Histone deacetylase 5 regulates glucose uptake and insulin action in muscle cells

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Abstract

The class IIa histone deacetylases (HDACs) act as transcriptional repressors by altering chromatin structure through histone deacetylation. This family of enzymes regulates muscle development and phenotype, through regulation of muscle-specific genes including myogenin and *MyoD* (*MYOD1*). More recently, class IIa HDACs have been implicated in regulation of genes involved in glucose metabolism. However, the effects of HDAC5 on glucose metabolism and insulin action have not been directly assessed. Knockdown of *HDAC5* in human primary muscle cells increased glucose uptake and was associated with increased *GLUT4* (*SLC2A4*) expression and promoter activity but was associated with reduced *GLUT1* (*SLC2A1*) expression. There was no change in *PGC-1 α* (*PPARGC1A*) expression. The effects of *HDAC5* knockdown on glucose metabolism were not due to alterations in the initiation of differentiation, as knockdown of *HDAC5* after the onset of differentiation also resulted in increased glucose uptake and insulin-stimulated glycogen synthesis. These data show that inhibition of HDAC5 enhances metabolism and insulin action in muscle cells. As these processes in muscle are dysregulated in metabolic disease, HDAC inhibition could be an effective therapeutic strategy to improve muscle metabolism in these diseases. Therefore, we also examined the effects of the pan HDAC inhibitor, Scriptaid, on muscle cell metabolism. In myotubes, Scriptaid increased histone 3 acetylation, *GLUT4* expression, glucose uptake and both oxidative and non-oxidative metabolic flux. Together, these data suggest that HDAC5 regulates muscle glucose metabolism and insulin action and that HDAC inhibitors can be used to modulate these parameters in muscle cells.

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Introduction

The class IIa histone deacetylases (HDACs) act as transcriptional repressors by altering chromatin structure to a state that impairs transcription (Li *et al.* 2007). This primarily occurs through deacetylation of histone lysine residues, which modifies the spatial relationship between histones and DNA (Li *et al.* 2007). This ultimately restricts initiators of transcription from accessing gene promoter regions, resulting in transcriptional repression (Li *et al.* 2007). The class IIa HDACs consist of isoforms 4, 5, 7 and 9 and are highly enriched in skeletal muscle, the heart and the brain (McKinsey *et al.* 2001). These transcriptional repressors act by associating with DNA binding transcription factors, such as the myocyte enhancer factor 2 (MEF2) to repress specific promoter regions (Lu *et al.* 2000). Loss-of-function animal models have shown that this family of enzymes regulates processes such as muscle development and phenotype (Potthoff *et al.* 2007), skeletogenesis (Vega *et al.* 2004), vascular

integrity (Chang *et al.* 2006) and stress-induced cardiac hypertrophy (Chang *et al.* 2004). At a cellular level, perhaps the most characterised functional role of the class IIa HDACs is regulation of muscle differentiation (Lu *et al.* 2000, McKinsey *et al.* 2000, Dressel *et al.* 2001, Zhang *et al.* 2001, 2002). This principally occurs through repression of *MEF2* that regulates the myogenin and *MyoD* promoters and hence suppresses their expression (Deng *et al.* 2005, Micheli *et al.* 2005). Inhibition of HDAC function increases myogenin and *MyoD* (*MYOD1*) expression and is sufficient to initiate the differentiation process that converts myoblasts to myotubes (Deng *et al.* 2005, Micheli *et al.* 2005). The class IIa HDACs are primarily regulated by phosphorylation-dependent nuclear export (McKinsey *et al.* 2000). Their phosphorylation provides binding sites for the 14-3-3 chaperone proteins, which export HDACs out of the nucleus via a CRM-1-dependent mechanism, which is sufficient to derepress the genes that they regulate (McKinsey *et al.* 2000). We have recently found that the AMP-activated protein kinase (AMPK) is a HDAC5

