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Abstract

Skeletal muscle is an insulin sensitive tissue and accounts for approximately 80% of post-prandial glucose disposal. This study describes the effects of insulin, delivered for 72 hours, to skeletal muscle myoblasts during differentiation or to skeletal muscle myotubes. After chronic treatment, cultures were acutely stimulated with insulin and analysed for total and phosphorylated Akt (Ser^{473}), mRNA expression of metabolic and myogenic markers and insulin-stimulated glucose uptake. Skeletal muscle cells differentiated in the presence of insulin chronically, reduced acute insulin stimulated phosphorylation of Akt Ser^{473}. In addition, there was a reduction in mRNA expression of Hexokinase II (HKII), GLUT4 and PGC-1α. Insulin-stimulated glucose uptake was attenuated when cells were differentiated in the presence of insulin. In contrast, myotubes exposed to chronic insulin showed no alterations in phosphorylation of Akt Ser^{473}. Both HKII and GLUT4 mRNA expression were reduced by chronic exposure to insulin; while PGC-1α was not different between culture conditions and was increased by acute insulin stimulation. These data suggest that there are differential responses in insulin signalling, transcription and glucose uptake of skeletal muscle cells when cultured in either the presence of insulin during differentiation or in myotube cultures.

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Introduction

In healthy individuals, an increase in post-prandial blood glucose concentration leads to an increase in plasma insulin levels (Goodyear et al., 1996). This increase enables effective glucose disposal in insulin-sensitive tissues such as adipose, liver and skeletal muscle thus maintaining blood glucose homeostasis (Wasserman, 2009). Skeletal muscle is responsible for the majority of post-prandial glucose uptake (DeFronzo et al., 1983), and a diminished response of skeletal muscle to insulin (insulin insensitivity) is a characteristic of metabolic diseases such as type II diabetes mellitus (Abdul-Ghani and Defronzo, 2010).

In states of metabolic disease, skeletal muscle is continuously exposed to abnormal systemic concentrations of glucose, fatty acids, insulin and cytokines (Pendergrass et al., 1998; Zierath et al., 1998). Specifically, hyperinsulinemia', has been considered both a consequence as well as driver of insulin resistance (Corkey, 2012), and can influence insulin signalling and glucose uptake in metabolic cells types such as adipocytes and skeletal muscle (Gonzalez et al., 2011; Kumar and Dey, 2003; Ricort et al., 1995). The precise mechanisms underpinning skeletal muscle insulin insensitivity in metabolic diseases is yet to be fully understood, however, the culture of skeletal muscle cells *in vitro* provides a system to understand cellular mechanisms regulating muscle adaptation and the pathogenesis of skeletal muscle insulin resistance, independent of the systemic environment (Aas et al., 2013). Various models of skeletal muscle insulin resistance have been developed using both cell lines and primary human cells (Aas et al., 2013; Jové et al., 2006; Nedachi et al., 2008). Indeed, these *in vitro* cellular models have investigated the effects of a variety of factors such as pro-inflammatory cytokines, fatty acids and insulin upon skeletal muscle insulin signalling and glucose uptake (del Aguila et al., 2011; Jové et al.,

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2006; Kumar and Dey, 2003; Philp et al., 2010). In particular, exposure to high levels of insulin, as are common in metabolic disease states, have been reported to impair proximal insulin signalling in skeletal muscle cells (Kumar and Dey, 2003).

Although progress has been made in establishing *in vitro* models of insulin resistant skeletal muscle, little attention has been paid to the potential influence of the myogenic programme. Both *in vivo* and *in vitro*, proliferating mononuclear myoblasts undergo myogenesis to form multinucleate terminally differentiated myotubes (Buckingham et al., 2003), characterised by elevations in the expression of the myogeninc regulatory factor Myogenin (Zammit, 2017). As such, two distinct phases exist whereby an insulin resistant muscle model could be established; either during myogeneis, such that the nascent myotubes are insulin resistant, or following myogenesis, where insulin resistance is induced in pre-existing myotube cultures. Indeed, although both methods have been used to investigate development of insulin insensitivity in muscle cells *in vitro* (del Aguila et al., 2011; Kumar and Dey, 2003), a consensus on the most suitable method for inducing this response is not currently clear.

Therefore, the current experiments sought to determine if culture of skeletal muscle cells in the presence of insulin, which is a common symptom during the onset of insulin resistance and diabetes, would lead to disturbances in insulin-stimulated intracellular signalling, mRNA expression of key myogenic and metabolic genes and glucose uptake in C2C12 skeletal muscle cells. In this report, we show that myogenic cells cultured in the presence of insulin affects these cells more during myogenesis, whereas there were minimal effects of insulin exposure in post-mitotic skeletal muscle myotubes.

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Methodology

Cell Culture

C2C12 myoblasts were grown using standard growth medium (GM) (Dulbecco's Modified Eagle's Medium (DMEM)) (Fisher –Scientific, Loughborough, UK), 20% fetal bovine serum (FBS) (Dutscher Scientific, EU approved, Essex UK), and, 1% Penicillin/Streptomycin (P/S) (Gibco, Invitrogen, Paisley, UK). Cells were harvested with the use of trypsin–EDTA (Sigma-Aldrich, Dorest, UK) at 80-90% confluence and subsequently counted using the trypan blue exclusion method and seeded at a density of 15,000 cells·cm^2 in 6 well plates. Conditions were conducted in triplicate wells and repeated across a minimum of three independent experiments.

