Title: Characterising hyperinsulinemia induced insulin resistance in human skeletal muscle cells.

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Running Head: Human skeletal muscle exposure to insulin in vitro.

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Abstract:

Hyperinsulinemia potentially contributes to insulin resistance in metabolic tissues, such as skeletal muscle. The purpose of these experiments was to characterise glucose uptake, insulin signalling and relevant gene expression in primary human skeletal muscle derived cells (HMDCs), in response to prolonged insulin exposure (PIE) as a model of hyperinsulinemia induced insulin resistance. Differentiated HMDCs from healthy human donors, were cultured with or without insulin (100nM) for three days followed by an acute insulin stimulation. HMDC’s exposed to PIE were characterised by impaired insulin stimulated glucose uptake, blunted IRS-1 phosphorylation (Tyr$^{612}$) and Akt (Ser$^{473}$) phosphorylation in response to an acute insulin stimulation. Glucose transporter 1 (GLUT1), but not GLUT4, mRNA and protein increased following PIE. The mRNA expression of metabolic (PDK4) and inflammatory markers (TNF-α) was reduced by PIE but did not change lipid (SREBP1 and CD36) or mitochondrial (UCP3) markers. These experiments provide further characterisation of the effects of PIE as a model of hyperinsulinemia induced insulin resistance in HMDCs.

Keywords: Hyperinsulinemia, Insulin Resistance, Diabetes Mellitus, Primary Skeletal Muscle Cells,
**Introduction:**

The inability to maintain glucose homeostasis in response to physiological insulin concentrations, leads to an increase in blood glucose (hyperglycaemia) and consequently prolonged raised insulin concentrations (hyperinsulinemia). Hyperinsulinemia has been causally linked to the onset of diabetes in the early stages of the insulin resistance and in type 2 diabetes mellitus (Corkey 2012; Templeman *et al.* 2017), negatively affecting insulin sensitive tissues such as liver, adipose and skeletal muscle (Page & Johnson 2018). In humans, prolonged administration of insulin can attenuate insulin responsiveness, independent of hyperglycaemia. This would therefore suggest a potential role of hyperinsulinemia as a cause of insulin resistance (Marangou *et al.* 1986; Del Prato *et al.* 1994).

*In vitro* research using human skeletal muscle derived cells (HMDCs) has routinely been used to investigate various aspects of metabolic physiology (Aas *et al.* 2013). Consequently, it has been possible to investigate some of the cellular and molecular characteristics of skeletal muscle insulin resistance (Ciaraldi *et al.* 1995; Henry *et al.* 1995), as well as the potential causes of insulin resistance in skeletal muscle cells in response to other cells types, fatty acids and inflammatory cytokines (Dietze *et al.* 2002; Mäkinen *et al.* 2017).

Despite the development of relevant *in vitro* models to study metabolic disease, there is limited information regarding the effects of chronic insulin exposure on glucose metabolism in human skeletal muscle cells. Models of hyperinsulinemia induced insulin resistance through prolonged insulin exposure (PIE), using murine C2C12 skeletal muscle cells, have been shown to impair downstream insulin signalling and glucose uptake (Kumar & Dey 2003; Turner *et al.* 2018; Cen *et al.*
In addition, work in HMDCs has shown that exposure to a chronic insulin exposure can ablate the fractional velocity of glycogen synthase activity (Henry et al. 1996; Gaster et al. 2001) and therefore, could contribute to the development of insulin resistance in skeletal muscle (Nikoulina et al. 1997).

Whilst previous literature has alluded to the physiological effects of hyperinsulinemia induced insulin resistance in human skeletal muscle tissue and primary cells (Del Prato et al. 1994; Gaster et al. 2001), currently the molecular characteristics which potentially underpin previously observed changes to PIE induced insulin resistance, are yet to be investigated. The aim of the current investigation was to determine how PIE would affect glucose uptake, insulin signalling and gene expression in HMDCs from healthy donors. In these experiments, it was found that PIE resulted in attenuated insulin signalling and glucose uptake, however did not alter the mRNA expression of genes involved in metabolism, which are putatively indicative of insulin resistant skeletal muscle.

