HC toxin (a HDAC inhibitor) enhances IRS1–Akt signalling and metabolism in mouse myotubes

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Abstract

Exercise enhances numerous signalling pathways and activates substrate metabolism in skeletal muscle. Small molecule compounds that activate these cellular responses have been shown to recapitulate the metabolic benefits of exercise. In this study, a histone deacetylase (HDAC) inhibitor, HC toxin, was investigated as a small molecule compound that activates exercise-induced adaptations. In C2C12 myotubes, HC toxin treatment activated two exercise-stimulated pathways: AMP-activated protein kinase (AMPK) and Akt pathways. HC toxin increased the protein content and phosphorylation of insulin receptor substrate 1 as well as the activation of downstream Akt signalling. The effects of HC toxin on IRS1–Akt signalling were PI3K-dependent as wortmannin abolishes its effects on IRS1 protein accumulation and Akt phosphorylation. HC toxin-induced Akt activation was sufficient to enhance downstream mTOR complex 1 (mTORC1) signalling including p70S6K and S6, which were consistently abolished by PI3K inhibition. Insulin-stimulated glucose uptake, glycolysis, mitochondrial respiration and fatty acid oxidation were also enhanced in HC toxin-treated myotubes. When myotubes were challenged with serum starvation for the induction of atrophy, HC toxin treatment prevented the induction of genes that are involved in autophagy and proteasomal proteolysis. Conversely, IRS1–Akt signalling was not induced by HC toxin in several hepatoma cell lines, providing evidence for a favourable safety profile of this small molecule. These data highlight the potential of HDAC inhibitors as a novel class of small molecules for the induction of exercise-like signalling pathways and metabolism.

Key Words

- ▶ histone deacetylase (HDAC)
- ▶ Akt
- ▶ insulin receptor substrate 1 (IRS1)
- ▶ metabolism
- ▶ exercise
- ▶ myotubes

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Introduction

Exercise plays a pivotal role in the prevention and treatment of metabolic diseases, including diabetes and obesity. Although exercise has a body-wide effect, it is believed that these benefits require metabolic adaptations in the skeletal muscle (Hawley 2004). Indeed, exercise enhances the utilisation and storage of metabolic

substrates by skeletal muscle, which, in turn, prevent the detrimental accumulation of excess macronutrients in the circulation (Hawley 2004, Abdul-Ghani & DeFronzo 2010). Although the molecular mechanisms underlying exercise-induced adaptations in muscle have not been fully elucidated, AMP-activated protein kinase (AMPK)

and Akt have been proposed to play a role. Studies have shown that exercise activates Akt in mouse and human skeletal muscle (Sakamoto *et al.* 2003, Deshmukh *et al.* 2006), and the overexpression of constitutively active Akt in L6 myotubes stimulates glucose uptake in the absence of insulin (Hajdуч *et al.* 1998). In contrast, pharmacological inhibition of Akt reduced contraction-stimulated glucose uptake in skeletal muscle cells (Wojtaszewski *et al.* 1996, Osorio-Fuentealba *et al.* 2013). Collectively, these studies suggest a role for Akt in exercise-induced glucose uptake.

Exercise promotes hypertrophy and prevents atrophy in skeletal muscle through the activation of mechanistic target of rapamycin complex 1 (mTORC1), a critical downstream target of Akt (Bodine *et al.* 2001). Inhibition of exercise-induced mTORC1 signalling by rapamycin blocked protein synthesis in both human and rodent skeletal muscle (Kubica *et al.* 2005, Drummond *et al.* 2009) and prevented muscle hypertrophy induced by overload or the overexpression of constitutively active Akt in mice (Bodine *et al.* 2001, Pallafacchina *et al.* 2002). Akt also inactivates Forkhead box Class O (FoxO) transcription factors, which induce muscle atrophy through the expression of numerous atrophy-related genes, including MuRF-1 and atrogin-1 (Zhao *et al.* 2007). The expression of dominant-negative FoxO in skeletal muscle prevented atrophy in cachectic mice and induced hypertrophy in normal mice (Reed *et al.* 2012). Conversely, the expression of a constitutively active mutant of FoxO3a induced atrophy in both C2C12 myotubes and mouse skeletal muscle (Sandri *et al.* 2004). Thus, exercise promotes muscle hypertrophy and suppresses atrophy through a coordinated change in the Akt-regulated anabolic and catabolic mediators, mTORC1 and FoxO, respectively.