Exposure of differentiating myoblasts to insulin

C2C12 myoblasts were grown to confluence before being changed to either a control differentiation media (CONTROL) (DMEM + 2% Horse Serum (HS) (Sigma-Aldrich, Dorset, UK)), or an insulin supplemented media (INSULIN) (DMEM + 2% HS and 100 nM Insulin). Human recombinant insulin was purchased from Sigma-Aldrich (Sigma-Aldrich, Dorset, UK). Myoblasts were incubated in their respective medias for three days with the media changed twice daily as described previously (Kumar and Dey, 2003) (*Figure 1a*). The culture media was changed one hour prior to acute insulin stimulation. Cells were then washed twice in Krebs Ringer HEPES (KRH) buffer (10 mM HEPES pH 7.4, 138 mM NaCl, 4.7 mM KCl, 1.25 mM $CaCl_2$, 1.25 mM MgSO, 5 mM Glucose and 0.05% BSA) and subsequently incubated twice in KRH buffer for 30 minutes before being stimulated for five minutes in KRH with insulin (100 nM) or left un-stimulated. Cells were then washed twice in PBS before being lysed for protein or RNA extraction.

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Exposure of skeletal muscle myotubes to insulin

C2C12 myoblasts were cultured until confluence, and differentiated in DM media for three days to encourage the formation of multinucleate myotubes. Subsets of wells, in triplicate, were acutely stimulated with insulin for five minutes prior to sampling for protein and RNA extraction (PRE). Further wells of myotubes, in triplicate, were either chronically treated with insulin (INSULIN) or kept in control DM for three days (CONTROL), prior to the acute five-minute insulin treatment as described above. Myotubes were sampled immediately following this acute insulin stimulation, for protein and RNA extraction. This protocol allowed for the examination of both basal chronic and acutely insulin-stimulated effects of chronic insulin treatment (*Figure 1b*).

[INSERT FIGURE 1]

Measurement of ^{3}H-Deoxy-D-Glucose uptake.

Glucose uptake was determined using ^{3}H-Deoxy-D-Glucose (^{3}H-2DG) as previously described (Nedachi and Kanzaki, 2006), with some slight modifications. Briefly, following 4 hr serum starvation, experimental plates (chronic treatments only) were washed twice in KRH buffer and incubated for 15 minutes in either the absence or presence of 100 nM insulin. Glucose uptake was determined by the addition of ^{3}H-Deoxy-D-Glucose (0.1 mM, 1μCi·mL; PerkinElmer Life and Analytical Science) for 30 minutes. After incubation, cells were washed in PBS and lysed in 0.2 M Sodium Hydroxide (NaOH) in phosphate buffered saline (PBS). Glucose uptake was assessed by liquid scintillation counting (Pakard-Bell). Data was normalised to total protein collected from parallel experimental plates.

Western Blotting

Cells were homogenised in lysis buffer (50 mM HEPES, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 50 mM NaF, 50 mM β-Glycerophosphate, 1 mM Na_3VO_4, 1%

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Triton X-100, 2 mM PMSF) and protein content of the samples was determined using the Pierce 660nm protein assay (Thermo-Fisher, Loughborough, UK). Prior to analysis, samples were mixed in Laemmli buffer (4 mL H_2O, 1mL 1M TRIS HCl pH 6.8, 0.4mL Glycerol, 0.4mL 2-β-mercaptoethanol, 0.05% bromophenol blue), and boiled for 5 minutes at 95ºC. Thereafter, 20µg of protein per sample was loaded into SDS-polyacrylamide gels (4% stacking and 12% resolving) for separation by electrophoresis (SDS-PAGE). Proteins were then wet transferred on to nitrocellulose membranes (Whatman Proton, Sigma-Aldrich, Dorset, UK), for 2 hr at a constant current of 0.35 Amps. Membranes were blocked for 1h with bovine serum albumin (BSA) (Sigma-Aldrich, Dorset, UK) at 4ºC and rinsed three times in Tris Buffered Saline with Tween (Tris, NaCl, pH 7.4, Tween-20, TBST), before being incubated with primary antibody overnight at 4ºC. Primary antibodies used in the experiments were, Akt, phospho Akt (Ser^{473}) and β-Actin (Cell Signalling, MA, USA). Following overnight primary antibody incubation, membranes were washed in TBST and subsequently incubated with anti-rabbit horseradish peroxidase-conjugated secondary antibody (Cell Signalling, MA, USA). Proteins were visualised using chemiluminescence substrate (Supersignal, ThermoFisher Scientific, Rockford, IIL, USA) and band densities were quantified using Quantity One image analysis software (Quality One 1-D analysis software version 4.6.8). Phosphorylation of proteins was normalised to the respective total protein and a housekeeping protein (β-Actin).

RNA extraction and qPCR analysis

RNA was extracted using TRI Reagent (Sigma-Aldrich, Dorset, UK) according to manufacturer's instructions and concentrations (ng/µL) and purity of RNA samples were determined using UV spectroscopy (NanoDrop, Fisher Scientific,

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Loughborough, UK). Gene expression was analysed by One-step reverse transcription-PCR using a Stratagene Mx3005p thermocycler (Agilent Technologies LDA UK Limited, Cheshire, UK). Primer sequences were synthesised by Sigma Aldrich (Sigma-Aldrich, Dorset, UK) as shown in Table 1. Each reaction consisted of 70 ng of RNA diluted in 9.5μL of nuclease free water, 0.15 μL forward primer, 0.15 μL reverse primer (100 μM), 10 μL Quantifast SYBR Green Mix (Qiagen, Crawley, UK) and 0.2 μL of reverse transcriptase (RT)-Mix (Qiagen, Crawley, UK) to constitute a final 20 μL reaction volume (Qiagen Chemistries, Crawley, UK). RT-PCR was conducted using the following steps: 10 min hold at 50 °C (reverse transcription), followed by a 5 min hold at 95 °C, and cycling between 95 °C for 10 s (denaturation) and 60 °C for 30 s (annealing and extension). Fluorescence was detected after every cycle (40 cycles) and data was analysed using the $\Delta\Delta C^T$ method using RNA polymerase II beta (POLR2B) as an endogenous control gene.