**Methods:**

**Participants**

Healthy male volunteers (age 24.4 ± 1.1 years, height 1.78 ± 0.04 cm, weight 70.6 ± 2.9 kg, body mass index (BMI) 22.3 ± 1.5 kg·m²) who did not report any family history of metabolic disease were recruited for this study. All procedures were conducted at Loughborough University, UK under ethical approval and in accordance with the Declaration of Helsinki, 2008.

**Isolation and culture of human muscle derived cells (HMDCs)**

Percutaneous skeletal muscle biopsies were obtained from the vastus lateralis by micro-biopsy technique (Acecut 11-gauge Biopsy Needle; TSK, Tochigi-Ken, Japan),
as previously described (Ferguson et al. 2018). Skeletal muscle biopsies were
scissor minced into small pieces, placed in tissue culture flasks coated in 0.2%
Gelatin/PBS and maintained at 37°C and 5% CO₂ in growth media (GM, consisting
of high glucose DMEM (Sigma, Dorset, UK) supplemented with 20% Foetal bovine
serum (Pan Biotech UK Ltd, Dorset, UK) and 1% penicillin/streptomycin, Fisher
Scientific, Loughborough, UK). The HMDCs which migrated out of the muscle tissue
and adhered to the tissue culture plastic were harvested and expanded through
serial passages to increase cell numbers prior to experimentation. For
experimentation, HMDCs were used between passages 3-7 (7-10 population
doublings).

**Experimental Protocol**

2,500 cells·cm⁻² of HMDCs were seeded into 12 well plates and cultured in GM until
80% confluent. Media was subsequently changed to low serum differentiation media
(DM) which consisted of high glucose DMEM (Sigma, Dorset, UK) supplemented
with 2% horse serum (Fisher Scientific, Loughborough, UK), and 1%
penicillin/streptomycin (Fisher Scientific, Loughborough, UK). Following
differentiation, (5-7 days) into multinucleate myotubes (as evidenced by light
microscopy), cultures were subjected to prolonged insulin exposure (PIE), consisting
of DM supplemented with the addition of 100nM human recombinant insulin (Sigma,
Dorset, UK). Cultures were then serum starved for 4 hours, before being acutely
stimulated with or without insulin (100nM) for 30 minutes.

**Protein quantification and immunoblotting**

HMDCs were washed with PBS before being lysed in RIPA buffer (Sigma, Dorset,
UK) containing a protease and phosphatase inhibitor cocktail mix (Fisher Scientific,
Loughborough, UK). Protein concentrations were determined using the Pierce
660nm protein assay (Fisher Scientific, Loughborough, UK) and thereafter samples were mixed with 4X Laemmli buffer (Bio-Rad, Herts, UK), boiled for 5 minutes at 95°C and separated by SDS-PAGE. Proteins were transferred onto nitrocellulose membranes (Whatman Proton, Sigma-Aldrich, Dorset, UK) and blocked for 1 hour at room temperature in 5% bovine serum albumin (BSA) in TBST (Sigma, Dorset, UK), before being incubated with primary antibody overnight at 4°C in BSA or Milk. Primary antibodies used for analysis were, Akt (#9272), phosphor Akt (Ser\textsuperscript{473}) (#4060), GSK-3β (#9315), GSK-3β (Ser\textsuperscript{9}) (#9336), AS160 (Ser\textsuperscript{588}) (#8730), Glucose Transporter 4 (GLUT4) (#2213) and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (#2118) purchased from Cell signalling (NEB, Herts, UK). Glucose transporter 1 (GLUT1) (#07-1401), Insulin Receptor Substrate-1 (IRS-1) (#05-784R) and phospho IRS-1 (Tyr\textsuperscript{612}) (#09-432) were purchased from Merck Millipore (Dorset, UK). Following overnight incubation, membranes were washed in TBST and subsequently incubated with anti-rabbit (#7074) or anti-mouse (#7076) horseradish peroxidase-conjugated secondary antibody (NEB, Herts, UK) at concentration of 1:2000 in milk. Proteins were visualised using chemiluminescence substrate (Bio-Rad, Herts, UK) and band densities were quantified using Quantity One image analysis software (Quality One 1-D analysis software version 4.6.8). Where appropriate, following visualisation of phosphorylated proteins, membranes were washed in TBST and incubated in stripping buffer (Fisher Scientific, Loughborough, UK) before being blocked and probed as outlined above for their corresponding total proteins. Phosphorylation was normalised to its corresponding total protein, with the exceptions of GLUT1, GLUT4 and AS160 (Ser\textsuperscript{588}) which were normalised to GAPDH.
RNA extraction and qPCR analysis

