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Characterising hyperinsulinemia-induced insulin resistance in human skeletal muscle cells

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Abstract

Hyperinsulinaemia 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 hyperinsulinaemia-induced insulin resistance. Differentiated HMDCs from healthy human donors were cultured with or without insulin (100 nM) for 3 days followed by an acute insulin stimulation. HMDCs exposed to PIE were characterised by impaired insulin-stimulated glucose uptake, blunted IRS-1 phosphorylation (Tyr⁶¹²) and Akt (Ser⁴⁷³) 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 (*PK4*) 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 hyperinsulinaemia-induced insulin resistance in HMDCs.

Key Words

- ▶ hyperinsulinaemia
- ▶ insulin resistance
- ▶ diabetes mellitus
- ▶ primary skeletal muscle cells

*Journal of Molecular
Endocrinology*
(2020) **64**, 125–132

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 (hyperinsulinaemia). Hyperinsulinaemia 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 hyperinsulinaemia 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 hyperinsulinaemia-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.* 2019). 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 hyperinsulinaemia-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, it 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, 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) supplemented with 20% foetal bovine serum (Pan Biotech UK Ltd, Dorset, UK) and 1% penicillin/streptomycin, Fisher Scientific). 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

2500 cells/cm² 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) supplemented with 2% horse serum (Fisher Scientific) and 1% penicillin/streptomycin (Fisher Scientific). 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 100 nM human recombinant insulin (Sigma). Cultures were then serum starved for 4 h before being acutely stimulated with or without insulin (100 nM) for 30 min.

Protein quantification and immunoblotting

HMDCs were washed with PBS before being lysed in RIPA buffer (Sigma) containing a protease and phosphatase inhibitor cocktail mix (Fisher Scientific). Protein concentrations were determined using the Pierce 660 nm protein assay (Fisher Scientific), and thereafter samples were mixed with 4× Laemmli buffer (Bio-Rad), boiled for 5 min at 95°C and separated by SDS-PAGE. Proteins were transferred onto nitrocellulose membranes (Whatman Proton, Sigma-Aldrich) and blocked for 1 h at room temperature in 5% BSA in TBST (Sigma) before being incubated with primary antibody overnight at 4°C in BSA or milk. Primary antibodies used for analysis

were Akt (#9272), phosphor Akt (Ser⁴⁷³) (#4060), GSK-3 β (#9315), GSK-3 β (Ser⁹) (#9336), AS160 (Ser⁵⁸⁸) (#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 (Tyr⁶¹²) (#09-432) were purchased from Merck Millipore. 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 a concentration of 1:2000 in milk. Proteins were visualised using chemiluminescence substrate (Bio-Rad) 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) before being blocked and probed as outlined previously for their corresponding total proteins. Phosphorylation was normalised to its corresponding total protein, with the exceptions of GLUT1, GLUT4 and AS160 (Ser⁵⁸⁸) which were normalised to GAPDH.

RNA extraction and qPCR analysis

RNA extraction was performed using TRI Reagent (Sigma) according to the manufacturer's instructions and quantified using UV spectroscopy (NanoDrop, Fisher Scientific). Gene expression was analysed by one-step RT-qPCR (Quantifast SYBR Green Mix (Qiagen)) using a Viia 7 thermocycler (Applied Biosystems). Each reaction consisted of 20 ng of RNA in a final 10 μ L reaction volume (Qiagen). 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 were analysed using the $\Delta\Delta C_t$ 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.

Cell based glucose uptake assay

HMDCs were plated into black, clear bottom 96-well plates (Fisher Scientific) 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). First, cultures were washed with PBS and incubated overnight in serum free media with/without 100 nM insulin. Briefly, HMDCs were washed with PBS before being stimulated with/without 1 nM insulin in PBS. 0.1 M 2DG was added to all of the wells for 30 min 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 660 nm protein assay (Fisher Scientific).

Statistical analysis

Statistical analysis was performed using SPSS (version 23). Insulin signalling proteins were 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 are presented as mean $\pm$ S.E.M. and statistical significance was set at $P < 0.05$.

Table 1 Primer sequences of genes analysed by qPCR.

Gene of interest	Symbol	Manufacturer	Assertion No.
RNA polymerase II	<i>POLR2B</i>	Sigma	NM_000938
Glucose Transporter 4	<i>GLUT4 (SLC2A4)</i>	Sigma	NM_001042
Hexokinase II	<i>HKII</i>	Qiagen	NM_000189
Pyruvate dehydrogenase lipoamide kinase isozyme 4	<i>PDK4</i>	Sigma	NM_002612
Glucose Transporter 1	<i>GLUT1 (SLC2A1)</i>	Qiagen	NM_006516
Uncoupling protein 3	<i>UCP3</i>	Qiagen	NM_003356
Glycogen Synthase Kinase 3 beta	<i>GSK3-β</i>	Qiagen	NM_001146156
Tumour necrosis factor alpha	<i>TNF-α</i>	Sigma	NM_000594
Insulin receptor substrate 1	<i>IRS-1</i>	Qiagen	NM_005544
Cluster of differentiation 36 (Fatty acid translocase)	<i>CD36</i>	Sigma	NM_000072
Sterol regulatory element binding protein factor 1	<i>SREBP1</i>	Qiagen	NM_004176

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 ($P < 0.05$, Fig. 1A). However, HMDCs cultured with PIE exhibited no significant increase in glucose uptake following acute insulin stimulation ($P > 0.05$, Fig. 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 ($P < 0.01$, Fig. 2A). In contrast, we observed elevated basal IRS-1 (Tyr⁶¹²) phosphorylation in PIE condition which was not increased further upon acute insulin stimulation ($P > 0.05$, Fig. 2). In addition, *IRS-1* mRNA expression was analysed; however, it was not different between control and PIE conditions ($P > 0.05$, Fig. 2B).