[INSERT TABLE 1]

Statistical Analysis

Data is presented as Mean ± S.E.M. Differences between conditions were analysed by ANOVA with post-hoc Bonferroni using SPSS (IBM SPSS, Version 19, NY, USA). When variances between conditions were found to be significant, Welch's F ratio was used to analyse significance with post-hoc Games-Howell to show differences between conditions. Significance level was set at equal to or less than $p < 0.05$.

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Results

^{3}H-Deoxy-D-Glucose uptake following acute and chronic insulin exposure in differentiating myoblasts and skeletal muscle myotubes

Firstly, the effect of chronic insulin treatment on insulin stimulated glucose uptake was investigated in C2C12 cells during myogenesis or in pre-existing myotubes.As expected, skeletal muscle cells differentiated in control media showed a significant increase in glucose uptake when stimulated with insulin for 30 minutes compared to basal control ($p < 0.05$) (*Figure 2a*). However, this response was not replicated in myoblasts differentiated in the presence of insulin ($p > 0.05$), which were found to have an increased basal level of glucose uptake. This increase in basal uptake was significantly greater than the basal uptake in myoblasts differentiated in control conditions ($p < 0.01$), suggesting that chronically elevated levels of insulin in this system results in elevated basal glucose uptake, independent of the action of exogenous insulin (*Figure 2a*). What is more there was no added effect of acute insulin stimulation upon glucose uptake following differentiation in the presence of insulin ($p > 0.05$).

Glucose uptake in skeletal muscle myotubes was analysed following differentiation and following culture in the presence of insulin (*Figure 2b*). Despite small increases in glucose uptake, there was no significant difference between basal and insulin stimulated myotubes differentiated for 3 days (PRE) ($p > 0.05$). Similarly, myotubes cultured in control media for a further 3 days (CONTROL) showed a small increase in glucose uptake following acute insulin stimulation compared to basal conditions however this was not statistically significant ($p > 0.05$). In myotubes cultured in the presence of insulin (INSULIN), there was an increase in basal uptake, similar to that

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observed in skeletal muscle cells differentiated in the presence of insulin, however the differences between conditions, and the effect of acute insulin stimulation, were not found to have any significant effect ($p > 0.05$).

[INSERT FIGURE 2]

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The effects of chronic insulin exposure on Akt Ser^{473} phosphorylation in myoblasts during differentiation.

To investigate the effect of insulin exposure on intracellular signalling proteins, expression of the phosphorylated Akt Ser^{473} was analysed due to its central role in recruitment of downstream insulin signalling proteins. Myoblast differentiation in the presence of insulin had a significant main effect upon the phosphorylation of Akt Ser^{473} ($p < 0.05$, *Figure 3*). Acute insulin stimulation increased the phosphorylation of Akt Ser^{473} compared to basal levels in skeletal muscle cells differentiated in control media ($p < 0.05$, *Figure 3*). However, stimulation with insulin did not increase phosphorylated Akt Ser^{473} in cells differentiated in the presence of insulin ($p > 0.05$, *Figure 3*), which remained similar to basal levels in the control untreated condition ($p > 0.05$, *Figure 3b*). This suggests that three days of insulin treatment throughout the differentiation period, contributes towards a reduced capacity for Akt Ser^{473} phosphorylation by acute insulin stimulation, compared to myoblasts differentiated in normal DM conditions.

[INSERT FIGURE 3]

mRNA expression in response to acute and chronic insulin exposure in skeletal muscle cells during myogenesis.

In order to investigate how the differences in insulin signalling between conditions had an effect upon transcriptional markers, the mRNA expression of genes which play a role in metabolism and myogenesis were analysed. Acute stimulation with insulin, significantly increased HKII mRNA expression in cells cultured in control media ($p <0.01$), however no change was observed in myoblasts differentiated in the

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presence of insulin ($p > 0.05$, *Figure 4*). GLUT4 mRNA expression reduced following acute insulin stimulation in skeletal muscle cells differentiated in control media ($p < 0.05$). Cells differentiated in the presence of insulin were found to express lower GLUT4 mRNA expression, compared to the expression in cells cultured in control media ($p < 0.05$, *Figure 4*) which was not affected by acute insulin stimulation. In contrast, PGC-1α mRNA expression was different between conditions ($p < 0.01$), increasing following acute insulin stimulation in cells cultured in control media to a level which approached significance ($p = 0.07$), whereas there was no difference in PGC-1α mRNA expression as a result of acute insulin stimulation in skeletal muscle cells differentiated in the presence of insulin ($p > 0.05$, *Figure 4*). In addition to investigating metabolic genes, the effect of insulin exposure on the myogenic transcription factor, myogenin, was investigated, as it is a pivotal regulator of skeletal muscle differentiation. Myoblasts differentiated in control media showed an increase in myogenin mRNA expression ($p < 0.05$), compared to basal when stimulated with insulin. In contrast, culture with insulin resulted in lower expression of myogenin mRNA when stimulated with insulin, compared to basal when differentiated in the presence of insulin ($p < 0.05$, *Figure 4*). This would suggest that acute insulin stimulation can induce changes in the mRNA expression of metabolic genes and subsequent culture in the presence of insulin can attenuate the induction of these genes by acute insulin stimulation. In contrast, myogenin expression is influence more by chronic culture in insulin rather than acute insulin stimulation.

[INSERT FIGURE 4]

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The effects of acute and chronic insulin exposure on the expression of key signalling proteins in post-mitotic skeletal muscle myotubes.