RNA extraction was performed using TRI Reagent (Sigma, Dorset, UK) according to the manufacturer’s instructions and quantified using UV spectroscopy (NanoDrop, Fisher Scientific, Loughborough, UK). Gene expression was analysed by one-step reverse transcription-qPCR (Quantifast SYBR Green Mix (Qiagen, Crawley, UK)) using a Viia 7 thermocycler (Applied Biosystems, Loughborough, UK). Each reaction consisted of 20ng of RNA in a final 10 µL reaction volume (Qiagen, Crawley, UK). Master mixes were made according to the manufacturer’s instructions using primers outlined in Table 1. Fluorescence was detected after every cycle (40 cycles) and data was analysed using the ΔΔCt method, using RNA polymerase II beta (POLR2B) as an endogenous control gene. Samples were normalised to each individual donor control sample, with each donor performed in duplicate for each condition and each sample was ran in triplicate.

[INSERT TABLE 1]

Cell Based Glucose uptake assay

HMDCs were plated into black, clear bottom 96 well plates (Fisher Scientific, Loughborough, UK) and cultured as described in the experimental protocol. The measurement of 2-deoxyglucose (2DG) uptake was performed using a commercially available Glucose Uptake-Glo™ Assay kit (Promega, Southampton, UK). Firstly, cultures were washed with PBS and incubated overnight in serum free media with/without 100nM insulin. Briefly, HMDC’s were washed with PBS before being stimulated with/without 1mM insulin in PBS. 0.1M 2DG was added to all of the wells for 30 minutes at 25°C. The reaction was arrested with the addition of stop and neutralization buffer, before the addition of 2DG6P detection reagent. Values were normalised to total protein concentration analysed on plates following glucose uptake.
measurement, using the Pierce 660nm protein assay (Fisher Scientific, Loughborough, UK).

Statistical Analysis

Statistical analysis was performed using SPSS (Version 23). Insulin signalling proteins was analysed by one-way ANOVA with Bonferroni post-hoc correction. Differences in glucose uptake and gene expression between control and PIE conditions, were analysed by independent samples t-test. The number of donors used for each analysis is outlined in the figure legends. Data is presented and mean ± standard error the mean (S.E.M) and statistical significance was set at p < 0.05.

Results:

Glucose Uptake in human skeletal muscle cells following insulin exposure

To investigate the physiological effects of exposure to PIE, we measured glucose uptake using a commercially available assay. Acute insulin-stimulation increased glucose uptake by approximately 1.5-fold in control HMDCs (p <0.05, figure 1a). However, HMDCs cultured with PIE exhibited no significant increase in glucose uptake following acute insulin stimulation (p >0.05, figure 1b).