kinase (McGee *et al.* 2008). AMPK is a cellular energy gauge, which is activated by alterations in the cellular AMP to ATP ratio and by hormones that signal low energy status (Kahn *et al.* 2005). In skeletal muscle, AMPK is thought to control metabolism not only by acute regulation of metabolic pathways but also by transcriptional control of these processes (McGee & Hargreaves 2010). Indeed, we have shown that phosphorylation of HDAC5 by AMPK increases *GLUT4* (*SLC2A4*) gene expression (McGee *et al.* 2008). This suggests that in addition to their role in repressing muscle differentiation, the class IIa HDACs could also have an important role in muscle glucose metabolism. Supporting this are experiments showing that cardiac expression of mutant *HDAC5* that cannot be phosphorylated suppressed numerous metabolic genes, including those involved in glucose, fatty acid and oxidative metabolism (Czubryt *et al.* 2003). Together, these data suggest that HDAC5 could be a central regulator of metabolic genes. Although we have shown that HDAC5 regulates the *GLUT4* gene, the functional effects of HDAC5 on glucose metabolism have not been directly assessed in skeletal muscle cells. Furthermore, the role of HDAC5 in insulin action has also not been assessed. Therefore, the purpose of this study was to examine whether knockdown of *HDAC5* regulates glucose uptake and insulin action in muscle cells and to determine whether this is independent of its effects on the initiation of differentiation. Furthermore, in proof-of-principle experiments, we also sought to determine whether pharmacological HDAC inhibition can regulate these same processes, using the hydroxamate broad-spectrum class I and class II HDAC inhibitor Scriptaid. As therapies aimed at restoring metabolic homeostasis in states of overnutrition will likely have to increase cellular energy expenditure (Anderson *et al.* 2009), we also examined myotube bioenergetics and metabolic flux in response to Scriptaid treatment.

Materials and methods

Reagents

DMEM, Opti-MEM, fetal bovine serum (FBS), penicillin/streptomycin (pen/strep), TRIzol, High-Capacity cDNA RT Kit and Lipofectamine LTX were from Invitrogen. Human myoblasts and myoblast media were from Lonza (Basel, Switzerland). Delta 8.9 and vsv-g plasmids were from Research Biolabs (Singapore) and pLKO.1 shRNA plasmids, horse serum (HS) and Scriptaid were from Sigma. RNeasy Mini Kits were purchased from Qiagen. TaqMan Universal PCR Master Mix and TaqMan probes for HDAC5 (Mm00515941_g1), GLUT4 (Mm01245507_g1), GLUT1 (Mm01192270_m1), MyoD (Mm00440387_m1), myogenin (Mm00446195_g1),

PGC-1 α (PPARGC1A) (Mm00447187_g1) and 18S (Hs999999901_s1) were from Applied Biosystems. Seahorse XF analyser culture plates and assay cartridges were from Seahorse Bioscience (Bellerica, MA, USA).

Lentivirus production

A shRNA sequence recognising bases 1324–1345 (CCATCGTGAGAATGGCTTTA) of human *HDAC5* cDNA (which shows 100% homology to mouse) in the pLKO.1 vector, or the empty pLKO.1 vector (control), were transfected with delta 8.9 and vsv-g plasmids at a ratio of 10:9:1 into HEK293-LentiX cells using Lipofectamine LTX at 3.75 μ l/ μ g of DNA. Transfected cells were incubated at 37 °C for 18 h before changing media to the target cell culture media's type including 10 mM sodium butyrate and were incubated for a further 8 h, before withdrawal of the sodium butyrate by media change. Cells were incubated overnight before collecting the viral supernatant. Media were replaced and the viral supernatant again collected 24 h later. All supernatants were filtered (0.45 μ m) and stored at –80 °C.

Cell culture

HEK293-LentiX, C2C12 myoblast and L6 myoblast cells were maintained in high-glucose DMEM supplemented with 10% FBS and 1% pen/strep. Human primary myoblasts were maintained in Human Myoblast Media supplemented with 10% FBS and 1% pen/strep. All myoblast cell lines were induced to differentiate to myotubes at confluence by replacing 10% FBS with 2% HS. All cultures were maintained in a humidified incubator at 37 °C with 5% CO₂. C2C12 and human primary myoblasts were infected with lentiviral supernatant at 3000 cell/cm². C2C12 myoblasts with stable knockdown of *HDAC5* were generated by selection with 1 μ g/ml puromycin for four passages (14 days) before experiments. Upon confluence, myoblasts were induced to differentiate to myotubes by exchanging media serum from 10% FBS to 2% HS. Scriptaid treatment was performed for 48 h at day 4 post-differentiation.