AMPK is an energy-sensing molecule that is activated during periods of energetic stress and has been identified as a key signalling molecule in exercise (Richter & Ruderman 2009). Numerous studies have demonstrated that pharmacological activation of AMPK produces metabolic responses and adaptations that are similar to those observed in exercise. Chronic administration of the AMPK activator, AICAR, improved skeletal muscle insulin sensitivity and prevented the development of hyperglycaemia in a diabetic rat model; these effects were similar to that observed in response to long-term exercise training (Pold *et al.* 2005). Similarly, both exercise and chronic AICAR treatment enhanced the expression of genes involved in lipid utilisation and insulin-stimulated glucose uptake in rat muscle (Jessen *et al.* 2003). Further evidence for the role of AMPK in exercise-induced effects comes from genetic

mouse models. The expression of kinase-dead AMPK in mouse skeletal muscle impaired contraction-stimulated glucose uptake (Mu *et al.* 2001), whereas knockout of the AMPK γ 3 subunit accelerated muscle fatigue and impaired transcriptional adaptations in response to exercise (Barnes *et al.* 2005). Thus, AMPK activation is a hallmark of exercise-induced signalling and could be used to recapitulate the beneficial effects of exercise.

Histone deacetylases (HDACs) are enzymes that catalyse the removal of acetyl groups from histones, which result in chromatin compaction and subsequent repression of gene expression (De Ruijter *et al.* 2003). Recent studies suggest that HDACs play a role in the regulation of skeletal muscle metabolism. Dietary supplementation of butyrate prevented diet-induced insulin resistance in mice, and this effect was associated with an inhibition of HDAC (Gao *et al.* 2009). In addition, it was recently shown that knockdown of HDAC5 in human muscle cells resulted in increased glucose uptake and insulin-stimulated glycogen synthesis (Raichur *et al.* 2012). This raises the possibility that HDAC inhibitors may be used to modulate exercise-induced signalling and adaptations in skeletal muscle.

In this study, we evaluated the potential of *Helminthosporium carbonum* (HC) toxin, a previously established HDAC inhibitor, as a small molecule that induces exercise-like adaptations in skeletal muscle. HC toxin is a cyclic tetrapeptide originally identified as a host-selective toxin from the maize pathogenic fungus *Cochliobolus* (*Helminthosporium*) *carbonum* (Mottamal *et al.* 2015), and it was shown to inhibit class I HDAC enzymes (HDAC1, -2, -3 and -8) in mammalian cells (Brosch *et al.* 1995, Hildmann *et al.* 2006). Herein, we report that HC toxin activates two exercise-activated pathways in C2C12 myotubes: AMPK and Akt pathways. These changes are associated with metabolic gene reprogramming as well as enhanced glucose and lipid metabolism. We also show that HC toxin treatment activates mTORC1 and suppresses atrophy-related genes in myotubes, an effect that is observed less in other reported exercise mimetics. These data highlight the potential of HDAC inhibitors as a novel class of small molecules for the induction of an exercise-like signalling and metabolic program.

Materials and methods

Materials

Antibodies against phospho-AMPK α (Thr172), AMPK α , phospho-Akt (Ser473), Akt, PDK1, phospho-p70S6K

(Thr389), p70S6K, phospho-S6 (Ser235/236), S6, phospho-IRS1 (Ser318), phospho-IRS1 (Ser612), IRS1, IRS2, phospho-FoxO3a (Ser253), FoxO3a and HRP-linked secondary anti-rabbit antibodies were from Cell Signaling Technology (Beverly, MA, USA). HC toxin was from Cayman Chemical (Ann Arbor, MI, USA). Wortmannin, dimethylsulfoxide, oligomycin, carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP), antimycin A, rotenone, sodium palmitate and real-time PCR primers were from Sigma-Aldrich (St Louis, MO, USA).