Prolonged exposure to insulin alters phosphorylation of insulin signalling proteins IRS-1 (Tyr612)

Following evidence of altered glucose uptake following PIE, we analysed the phosphorylation of both Insulin receptor substrate 1 (IRS-1) and Akt as critical nodes of insulin signalling. Acute insulin stimulation significantly increased tyrosine phosphorylation of IRS-1 (Tyr612) above basal levels in control HMDCs (p <0.01,
In contrast, we observed elevated basal IRS-1 (Tyr^{612}) phosphorylation in PIE condition which was not increased further upon acute insulin stimulation (p > 0.05, Figure 2). In addition, IRS-1 mRNA expression was analysed however was not different between control and PIE conditions (p > 0.05, Figure 2B).

Prolonged exposure to insulin alters phosphorylation of Akt (Ser^{473}). Akt (Ser^{473}) was responsive to acute insulin stimulation when HMDC’s were cultured in control conditions (p < 0.05 figure 3a), however this response was blunted following PIE (p > 0.05; Figure 3a). GSK-3β (Ser^{9}) or AS160 (Ser^{588}) phosphorylation was not different following acute stimulation or different between conditions (both p >0.05; Figure 3b and 3c respectively).

Exposure to insulin results in changes to GLUT1 but not GLUT4 mRNA and protein in HMDCs. PIE increased the mRNA expression of GLUT1 by approximately 1.6-fold above HMDCs cultured in control conditions (p < 0.01; figure 4a), but did not alter the mRNA expression of GLUT4 (p >0.05; figure 4b). Protein expression analysis of GLUT1 was also increased in the PIE condition compared to CON (p < 0.05 figure 4c), but there was no difference in GLUT4 protein expression between conditions (p > 0.05 figure 4d).
The effects of insulin exposure on the mRNA expression of metabolism markers.

Hexokinase II and pyruvate dehydrogenase kinase isoform 4 (PDK4), two enzymes which regulate glucose oxidation, have previously been shown to be regulated by insulin. Here, HKII mRNA expression did not change in HMDC’s exposed to PIE (p > 0.05), however PDK4 mRNA expression was significantly reduced following PIE (p < 0.01). Glycogen synthase kinase-3β (GSK-3β) mRNA expression was increased following PIE, but did not reach statistical significance (p = 0.054). The mRNA expression of the lipid metabolism markers, sterol regulatory element binding protein 1 (SREBP-1) and fatty acid translocase (cluster of differentiation 36 (CD36)), was not different between conditions (p > 0.05). Similarly, the mRNA expression of mitochondrial uncoupling protein -3 (UCP-3), was not different between conditions (p > 0.05). However, the mRNA expression of the pro-inflammatory marker TNF-α was significantly lower in PIE compared to control (p < 0.05, figure 5b).

Discussion:

Hyperinsulinemia is a symptom in the early stages of insulin resistance and type 2 diabetes mellitus (Shanik et al. 2008; Page & Johnson 2018). To decipher how prolonged exposure to high concentrations of insulin could contribute to skeletal muscle insulin resistance, in vitro experiments have been used to investigate some of the physiological effects of hyperinsulinemia in cell lines (Kumar & Dey 2003; Turner et al. 2018; Cen et al. 2019), and primary human skeletal muscle cells (Henry et al. 1996; Gaster et al. 2001). Our initial experiments used prolonged insulin exposure (PIE) as a model of hyperinsulinemia and demonstrated that this was able to induce impaired glucose uptake in response to an acute insulin stimulation, thus
confirming our own previous findings in C2C12 skeletal muscle cells (Turner et al. 2018) and those of Henry and colleagues who have shown hyperinsulinemia to impair glucose synthase activity and insulin stimulated glucose uptake in primary human skeletal muscle cells when chronically exposed to insulin (Ciaraldi et al. 1995) and is a phenomenon which occurs in both healthy and insulin resistant skeletal muscle.