After three days differentiation, acute insulin stimulation significantly increased the phosphorylation of Akt Ser^{473} compared to basal levels ($p < 0.05$). Myotubes cultured in control media for a further three days also increased phosphorylation of Akt Ser^{473} as a result of insulin stimulation compared to basal control; however, the differences between the basal control and insulin stimulation were not significantly different ($p > 0.05$). In contrast, myotubes cultured in the presence of insulin showed no changes in phosphorylated Akt Ser^{473} between basal and acute insulin stimulation ($p > 0.05$) (*Figure 5*). Together this suggests that, acute insulin stimulation was not effective in significantly inducing phosphorylation Akt in skeletal muscle myotubes cultured in control media or in the presence of insulin.

[INSERT FIGURE 5]

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mRNA expression of metabolic and myogenic markers in response to acute and chronic insulin exposure in skeletal muscle myotubes.

In myotube cultures, a significant main effect was observed in HKII mRNA expression ($p < 0.05$). Myotubes which were cultured in the presence of insulin showed a reduction in expression of HKII compared to differentiated myotubes ($p < 0.05$) (*Figure 6*), with a similar response observed for GLUT4 mRNA expression ($p < 0.05$) (*Figure 6*). Acute insulin stimulation had no effect upon HKII or GLUT4 mRNA expression in any of the conditions over basal control ($p > 0.05$), but acute insulin did significantly increase mRNA expression of PGC-1α ($p < 0.05$), independent of condition ($p > 0.05$) (*Figure 6*). Myogenin mRNA expression showed no significant main effects for culture condition or acute insulin stimulation ($p > 0.05$); however, there was a significant interaction effect ($p < 0.05$). Acute insulin stimulation significantly increased myogenin expression in differentiated skeletal muscle myotubes ($p < 0.05$), although this effect was not observed in myotubes cultured in control media ($p > 0.05$). There was however, a trend for a reduction in myogenin expression following acute insulin stimulation in myotubes cultured in the presence of insulin ($p = 0.06$) (*Figure 6*), demonstrating a divergent response in the early and late myotube conditions.

[INSERT FIGURE 6]

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Discussion

Insulin stimulates glucose uptake in a wide variety of cell types and contributes to a number of other cellular functions however, insulin stimulated glucose disposal is highest in metabolic tissues such as skeletal muscle (DeFronzo et al., 1983). Despite of the fact that insulin-mediated glucose utilisation is high in metabolic tissues such as skeletal muscle, continuous exposure to elevated levels of insulin, such that occur in metabolic diseases, can have negative effects on cellular metabolism (Kumar and Dey, 2003; Pagel-Langenickel et al., 2008). The development of *in vitro* models of skeletal muscle insulin resistance are undoubtedly useful tools for defining the underpinning mechanisms, and as such the most suitable methods to induce this phenotype should be explored.

Using a model of hyperinsulinemia to induce insulin resistance, we investigated the impact of exposure to insulin during myogenesis of skeletal muscle cells and in differentiated skeletal muscle myotubes. Our results indicate that insulin stimulated glucose uptake was altered as a result of exposure to insulin during myogenesis. Similar responses have been observed previously in C2C12 skeletal muscle cells using a similar model of hyperinsulineamic induced insulin resistance (Kumar and Dey, 2003, 2002). The similar findings between our work and those of Kumar and Dey provides validation of the use of this method of exposing skeletal muscle cells to insulin during myogenesis for induction of insulin insensitivity. To see if the findings could be translated into skeletal muscle myotubes, we next investigated the method in inducing skeletal muscle insulin resistance following myogenic differentiation. While the trends were similar to those observed in skeletal muscle cells during myogenesis, the changes were not significant, suggesting that the changes in

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glucose uptake following hyperinsulinemic induced insulin resistance were more prominent during the differentiation of skeletal muscle cells.

In order to investigate the impact of the changes in glucose uptake which were observed, analysis of the phosphorylation of Akt (Ser^{473}) was investigated. Acute insulin stimulation significantly augmented Akt phosphorylation in skeletal muscle cells following myogenesis; however, phosphorylation of Akt was not increased following acute insulin stimulation following chronic culture in control or insulin media during cell differentiation. Often an indicator of insulin signalling activation, impaired Akt phosphorylation is attenuated in insulin resistant skeletal muscle (Karlsson et al., 2005). Other in vitro research in skeletal muscle and adipocyte cells has reported impaired proximal insulin signalling, specifically at the site of the insulin receptor, in a model of hyperinsulineamic induced insulin resistance (Kumar and Dey, 2003; Ricort et al., 1995). Therefore, the changes in Akt phosphorylation observed in skeletal muscle cells following insulin exposure are most likely a result of impaired signalling upstream of Akt.

In order to further characterise the model of hyperinsulinemia induce insulin resistance in skeletal muscle, important genes involved in metabolism, which are differentially expressed in states of insulin resistance were analysed (Ducluzeau et al., 2001). Hexokinase II is important in metabolic regulation and can be seen as a potential bio-marker of insulin resistance, due to the reduction in expression observed in skeletal muscle of insulin resistant subjects (Mandarino et al., 1995; Pendergrass et al., 1998; Vestergaard et al., 1995). While skeletal muscle cells differentiated in the presence of insulin showed an attenuated response in HKII mRNA expression following acute insulin stimulation, skeletal muscle myotubes did not respond in a similar manner. Insulin has been shown to act as a regulator of HKII

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mRNA expression in skeletal muscle (Printz et al., 1993), specifically through a PI-3 kinase dependent pathway (Osawa et al., 1996).

