That is, animals fed a high fat diet had restored IRS-1 total tyrosine phosphorylation after a single bout of exercise and this occurred without a reduction in the increased Ser^{307} phosphorylation [34]. Hence it may be that under certain circumstances, as in the current study, there isn't a linear relationship between serine and tyrosine phosphorylation of IRS-1. Lastly, the reciprocal variation between AMPKα2 activity and elevated mTOR and p70S6 K, approximately coincident in time, supports a possible inverse cyclic interaction between these two pathways, as supported by recent genetic downregulation studies of S6 kinase [35].

Due to the lack of alterations in the phosphorylation state of proximal signaling intermediates (IR, IRS-1) it was unclear what caused the decreased Akt phosphorylation observed in the RQ after 8 h of glucose infusion. The selectivity of this defect at Akt is similar to that seen in cells treated with palmitate, or ceramide [36,37]. We observed a ~50% increase in mean ceramide content in red gastrocnemius in the current study; however, neither the increase in ceramide nor its correlation with Akt phosphorylation was statistically significant.

Our data indicates that white glycolytic muscle may be less susceptible to the onset of insulin resistance than red oxidative muscle. White quadriceps showed a significant delay in development of insulin resistance compared to red quadriceps (after 8 vs 5 h infusion). This is consistent with less glucose uptake into white muscle under equivalent conditions of insulin elevation [9]. Importantly and consistent with this finding was a delay in suppression of AMPKα2 activity in white versus red quadriceps muscle.

In conclusion, this study adds to the growing body of literature [6,27,38] that suggests that defects in the insulin signaling cascade and glucose transport are not always associated with impaired glucose uptake and metabolism in skeletal muscle. Thus other mechanisms appear to be responsible for the decrease in muscle glucose uptake and glycogen storage during the early stages of a glucose infusion (i.e. prolonged hyperglycemia and hyperinsulinemia). The results suggest that reduced AMPK activity and increased glycogen are possible contributors to these mechanisms.

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