Real time RT-PCR

Total RNA was isolated by collecting cells in TRIzol and taking the upper aqueous phase after incubation with chloroform. An equal volume of 70% ethanol was added and RNA was extracted using the RNeasy Mini Kit columns. RNA was eluted in 50 μ l RNase-free water and the concentration of RNA was estimated by spectrophotometry. cDNA (2 μ g) was synthesised using the High-Capacity cDNA RT Kit. Gene expression was determined using TaqMan probes for *HDAC5*,

GLUT4, *GLUT1* (*SLC2A1*), *MyoD*, myogenin, *PGC-1 α* and *18S* and TaqMan Universal PCR Master Mix. Gene expression levels were determined using the $\Delta\Delta CT$ method using the housekeeping gene *18S*. *18S* expression did not change in any of the interventions tested (data not shown).

GLUT4 reporter assay

Each 96-well plate of control and stable HDAC5 k/d C2C12 myoblasts and human primary myoblasts (~60% confluence) were transfected with a total of ~2.0–3.0 μ g DNA per plate using the Lipofectamine (Invitrogen)-mediated transfection. Procedure was followed as per the manufacturer's instructions. Briefly, pSGG-Prom reporter vector (Switchgear Genomics, Menlo Park, USA) containing ~900 bp human *Glut4* promoter was transfected with or without *HDAC5* shRNA. The transfection efficiencies were normalised with Renilla luciferase vector. The culture medium was changed 24 h later and upon reaching 100% confluency cells were differentiated for 2 days. Subsequently, cells were harvested for the dual luciferase assay. Fold activation was expressed relative to activity obtained after co-transfection of the promoter-reporter and control shRNA vectors only, arbitrarily set at 1.

Glucose uptake

Cell media from myoblast and myotube cultures in 96-well plates were removed and cells were washed twice with Krebs–Ringer HEPES buffer (130 mM NaCl, 5 mM KCl, 2.5 mM CaCl_2 , 20 mM HEPES, 1.2 mM MgSO_4 , 1.2 mM KH_2PO_4 and 0.1% BSA), before being incubated in this buffer for 60 min at 37 °C with 5% CO_2 . Cells were then incubated with labelled deoxy-[^3H]-D-glucose (0.25 μ Ci; 5 μ M) and unlabelled 2-deoxy-D-glucose (2 mM) for 15 min at 37 °C with 5% CO_2 before being washed twice with Krebs–Ringer HEPES buffer. Cells were solubilised with 30 μ l 0.1% SDS and 20 μ l was transferred to an Isoplate-96 Microplate. Scintillation (100 μ l) cocktail was added to each well and incubated at room temperature for 2 h before measurement with a Beta Counter. The remaining 10 μ l cell lysate were used to determine protein concentration, with deoxy-[^3H]-D-glucose normalised to protein content.

Glycogen synthesis

Assays were performed on myotubes after 4 days of differentiation. Differentiation media were removed and myotubes were serum starved (DMEM and 0.2% BSA) for 3 h before the addition of 50 nM insulin or vehicle and [^3H]-D-glucose (1 μ Ci) followed by further incubation for 120 min in the culture incubator.

Cells were washed twice with PBS before being lysed in 300 μ l 1 M NaOH. Lysates were left to incubate overnight at room temperature, followed by the addition of 10 mg/ml cold glycogen (1:1, v/v) and 100% ethanol (1:1, v/v). Samples were incubated at –20 °C for 30 min before being spun in a centrifuge at 12 000 g for 10 min at 4 °C. The pellet was solubilised with 100 μ l deionised water and 20 μ l were transferred to an Isoplate-96 Microplate. Scintillation (100 μ l) cocktail was added to each well and incubated at room temperature for 2 h, before measurement with a Beta Counter.

Western blotting

Cells were collected in RIPA buffer and the total protein content of cell lysates was determined using the DC Protein Assay Kit (Bio-Rad). Protein (30 μ g) was mixed with NuPAGE LDS Sample Buffer (Invitrogen) and was separated by SDS–PAGE. Protein was transferred to membranes using the iBlot System (Invitrogen), blocked with 5% BSA in Tris-buffered saline with 0.25% Tween 20 (TBST) for 30 min at RT. Membranes were exposed to primary antibodies overnight at 4 °C, washed in TBST, exposed to secondary antibodies for 60 min at RT and washed again. Antibody binding was visualised using the Li-Cor Odyssey System.