Figure 1

2-Deoxyglucose (2DG) uptake and phase contrast images of HMDCs cultured in control (CON) or prolonged insulin exposure (PIE) for 3 days. Cells were stimulated without (open bar) or with (dotted bar) insulin. Data are mean $\pm$ s.e.m. from three donors. *Significant difference between basal and stimulation ($P < 0.05$). A full colour version of this figure is available at <https://doi.org/10.1530/JME-19-0169>.

Figure 2

(A) IRS-1 phosphorylation (Tyr⁶¹²) of HMDC cultured in CON or PIE media. Cells were stimulated without (open bar) or with (dotted bar) insulin (100 nM) for 30 min. (B) *IRS-1* mRNA expression ($\Delta\Delta C_t$) of basal samples cultured in CON or PIE media. Data are mean $\pm$ s.e.m. from two to four donors. *Significant difference between basal and acute insulin stimulation ($P < 0.05$).

Prolonged exposure to insulin alters phosphorylation of Akt (Ser⁴⁷³)

Akt (Ser⁴⁷³) was responsive to acute insulin stimulation when HMDCs were cultured in control conditions ($P < 0.05$; Fig. 3A); however, this response was blunted following PIE ($P > 0.05$; Fig. 3A). GSK-3 β (Ser⁹) or AS160 (Ser⁵⁸⁸) phosphorylation was not different following

acute stimulation or different between conditions (both $P>0.05$; Fig. 3B and C 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 ($P<0.01$; Fig. 4A), but did not alter the mRNA expression of *GLUT4* ($P>0.05$; Fig. 4B). Protein expression analysis of GLUT1 was also increased in the PIE condition compared to CON ($P<0.05$; Fig. 4C), but there was no difference in GLUT4 protein expression between conditions ($P>0.05$; Fig. 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,

Figure 3

(A) Akt phosphorylation (Ser⁴⁷³), (B) GSK-3β phosphorylation (Ser⁹) of HMDC cultured in CON or PIE media, (C) AS160 phosphorylation (Ser⁵⁸⁸) of HMDCs cultured in CON or PIE media before being stimulated without (open bar) or with (dotted bar) insulin (100 nM) for 30 min. Data are mean $\pm$ s.e.m. from two to five donors as represented by the graph. *Significant difference between basal and acute insulin stimulation ($P<0.05$).

Figure 4

(A) Glucose transporter 1 (*GLUT1*) and (B) glucose transporter4 (*GLUT4*) mRNA expression ($\Delta\Delta C_t$). (C) GLUT1 protein expression and (D) GLUT4 protein expression normalised to GAPDH in HMDC cultured CON or PIE media. Data are mean $\pm$ s.e.m. from three donors. Significant difference between conditions **($P<0.01$).

have previously been shown to be regulated by insulin. Here, *HKII* mRNA expression did not change in HMDCs exposed to PIE ($P>0.05$); however, *PDK4* mRNA expression was significantly reduced following PIE ($P<0.01$) and 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 (*UCP3*) 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 ($P<0.05$, Fig. 5B).

Discussion

Hyperinsulinaemia 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,

Figure 5

mRNA expression ($\Delta\Delta C_t$) of genes in HMDs cultured CON or PIE media. Data are mean $\pm$ S.E.M. from three to four donors. Significantly different between conditions *($P < 0.05$), **($P < 0.01$).

in vitro experiments have been used to investigate some of the physiological effects of hyperinsulinaemia 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 hyperinsulinaemia 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 hyperinsulinaemia 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 as 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 (Tyr⁶¹²) 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 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, its 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 it is linked to the increase in *GLUT1* mRNA and protein expression. In contrast, *GLUT4* mRNA and protein expression were not altered in response to PIE, which has previously been reported in human skeletal muscle following hyperinsulinaemia-euglycaemia clamp (Postic *et al.* 1993). Despite contradictory findings in rodent skeletal muscle (Cusin *et al.* 1990), our findings provide further evidence that that hyperinsulinaemia 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 its 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 that while hyperinsulinaemia can induce insulin

Figure 6

Summary of human muscle-derived cell (HMDC) responses to prolonged insulin exposure (PIE) as a model of hyperinsulinaemia-induced insulin resistance.

resistance in healthy HMDCs, 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 hyperinsulinaemia 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 induce compensatory changes in glucose transporter expression in HMDCs from healthy donors (Fig. 6). While we appreciate that the concentrations of insulin used in these experiments are in excess of physiological hyperinsulinaemia 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 hyperinsulinaemia-induced insulin resistance in primary human skeletal muscle cells.

Declaration of interest

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

Funding

The research was supported by the National Institute for Health Research (NIHR) Leicester Biomedical Research Centre. The views expressed are those of the authors and not necessarily those of the NHS, the NIHR or the Department of Health and Social Care.

Author contribution statement

M C T performed the experiments. M C T and M P L developed the experiments. M C T, E A, D J P, C J H and N R W M analysed the data. R A F and P W took the skeletal muscle biopsies from which M C T, D J P and N R W M extracted and cultured the cells. All authors read and approved the final manuscript for submission.

Acknowledgements

The authors would like to thank all the subjects for their time and participation in this research and Dr Carl Hulston for providing the IRS-1 antibodies and input into the manuscript.

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Received in final form 20 December 2019

Accepted 10 January 2020

Accepted Manuscript published online 10 January 2020