To examine the molecular responses to this impaired insulin-stimulated glucose uptake we initially examined critical nodes of the insulin signalling cascade. Indeed, impaired insulin signalling contributes to skeletal muscle insulin resistance (Taniguchi et al. 2006). While the physiological changes in human skeletal muscle cells have been previously described (Henry et al. 1996; Gaster et al. 2001), we observed an attenuated IRS-1 phosphorylation (Tyr{superscript}612) to acute insulin stimulation. This was due to an increase in basal phosphorylation which has previously been reported in rat skeletal muscle (Kanety et al. 1994). This response has been attributed to multisite phosphorylation of the insulin receptor in a cell lines, including skeletal muscle (Kumar & Dey 2003; Cen et al. 2019), although further experiments would be required to determine the phosphorylation sites in human skeletal muscle cells. The attenuation in the phosphorylation of Akt is indicative of insulin resistance (Krook et al. 1998a; Karlsson et al. 2005), which has been reported by our group in C2C12 skeletal muscle cells following sustained exposure to insulin (Turner et al. 2018). In addition, while our findings did not show any changes in phosphorylation of downstream target AS160, it’s phosphorylation of multiple serine sites in response to insulin (Kramer et al. 2006) mean that further investigation is required to elucidate what sites are specifically inhibited following prolonged insulin.
Exposure to insulin can increase glucose transporter (GLUT) mRNA and protein expression (Walker et al. 1989, 1990), a finding which has also been shown in primary human skeletal muscle cells (Ciaraldi et al. 1995). The increase in expression is mostly likely due to an increase in GLUT1 mRNA and protein expression, which was increased following exposure to PIE in the present set of experiments. Basal glucose uptake has previously been observed in human (Ciaraldi et al. 1995), and murine skeletal muscle cells (Turner et al. 2018), and is linked to the increase in GLUT1 mRNA and protein expression. In contrast, GLUT4 mRNA and protein expression was not altered in response to PIE, which has previously been reported in human skeletal muscle following hyperinsulinemia-euglycemia clamp (Postic et al. 1993). Despite contradictory findings in rodent skeletal muscle (Cusin et al. 1990), our findings provide further evidence that that hyperinsulinemia is not a mediator of GLUT4 protein or mRNA expression in human skeletal muscle. Insulin regulates the expression of genes involved in skeletal muscle metabolism (Rome et al. 2003), of which the expression is altered in metabolic disease (Ducluzeau et al. 2001). HKII mRNA expression has previously been shown to be sensitive to insulin stimulation (Osawa et al. 1996), and it’s response is attenuated in insulin resistant skeletal muscle (Ducluzeau et al. 2001). However, this is in contrast to our experiments, which may reflect the differences in metabolic demands of cells and tissues. The reduction in PDK4 mRNA expression in these experiments, could be considered indicative of a physiological and not pathophysiological response to insulin in skeletal muscle (Kim et al. 2006; McAinch et al. 2015). In addition, a number of genes which are associated with skeletal muscle insulin resistance, such as SREBP1, CD36 and UCP3 (Krook et al. 1998b; Ducluzeau et al. 2001; Wallberg-Henriksson et al. 2007), were not altered in these experiments. This indicates while
hyperinsulinemia can induce insulin resistance in healthy HMDC’s, it might not
contribute to the transcriptional changes which have previously been observed in
disease states (Ducluzeau et al. 2001). These changes could be mediated by other
factors, such as low grade chronic inflammation and the expression of pro-
inflammatory cytokines (Ruge et al. 2009).

With the previously documented role of hyperinsulinemia in skeletal muscle insulin
resistance, for the first time these experiments were able to confirm previous findings
that PIE can attenuate insulin stimulated glucose uptake, attenuate insulin signalling
and the induce compensatory changes in glucose transporter expression in HMDC’s
from healthy donors. While we appreciate that the concentrations of insulin used in
these experiments are in excess of physiological hyperinsulinemia in humans and
therefore further experiments would elude to impact of lower insulin concentrations
upon skeletal muscle insulin sensitivity in vitro, these findings provide insight into the
specific impact of hyperinsulinemia induced insulin resistance in primary human
skeletal muscle cells.

[INSERT FIGURE 6]

Conflict of Interest:
The authors declare that there is no conflict of interest that could be perceived as
prejudicing the impartiality of the research reported.