The transcriptional coactivator PGC1alpha appears to play a key role in energy metabolism (Wu et al., 2016). Overexpression of PGC1alpha increases GLUT4 expression and glucose uptake in skeletal muscle (Benton et al., 2008) whereas its deletion leads to abnormal glucose homeostasis (Handschin and Spiegelman, 2006). The reduction in PGC-1α mRNA expression in response to chronic exposure to insulin in differentiating skeletal muscle cells was somewhat expected based on Akt's role in mediating Foxo1 localisation with PGC1 in the nucleus (Brunet et al., 1999; Fernandez-Marcos and Auwerx, 2011). This finding lends further support to the notion that this method represents a strong model for inducing insulin resistance since PGC1 alpha levels are attenuated in diabetic skeletal muscle. In contrast, the increase in PGC1alpha in response to acute insulin stimulation in both differentiating control cells and pre-existing myotubes regardless of chronic insulin treatment was surprising. Ling and colleagues have previously observed an increase in PGC1alpha mRNA levels following a hyperinsulinaemic euglycaemic clamp in young and elderly twins (Ling et al., 2004), however the mechanisms which underpin this observation are not well understood and are beyond the scope of the present investigation. Interestingly however, in our study, the transcription of PGC1alpha in myoblasts differentiated in the presence of insulin was impaired, further suggesting that metabolic control is somewhat supressed with this intervention.

In the current set of experiments, differentiating myoblasts but not myotubes, showed a reduction in GLUT4 mRNA expression following chronic exposure to insulin. The repression of GLUT4 mRNA transcription is a common physiological response following response exposure to a hyperinsulinemic environment in insulin

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sensitive tissues (Flores-Riveros et al., 1993), leading to a reduction in insulin stimulated glucose uptake. Importantly hyperinsulinaemia has been shown to diminish GLUT4 translocation upon insulin stimulation and increased basal glucose uptake in L6 skeletal muscle cells exposed to high glucose and insulin (Huang et al., 2002). In addition, although not measured in our experiments, other glucose transporters such as GLUT1 have been reported to increase following prolonged exposure to insulin (Taha et al., 1999). This change in glucose transporter expression could explain the increased basal uptake observed in C2C12 skeletal muscle cells cultured in the presence of insulin during differentiation and to an extent, in differentiated myotubes.

In conclusion, these experiments show that alterations in glucose uptake, insulin signalling, and gene transcription were affected by exposure to insulin during differentiation. This therefore describes a putative model of hyperinsulineamic induced skeletal muscle cell insulin resistance. This contrasts with the effects of hyperinsulineamia on post-mitotic skeletal myotube cultures, myotubes, where by alterations in gene transcription and glucose uptake were not characteristic of an insulin resistant phenotype. Therefore, based on our findings and the biomarkers used as indicators of potential insulin resistance (*Table 2*), skeletal muscle cells exposed to insulin during differentiation appears to be an effective method for the development of a hyperinsulinemia-induced insulin resistant skeletal muscle. This model has potential to be used as a method for inducing skeletal muscle insulin resistance *in vitro,* which could be used to investigate potential lifestyle interventions such as exercise, upon insulin resistant skeletal muscle.

[INSERT TABLE 2]

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Figure Legends

Figure 1. Protocol schematic for investigating chronic insulin exposure upon A; skeletal muscle cells during myogenesis and B; post-mitotic skeletal muscle myotubes.

Figure 2. **A**; ^{3}H-Deoxy-D-Glucose uptake in C2C12 skeletal muscle cells during myogenesis.**B** C2C12 skeletal muscle myotubes. Skeletal muscle myotubes were exposed to low serum media supplemented without (Control) or with (Insulin) the addition of insulin (100 nM). Open bars represent unstimulated filled bars represented acute 30 minutes insulin stimulation. * Significantly different to control ($p<0.05$). † Significantly different to control unstimulated ($p<0.05$). * Significantly different from control unstimulated sample ($p<0.05$).

Figure 3. Akt (Ser^{473}) phosphorylation following insulin exposure in C2C12 skeletal muscle cells during myogenesis. Open bars represent unstimulated filled bars represented acute 30 minutes insulin stimulation. * Significantly different to control ($p<0.05$). † Significantly different to control unstimulated ($p<0.05$).

Figure 4. Gene expression analysis ($\Delta\Delta C_t$) of C2C12 skeletal muscle cells differentiated in control media (CON) or chronically exposed to insulin (100 nM) (IT). **A**; GLUT4, **B**; HKII, **C**; PGC-1α, **D**; MyoG. Open bars represent unstimulated filled bars represented acute 30 minutes insulin stimulation. * Significantly different from control unstimulated sample ($p<0.05$).

Figure 5. Akt (Ser^{473}) phosphorylation C2C12 skeletal muscle myotubes following differentiation (PRE), control media (CON) or chronically exposed to insulin (100 nM) (IT). Open bars represent unstimulated filled bars represented acute 30 minutes insulin stimulation. * Significantly different to control ($p<0.05$). † Significantly different

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to control unstimulated ($p<0.05$). * Significantly different from control unstimulated sample ($p<0.05$).

Figure 6. Gene expression analysis ($\Delta\Delta C_t$) of C2C12 skeletal muscle myotubes following differentiation (PRE) and chronic incubation in control media (CON) or high insulin (100 nM) media (IT). **A**; GLUT4, **B**; HKII, **C**; PGC-1α, **D**; MyoG. Open bars represent unstimulated filled bars represented acute 30 minutes insulin stimulation. # significantly different to unstimulated sample ($p<0.05$). * Significantly different to pre-unstimulated sample ($p<0.05$). § Significantly different to Pre ($p<0.05$).