Bioenergetics and mitochondrial function assays

Bioenergetic and mitochondrial function analyses were performed using the Seahorse XF24 Extracellular Flux Analyser (Seahorse Bioscience). L6 myoblasts were seeded into 24-well Seahorse V7 plates at 2.5×10^4 cells/well. The following day, cells were differentiated to myotubes by serum withdrawal. After 4 days of differentiation, myotubes were treated with 0.1 or 1 μ M Scriptaid, or vehicle (PBS and 10% DMSO) for 60 min, twice per day, for 2 days. Myotubes were assayed on day 6 of differentiation, 16 h after the final Scriptaid treatment. Before assay, cells were washed twice with assay running media (unbuffered DMEM, 25 mM glucose, 1 mM glutamine and 1 mM sodium pyruvate), before being resuspended in 675 μ l running media. Cells were equilibrated in a non- CO_2 incubator for 60 min before assay. The assay protocol consisted of repeated cycles of 4-min mix, 2-min wait and 2-min measurement periods, with oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) measured simultaneously through each measurement period by excitation of fluorophores for O_2 and H^+ . This gives measurement of oxidative and non-oxidative metabolism respectively. Basal energetics were established after three of these cycles, followed by sequential exposure of the ATP synthase inhibitor oligomycin, the proton ionophore carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone

(FCCP) and the complex III inhibitor antimycin A, all to a final concentration of 1 μ M. These compounds were introduced to the cell media by the Seahorse injection system. Three mix, wait and measurement cycles separated each compound injection. Respiration due to ATP turnover was determined by subtracting the mean respiration values following oligomycin addition from basal respiration rates. Uncoupled respiration was determined by subtracting the mean respiration rate following antimycin A addition from that following oligomycin addition. Maximal respiratory capacity was determined by respiration following FCCP addition. Spare respiratory capacity was established by subtracting basal respiration rate from maximal respiration. All calculations were performed on values obtained in each well and all treatment conditions were analysed as ten replicates, over at least two independent experiments and data were pooled to give average values for each treatment.

Statistical analysis

All values are reported as mean $\pm$ S.E.M. and were evaluated for statistically significant differences using *t*-tests, ANOVA or two-way ANOVA where appropriate. Differences between groups were considered statistically significant where $P < 0.05$.

Results

Knockdown of HDAC5 increases glucose uptake and regulates GLUT expression in human primary myotubes

We have previously shown that HDAC5 regulates the *GLUT4* gene (McGee *et al.* 2008). To assess the role of HDAC5 on metabolism and insulin action, we infected human primary myoblasts with a lentivirus expressing *HDAC5* shRNA. Myoblasts were then differentiated to myotubes for 4 days before analysis of gene expression and glucose uptake. We achieved $\sim 90\%$ knockdown of *HDAC5* gene expression following infection of human primary myoblasts with the *HDAC5* shRNA lentivirus (Fig. 1A). *HDAC5* knockdown significantly increased basal glucose uptake (Fig. 1B), which was associated with enhanced expression of the glucose transport isoform 4 (*GLUT4*) gene (Fig. 1C). To determine whether the regulation of *GLUT4* expression was associated with regulation of the *GLUT4* promoter, gene reporter assays were performed using a plasmid expressing the luciferase gene driven by ~ 900 bp of the *GLUT4* promoter. A titrated knockdown of *HDAC5* resulted in reciprocal increases in *GLUT4* promoter activity (Fig. 1D). We also assessed *GLUT1* gene expression, which is a contributor to basal glucose

Figure 1 Knockdown of *HDAC5* increases glucose uptake and regulates GLUT expression in human primary myotubes (A) *HDAC5* mRNA expression in control and *HDAC5* knockdown human primary myotubes; (B) glucose uptake in control and *HDAC5* knockdown human primary myotubes; (C) *GLUT4* mRNA in control and *HDAC5* knockdown human primary myotubes; (D) *GLUT4* promoter activity in control and *HDAC5* knockdown human primary myotubes; (E) *GLUT1* mRNA in control and *HDAC5* knockdown human primary myotubes; and (F) *PGC-1 α* mRNA in control and *HDAC5* knockdown human primary myotubes. Data are expressed as mean $\pm$ S.E.M. $n = 3-6$ per group. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs control knockdown.

uptake, and found that *HDAC5* knockdown resulted in a small but statistically significant decrease in *GLUT1* mRNA (Fig. 1E). We also assessed the expression of *PGC-1 α* , which is thought to be a central regulator of metabolic gene expression (Lin 2009) and a target of HDAC5 (Czubryt *et al.* 2003), and found no change in *PGC-1 α* gene expression (Fig. 1F).