Cell culture

Mouse C2C12 myoblasts, human HepG2 hepatoma cells, rat H4IIE hepatoma cells (ATCC, Manassas, VA, USA), human Huh7 and Huh6 hepatoma cells (JCRB Cell Bank, Osaka, Japan) were maintained in DMEM (SH30249; GE Healthcare Life Sciences HyClone Laboratories, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin and 1 mM sodium pyruvate. The cells used were <20 passages. For C2C12 myoblasts, media was switched to differentiation media (DMEM supplemented with 2% horse serum, 100 U/ml penicillin, 100 µg/ml streptomycin and 1 mM sodium pyruvate) over 3 days to initiate the differentiation of myoblasts to myotubes. C2C12 myotubes in differentiation media and hepatoma cells in serum-free media (supplemented with 0.2% BSA) were treated with HC toxin at the times and concentrations indicated. For PI3K inhibition experiments, 200 nM wortmannin was added during the last 2 h of the HC toxin treatment.

Immunoblotting

Cells were homogenized on ice in 0.2 ml of ice-cold lysis buffer containing 25 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% IGEPAL, 24 mM sodium deoxycholate, 0.1% SDS, 5 mM β-glycerophosphate, 5 mM NaF, 5 mM Na₃VO₄ and 1% Halt protease inhibitor cocktail (Life Technologies, Carlsbad, CA, USA). Lysates were subjected to brief sonication and centrifuged at 13 000 *g* for 10 min to remove insoluble cell debris, and the supernatant was diluted in Laemmli sample buffer. Proteins (30 µg/well) were separated by SDS-PAGE and transferred to PVDF membranes (Bio-Rad Laboratories). Membranes were blocked with 5% non-fat milk, incubated overnight with primary antibodies, washed and then incubated with secondary antibodies. Proteins were visualized by chemiluminescence and quantified by densitometry using ImageJ (NIH, Bethesda, MD, USA).

Quantitative real-time PCR

Total RNA was extracted from myotubes using the ReliaPrep RNA Cell Miniprep System (Promega) following the manufacturer's protocol. cDNA was prepared using SuperScript III First-Strand Synthesis System for RT-PCR (Life Technologies). Quantitative real-time PCR (qRT-PCR) reaction consisted of 1× SYBR Select Master Mix (Life Technologies), 10 µM each of gene-specific forward and reverse primers and 30 ng cDNA per reaction. qRT-PCR was performed in a 96-well plate format, and reactions were carried out on the Applied Biosystems 7300 Real-Time PCR System. The abundance of target gene mRNA was normalized to housekeeping genes 36B4 or β-actin.

Glucose uptake assay

C2C12 myotubes were incubated with or without HC toxin in DMEM supplemented with 2% horse serum for 24 h. Myotubes were then washed with warm PBS and subsequently incubated in KRHB (130 mM NaCl, 5 mM KCl, 1.3 mM MgSO₄, 1.3 mM CaCl₂, 25 mM HEPES, pH 7.4) with or without insulin for 20 min, followed by incubation with 0.05 mM 2-deoxy-D-glucose and 1.85 × 10⁴ Bq/ml 2-deoxy-D-[1,2-³H] glucose for 10 min. Radioactivity was determined by liquid scintillation counting (Perkin Elmer Trilu, Waltham, MA, USA).

Measurements of extracellular acidification rate and oxygen consumption rate

C2C12 myoblasts were plated and differentiated on XF24 cell culture microplates. Myotubes were then treated with HC toxin at the times and concentrations as indicated. Media was then switched to unbuffered DMEM (D5030; Sigma-Aldrich) supplemented with 25 mM glucose, 4 mM glutamine and 2% horse serum. For extracellular acidification rate (ECAR) experiments, 1 mM sodium pyruvate was added. Myotubes were subsequently incubated in a non-CO₂ incubator for 1 h. ECAR and oxygen consumption rate (OCR) measurements were then performed using the XF^e24 flux analyser (Seahorse Bioscience, North Billerica, MA, USA). During ECAR measurements, 1 µM oligomycin, 0.7 µM carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) and 1 µM antimycin A/rotenone were sequentially added. Each cycle consisted of 3 min of mixing, 2 min of pre-incubation and 3 min of measurement. For the measurement of fatty acid oxidation (FAO), the XF FAO assay was performed following manufacturer's instructions. Briefly, treated myotubes were switched to