Table Legend

Table 1. Primer sequences of mouse mRNA genes used for one-step qPCR

Table 2. Summary of findings in skeletal muscle cells during myogenesis

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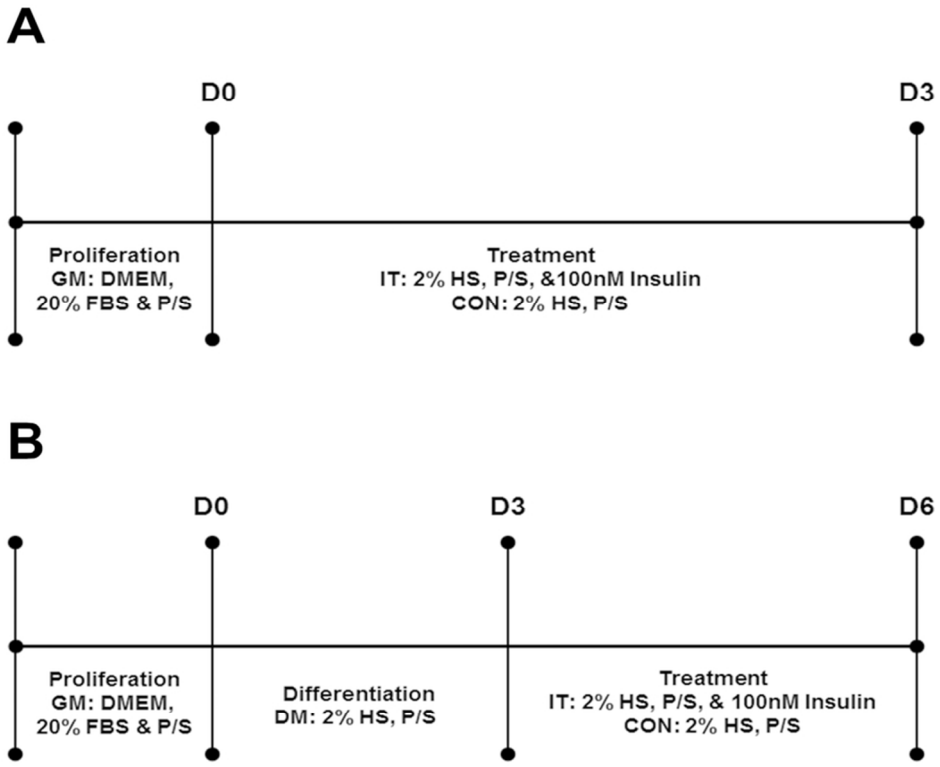


Figure 1. Protocol schematic for investigating chronic insulin exposure upon A; skeletal muscle cells during myogenesis and B; post-mitotic skeletal muscle myotubes.

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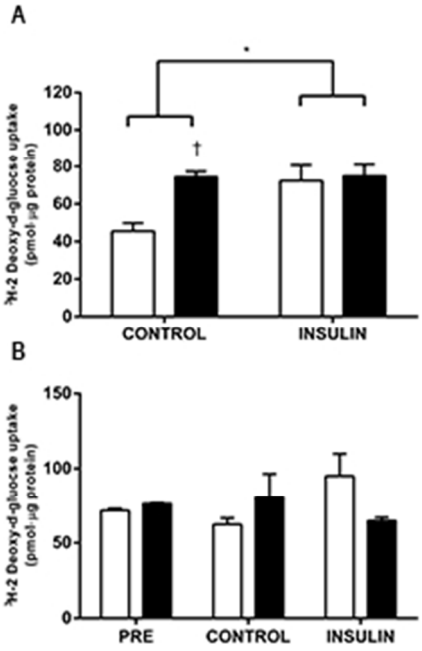


Figure 2. A; 3H-Deoxy-D-Glucose uptake in C2C12 skeletal muscle cells during myogenesis.B C2C12 skeletal muscle myotubes. Skeletal muscle myotubes were exposed to low serum media supplemented without (Control) or with (Insulin) the addition of insulin (100 nM). Open bars represent unstimulated filled bars represented acute 30 minutes insulin stimulation. * Significantly different to control ($p<0.05$). † Significantly different to control unstimulated ($p<0.05$). * Significantly different from control unstimulated sample ($p<0.05$).

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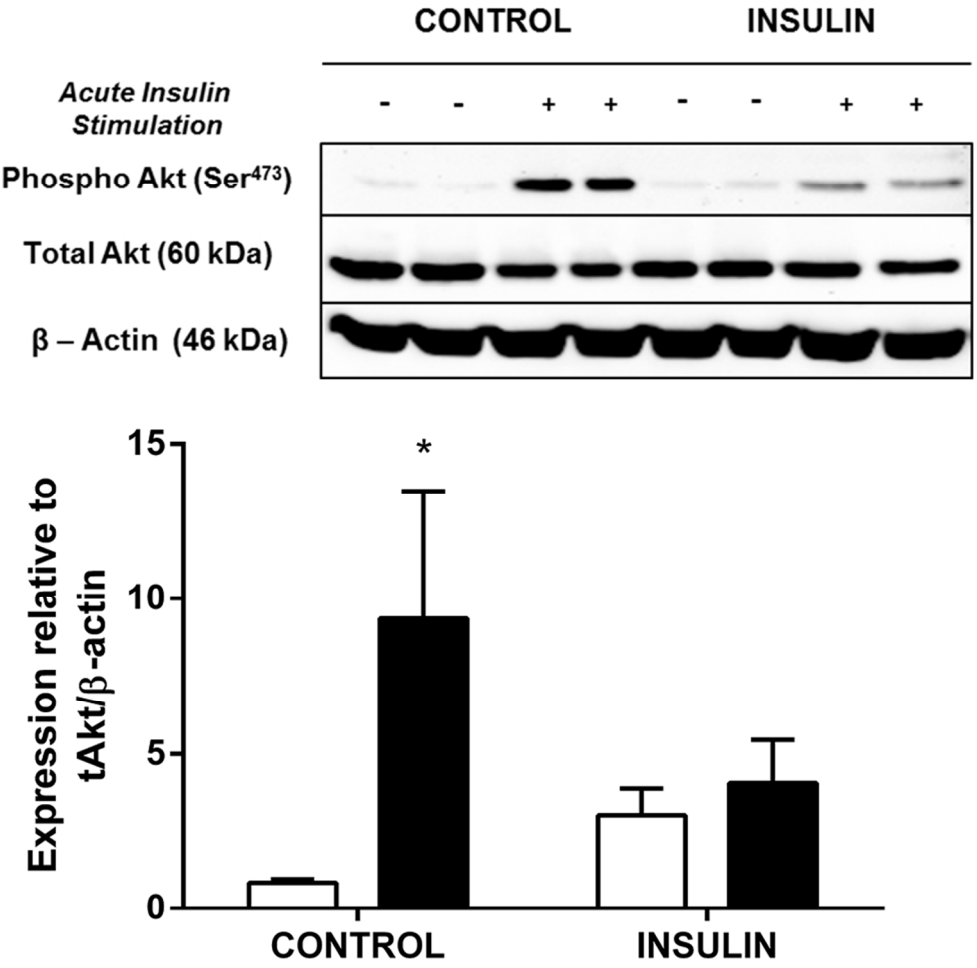