Enhanced glucose uptake is independent of differentiation and HDAC5 knockdown enhances insulin action

The differentiation of myoblasts to myotubes is known to increase cellular metabolism and metabolic capacity

(Franko *et al.* 2008). To determine whether the increased basal glucose uptake was due to alterations in the initiation of differentiation regulated by HDAC5, we infected human myoblasts with the *HDAC5* shRNA lentivirus 48 h after the onset of differentiation and then assessed basal glucose uptake 48 h after infection. Glucose uptake was again enhanced following *HDAC5* knockdown using this protocol (Fig. 2A). To assess whether HDAC5 regulates insulin action, we also performed basal and insulin-stimulated glycogen synthesis in human primary myotubes using this same differentiation/knockdown procedure. *HDAC5* knockdown had a small but significant effect on basal glycogen synthesis; however, insulin-stimulated glycogen synthesis was increased by 46% in *HDAC5* knockdown cells, suggesting enhanced insulin action (Fig. 2B).

Figure 2 Enhanced glucose uptake is not due to altered differentiation and *HDAC5* knockdown enhances insulin action. (A) Glucose uptake in control and *HDAC5* knockdown human primary myotubes, infected after the initiation of differentiation and (B) basal and insulin-stimulated (50 nM) glycogen synthesis in control and *HDAC5* knockdown human primary myotubes, infected after the initiation of differentiation. Data are expressed as mean $\pm$ S.E.M. $n=6-8$ per group. ** $P<0.01$ and *** $P<0.001$.

The effects of HDAC5 knockdown on GLUT4 promoter activity and glucose uptake are consistent in two different muscle cell types

To determine whether the observed effects of *HDAC5* knockdown on GLUT4 regulation and glucose uptake were specific for human primary muscle cells, we assessed GLUT4 promoter activity and glucose uptake in C2C12 myotubes following *HDAC5* knockdown. Infection of C2C12 myoblasts with *HDAC5* shRNA lentivirus before differentiation resulted in 62% knockdown of *HDAC5* in myotubes (Fig. 3A). Knockdown of *HDAC5* also increased GLUT4 promoter activity (Fig. 3B) and glucose uptake (Fig. 3C) in C2C12 myotubes. These data show that HDAC5 is a regulator of GLUT4 and glucose uptake in two different muscle cell types.

Scriptaid, a broad-spectrum HDAC inhibitor, also regulates GLUT4 gene expression, glucose uptake and mitochondrial function

The data presented to date show that inhibition of HDAC5 can enhance glucose metabolism and insulin action in muscle cells. Therefore, we also examined the effects of the broad-spectrum HDAC inhibitor, Scriptaid, on metabolism. Forty-eight hours of Scriptaid exposure increased histone 3 acetylation in a dose-dependent manner in L6 myotubes (Fig. 4A). This was associated with significant increases in *GLUT4* expression at Scriptaid concentrations of 0.5 μ M and higher (Fig. 4B). Incubation of L6 myotubes with 1 μ M Scriptaid for 48 h also increased basal glucose uptake (Fig. 4C), with no change in *GLUT1* gene expression (data not shown). We also assessed the ability of Scriptaid to alter cellular bioenergetics using the Seahorse XF analyser. Myotubes were exposed to Scriptaid or vehicle for 1 h, twice per day for 2 days before assay. This protocol was used to mimic potential drug treatment and pharmacokinetics *in vivo*. Incubation of myotubes with 0.1 μ M Scriptaid increased basal OCR, while 1 μ M Scriptaid increased both basal OCR and ECAR (Fig. 4D), suggesting that Scriptaid increases cellular energy expenditure. To determine the metabolic parameter(s) contributing to the increase in basal oxidative metabolism, we also performed mitochondrial function tests on these cells. Scriptaid increased basal ATP turnover (Table 1), without any change in uncoupled respiration, which are the two parameters contributing to basal OCR. Scriptaid also increased maximal and spare respiratory capacity, while 1 μ M Scriptaid also increased non-mitochondrial oxygen consumption (Table 1).