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Author Contributions:

MCT performed the experiments. MCT and MPL developed the experiments. MCT, EA, DJP, CJH and NRWM analysed the data. RAF and PW took the skeletal muscle biopsies from which MCT, DJP, NRWM and extracted and cultured the cells. All authors read and approved the final manuscript for submission.

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Suppl 2 S262-8. (doi:10.2337/dc08-s264)


Figure Legends

**Figure 1**: 2-deoxyglucose (2DG) uptake and phase contrast images of HMDCs cultured in control (CON) or prolonged insulin exposure (PIE) for three days. Cells were stimulated without (open bar) or with (dotted bar) insulin. Data is mean ± s.e.m from 3 donors. *Significant difference between basal and stimulation (p < 0.05).

**Figure 2**: A; IRS-1 phosphorylation (Tyr^{612}) of HMDC cultured in CON or PIE media. Cells were stimulated without (open bar) or with (dotted bar) insulin (100nM) for 30 minutes. B; IRS-1 mRNA expression (ΔΔCt) of basal samples cultured in CON or PIE media. Data is mean ± s.e.m from 2-4 donors. *Significant difference between basal and acute insulin stimulation (p < 0.05).

**Figure 3**: A; Akt phosphorylation (Ser^{473}), B; GSK-3β phosphorylation (Ser^{9}) of HMDC cultured in CON or PIE media, C; AS160 phosphorylation (Ser^{588}) of HDPC’s cultured in CON or PIE media before being stimulated without (open bar) or with (dotted bar) insulin (100nM) for 30 minutes. Data is mean ± s.e.m from 2-5 donors as represented by in the graph. *Significant difference between basal and acute insulin stimulation (p < 0.05).

**Figure 4**: A; glucose transporter 1 (GLUT1) and B; glucose transporter 4 (GLUT4) mRNA expression (ΔΔCt). C; GLUT1 protein expression and D; GLUT4 protein expression normalised to GAPDH in HMDC cultured CON or PIE media. Data is mean ± s.e.m from 3 donors. Significant different between conditions ** (p < 0.01).

**Figure 5**: mRNA expression (ΔΔCt) of genes in HMDCs cultured CON or PIE media. Data is mean ± s.e.m from 3-4 donors. Significantly different between conditions * (p <0.05), ** (p <0.01).
Figure 6: Summary of human muscle derived cell (HMDC) responses to prolonged insulin exposure (PIE) as a model of hyperinsulinemia induced insulin resistance.

Table 1: Primer sequences of genes analysed by qPCR.
Glucose Uptake (Relative to Basal)

**Condition**

- **Basal**
  - **CON**
  - **PIE**

- **Stimulated**
  - **CON**
  - **PIE**

*Significant difference between conditions.*
**A**

**Condition**

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* p < 0.05

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Stimulation

- CON  - PIE

p-GSK3-\(\beta\) (Ser9) (46kDa)
GSK3-\(\beta\) (46kDa)
GAPDH (37kDa)

C

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Stimulation

- CON  - PIE

p-AS160 (Ser588) (160kDa)
GAPDH (37kDa)
Changes to HMDCs following PIE

Physiological Response To PIE
- ↓ Insulin Stimulated Glucose Uptake

Phosphorylation to acute insulin stimulation following PIE

CON
- ↑ IRS-1 phosphorylation
- ↑ Akt phosphorylation
- - GSK3-β phosphorylation
- - AS160 phosphorylation

PIE
- - IRS-1 phosphorylation
- - Akt phosphorylation
- - GSK3-β phosphorylation
- - AS160 phosphorylation

Total Protein Response to PIE
- ↑ GLUT1, ↓ GLUT4

Gene Expression Response to PIE
- ↑ GLUT1
- HKII, GLUT4, SREBP1, CD36, GSK3-β, UCP3
- ↓ PDK4, TNFα
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