Figure 3. Akt (Ser473) phosphorylation following insulin exposure in C2C12 skeletal muscle cells during myogenesis. Open bars represent unstimulated filled bars represented acute 30 minutes insulin stimulation. * Significantly different to control ($p<0.05$). † Significantly different to control unstimulated ($p<0.05$).

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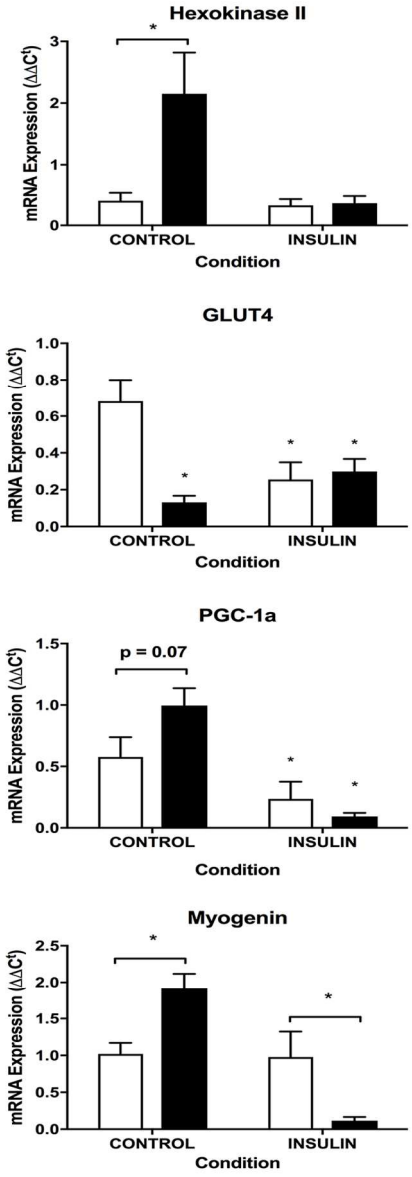


Figure 4. Gene expression analysis (ΔΔCt) of C2C12 skeletal muscle cells differentiated in control media (CON) or chronically exposed to insulin (100 nM) (IT). A; GLUT4, B; HKII, C; PGC-1α, D; MyoG. Open bars represent unstimulated filled bars represented acute 30 minutes insulin stimulation. * Significantly different from control unstimulated sample ($p<0.05$).

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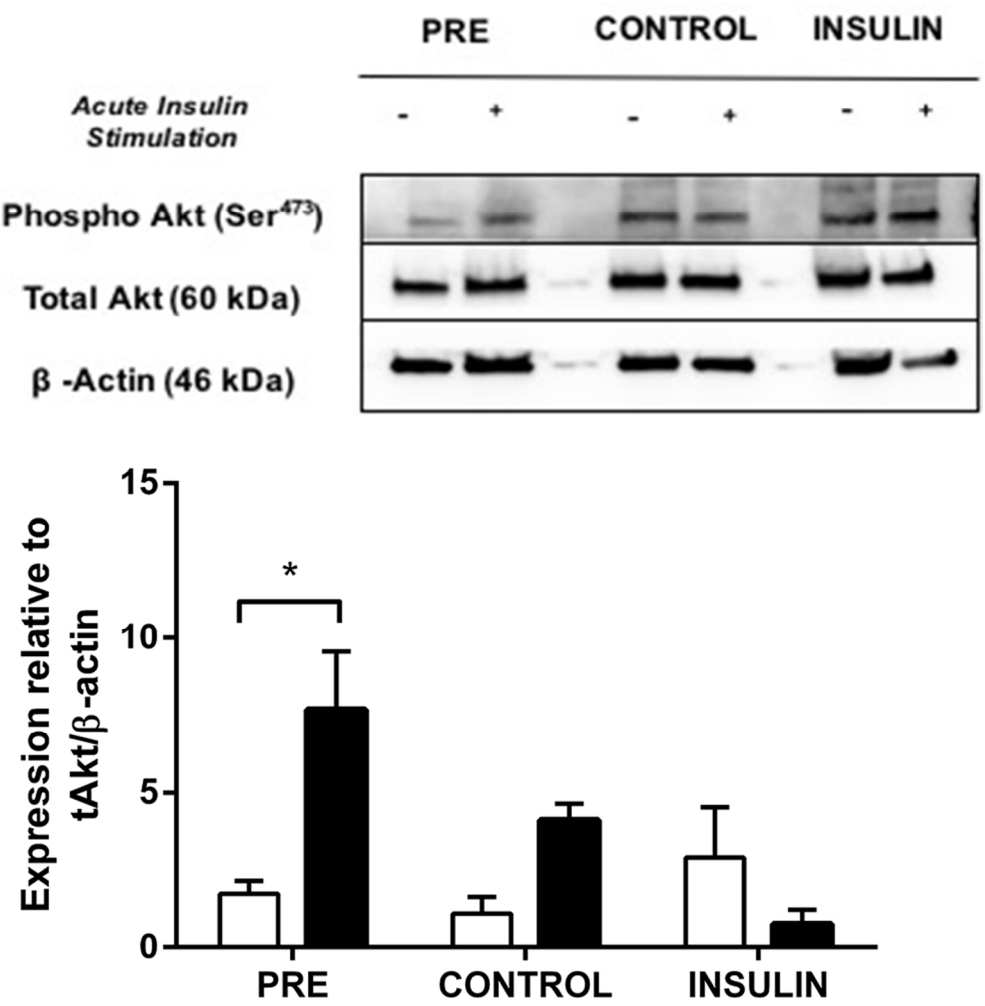