Figure 3 Knockdown of *HDAC5* increases GLUT4 promoter activity and glucose uptake in C2C12 myotubes. (A) *HDAC5* mRNA expression in control and *HDAC5* knockdown C2C12 myotubes; (B) GLUT4 promoter activity in control and *HDAC5* knockdown C2C12 myotubes; and (C) glucose uptake in control and *HDAC5* knockdown human primary myotubes; data are expressed as mean $\pm$ s.e.m. $n=3-6$ per group. ** $P<0.01$ and *** $P<0.001$ vs control knockdown.

Discussion

We have previously shown that HDAC5 represses the *GLUT4* gene (McGee *et al.* 2008). However, to date, no study has examined whether members of the class IIa HDACs actually regulate glucose metabolism in skeletal muscle cells. Our data show that knockdown of *HDAC5* is sufficient to increase glucose uptake in both human primary myotubes and mouse C2C12 myotubes. This was associated with increased GLUT4 promoter activity in the two cell types examined. Whether the increase in glucose uptake with *HDAC5* knockdown is solely due to enhanced *GLUT4* expression remains to be determined; however, HDAC5 has been shown to repress a number of genes involved in glucose metabolism, including hexokinase II and glycogen phosphorylase (Czubryt *et al.* 2003). This study also suggests that the effect of *HDAC5* knockdown on glucose uptake occurs independently of any increase in *PGC-1 α* expression. This is an interesting finding given that the class IIa HDACs are thought to regulate the *PGC-1 α* promoter and that many of the muscle phenotypic effects of the class IIa HDACs have been ascribed to regulation of *PGC-1 α* (Czubryt *et al.* 2003). However, HDAC5 is known to directly repress transcription factors such as *MEF2* (Lu *et al.* 2000), serum response factor (SRF; Xing *et al.* 2006) and the nuclear factor of activated T-cells (Dai *et al.* 2005), which are also known to exert phenotypic effects in muscle. Data from this study also show that *HDAC5* knockdown enhances insulin action. As cultured myotubes show limited insulin responsiveness as assayed by glucose uptake, we assessed insulin-stimulated glycogen synthesis in these cells. Using this method, we found that the fold increase in insulin action was 46% greater in *HDAC5* knockdown cells. It remains to be determined whether *HDAC5* knockdown enhances insulin action *per se*, or whether it regulates the expression of rate-limiting enzymes in this pathway. However, the findings that HDAC5 regulates glucose uptake and insulin action are important, as these processes are dysregulated in metabolic diseases such as obesity and type 2 diabetes (Savage *et al.* 2007). Therefore, targeting the class IIa HDACs in muscle could be an effective treatment strategy to normalise these defects in metabolic disease states.

With this in mind, we also tested the efficacy of the broad spectrum class I and class II HDAC inhibitor Scriptaid, in proof-of-principle experiments, to determine whether HDAC inhibition also regulates metabolic processes in myotubes. These data revealed that *GLUT4* expression and glucose uptake were also increased following exposure to Scriptaid. These glucose uptake data are similar to those reported by Takigawa-Imamura *et al.* (2003). However, that paper showed no effect of Scriptaid on *GLUT4* gene expression (Takigawa-Imamura *et al.* 2003), while we

Figure 4 Scriptaid regulates *GLUT4* gene expression, glucose uptake and mitochondrial function. (A) Histone 3 acetylation in response to increasing concentrations of Scriptaid for 48 h in L6 myotubes; (B) *GLUT4* mRNA in response to increasing concentrations of Scriptaid for 48 h in L6 myotubes; (C) glucose uptake in response to 1 μM Scriptaid for 48 h in L6 myotubes and (D) basal OCR and ECAR in L6 myotubes in response to multiple treatments with 0.1 and 1 μM Scriptaid. Data are expressed as mean ± S.E.M. *n* = 20 per group, across two independent experiments. **P* < 0.05, ***P* < 0.01 and ****P* < 0.001 vs DMSO, †*P* < 0.05 vs DMSO for OCR and #*P* < 0.05 vs DMSO for OCR and ECAR.