Figure 5. Akt (Ser473) phosphorylation C2C12 skeletal muscle myotubes following differentiation (PRE), control media (CON) or chronically exposed to insulin (100 nM) (IT). Open bars represent unstimulated filled bars represented acute 30 minutes insulin stimulation. * Significantly different to control ($p<0.05$). † Significantly different to control unstimulated ($p<0.05$). * Significantly different from control unstimulated sample ($p<0.05$).

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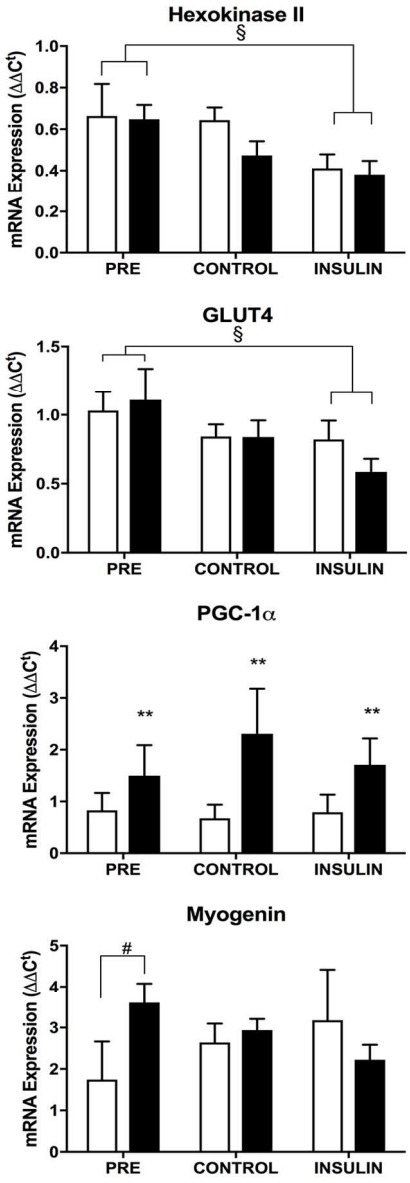


Figure 6. Gene expression analysis (ΔΔCt) of C2C12 skeletal muscle myotubes following differentiation (PRE) and chronic incubation in control media (CON) or high insulin (100 nM) media (IT). A; GLUT4, B; HKII, C; PGC-1α, D; MyoG. Open bars represent unstimulated filled bars represented acute 30 minutes insulin stimulation. # significantly different to unstimulated sample ($p<0.05$). * Significantly different to pre-unstimulated sample ($p<0.05$). § Significantly different to Pre ($p<0.05$).

63x160mm (300 x 300 DPI)

Table 1 Primer Sequences of mouse mRNA used for one-step PCR

Gene	Primer Sequence	Accession No:
RNA Polymerase II -Beta (POLR2B)	F: 5'-GGTCAGAAGGGAACTTGTGGTAT	NM_153798
	R: 5'-GCATCATTAAATGGAGTAGCGTC	
Myogeninn (MyoG)	F: CCAACTGAGATTGTCTGTC R: GGTGTTAGCCTTATGTGAAT	NM_031189
Peroxisome Proliferator-Activated coactivator (PGC1-α)	F: 5'-AGACTATTGAGCGAACCT	NM_008904.2
	R: 5'-TATGAGGAGGAGTTGTGG	
Hexokinase II (HKII)	F: 5'- GGAGGAGGAGCAGTATGG	NM_013820
	R: 5'-TTCAGCCGTGTGAGGTAA	
GLUT4	F: 5'-CATCAGGATAAACAGCAGGG R: 5'-GGAGGCAGGGCTAGATTT	NM_011480

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Table 2 Findings summary

Findings Summary
• Chronic exposure to insulin impaired phosphorylation of insulin signalling proteins in differentiating skeletal muscle cells. • The mRNA expression of metabolic genes was impaired following acute stimulation in differentiating skeletal muscle cells chronically exposed insulin. • Basal glucose uptake in differentiating skeletal muscle cells was increased following chronic insulin exposure. This increase hindered any observation of insulin stimulated glucose uptake in these cultures. • Acute insulin stimulation did not change mRNA expression of skeletal muscle myotubes. • Chronic insulin exposure increased GLUT4 mRNA expression while reducing HKII mRNA expression. • Chronic insulin exposure in skeletal muscle myotubes did not alter insulin stimulated glucose uptake.

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