observed a correlation between glucose uptake and *GLUT4* expression. Nonetheless, these data show that HDAC inhibition studies in whole-body models of metabolic disease are warranted. Scriptaid has similar inhibitory activities towards HDAC5 (*IC*₅₀ = ~2 μM) and the class I HDACs (*IC*₅₀ = ~0.6 μM; Bradner *et al.* 2010), and thus, we cannot conclude that the Scriptaid effect on metabolism is mediated entirely through HDAC5. Indeed, there is good rationale for inhibiting both HDAC5 and the class I HDACs, as the class IIa HDACs are thought not to contain intrinsic HDAC activity, but instead gain HDAC activity via recruitment of HDAC3 as part of a multi-protein repressive complex

(Fischle *et al.* 2002). It has been proposed that HDAC inhibitors impair class IIa activity by dissociating this repressive complex from class IIa HDACs (Bradner *et al.* 2010). These experiments provide the impetus to develop more specific class IIa-specific inhibitors to determine the specificity requirements for these compounds to increase *GLUT4* and metabolic gene expression.

However, it is generally accepted that any therapy designed to completely resolve skeletal metabolic dysregulation seen in response to the caloric excess observed in many metabolic disease states will likely need to increase basal skeletal muscle energy

Table 1 Mitochondrial function (Mt Fx) parameters in response to Scriptaid. Data are expressed as mean ± S.E.M. *n* = 8 per group

Mt Fx parameter (OCR pmol/min)	Scriptaid		
	0 μM	0.1 μM	1 μM
ATP turnover	236.4 ± 6.5	275.0 ± 7.8 [†]	283.0 ± 7.7 [†]
Uncoupled respiration	47.2 ± 3.3	47.8 ± 2.3	50.6 ± 2.6
Maximal respiratory capacity	866.5 ± 37.3	1069.8 ± 42.5 [†]	1038.0 ± 41.0 [†]
Spare respiratory capacity	497.0 ± 28.8	652.1 ± 33.1 [†]	607.5 ± 32.6 [†]
Non-mitochondrial O ₂ consumption	86.0 ± 3.2	94.9 ± 4.0	97.8 ± 3.4*

**P* < 0.05 vs DMSO, †*P* < 0.001 vs DMSO.

expenditure (Anderson *et al.* 2009). We assessed whether Scriptaid also regulated basal energy expenditure by measuring cellular OCR and ECAR using the Seahorse XF analyser. Scriptaid increased basal OCR, and at higher concentrations also increased ECAR, a proxy measure of anaerobic glycolysis. This suggests that Scriptaid can increase basal energy expenditure through increased oxidative and non-oxidative metabolism. To determine the mechanism by which Scriptaid increases oxidative metabolism, we also performed mitochondrial function tests on these cells. These analyses revealed that the increase in basal respiration was due to enhanced ATP turnover, while there was no contributing effect from uncoupled respiration. Future studies will be required to determine the mechanism resulting in higher ATP turnover. These data complement recent findings that hepatic glucose production is under the control of the class IIa HDACs (Mihaylova *et al.* 2011). Together, these studies highlight that further studies examining the utility of HDAC inhibition as a therapeutic strategy in the treatment of metabolic diseases such as obesity and type 2 diabetes are warranted.

Importantly, we also showed that the effects of HDAC5 knockdown on glucose uptake were independent of myoblast differentiation. As metabolic processes such as glucose uptake and mitochondrial capacity are increased throughout myogenic differentiation (Franko *et al.* 2008), it was possible that the increased glucose uptake observed with HDAC5 knockdown was simply due to enhanced myogenesis. However, we showed that the increase in glucose uptake was preserved when HDAC5 knockdown was performed after the initiation of myogenic differentiation. These data suggest that targeting the class IIa HDACs in fully differentiated muscle cells *in vitro* is effective in modulating metabolism. This is further supported by data showing that Scriptaid is able to increase metabolic parameters in fully differentiated myotubes.

In conclusion, this study shows that HDAC5 regulates glucose metabolism and insulin action in both human and mouse myotubes. We have also shown that HDAC inhibition in myotubes can increase glucose uptake and basal energy expenditure. As glucose metabolism is dysregulated in metabolic disease states such as obesity and type 2 diabetes, these data indicate that further experiments targeting HDAC5 as a therapeutic strategy for the treatment of these diseases are needed.

Declaration of interest

S R, S H T, K O, Y C L and J K were employees of Eli Lilly.

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