KHB buffer (111 mM NaCl, 4.7 mM KCl, 1.25 mM CaCl₂, 2 mM MgSO₄, 1.2 mM NaH₂PO₄) supplemented with 2.5 mM glucose, 0.5 mM carnitine, 25 mM HEPES and 2% horse serum for 1 h in a non-CO₂ incubator. OCR measurements were then performed. FAO was calculated as the difference between basal OCR and OCR after injection of 200 μ M palmitate conjugated to BSA.

Results

HC toxin treatment induces AMPK and Akt-mTORC1 signalling in a dose-dependent manner

The AMPK and Akt signalling pathways are two major signalling networks that are involved in exercise-mediated skeletal muscle adaptations. To evaluate the effect of HC toxin on AMPK and Akt signalling in skeletal muscle, C2C12 myotubes were treated with 0, 10, 30 and 100 nM HC toxin for 48 h. We found that HC toxin treatment increased p-AMPK (T172) and p-Akt (S473) in a dose-dependent manner (Fig. 1A and B). Consistent with the activation of Akt, downstream mTOR signalling, as assessed by pS6 (S235/236), was also enhanced at the concentration of 100 nM (Fig. 1C). The upstream signalling component of insulin/Akt signalling, IRS1, was also increased in a dose-dependent manner (Fig. 1D).

HC toxin-induced IRS1-Akt signalling requires intact PI3K activity

IRS1 plays a critical role in insulin-like signalling and it is involved in the enhancement of insulin sensitivity after exercise (Henriksen 2002). Given that HC toxin led to a

robust increase in IRS1 protein, we further assessed changes in the IRS1 signalling pathway in response to HC toxin. We found that treatment with HC toxin resulted in increased p-IRS1 levels at S318 and S612, suggesting that both IRS1 phosphorylation and total protein levels are enhanced in response to HC toxin (Fig. 2A). Inhibition of PI3K/Akt by wortmannin caused a depletion in IRS1 protein levels and hampered the effects of HC toxin on the accumulation of the adaptor protein. As expected, wortmannin treatment reduced basal levels of S318 and S612 phosphorylation. However, HC toxin treatment was able to restore the phosphorylation of these PI3K-sensitive serine residues (Fig. 2A). Because IRS2 has also been identified as a mediator of insulin signalling, we assessed changes in the IRS2 pathway but found that HC toxin treatment did not alter IRS2 protein levels (Fig. 2A).

We next sought to determine whether other components in the insulin-like signalling pathway were affected by HC toxin treatment. Indeed, we found that HC toxin increased PDK1 (Fig. 2B), which initiates the activation of Akt (Manning & Cantley 2007). Consistently, we observed an increase in the phosphorylation of Akt at S473 (Fig. 2C), which is required for its full activation. Wortmannin treatment abolished p-Akt (S473) (Fig. 2C) in the presence or absence of HC toxin, indicating that HC toxin-induced Akt activation is PI3K dependent. HC toxin also significantly increased the phosphorylation of the mTORC1 substrate p70S6K (T389) and its direct substrate S6 (S235/236), and, again, these effects were abolished by wortmannin and unable to be recovered by HC toxin (Fig. 2D and E), suggesting that the HC toxin-mediated mTORC1 signalling axis is PI3K-dependent.

Figure 1

HC toxin enhances Akt and AMP-activated protein kinase (AMPK) signalling in C2C12 myotubes. C2C12 myotubes were incubated with HC toxin at various concentrations (0, 10, 30 and 100 nM) for 48 h. Changes in the levels of p-AMPK (A), p-AKT (B), p-S6 (C) and total insulin receptor

substrate 1 (IRS1; D) were assessed by western blot. Representative blots and quantification are presented. Bars indicate the mean $\pm$ S.E.M. * P < 0.05 vs the control group; n = 3.

Figure 2

HC toxin-induced IRS1–Akt signalling requires intact PI3K activity. C2C12 myotubes were treated with HC toxin (100 nM) for 48 h in the presence or absence of wortmannin (200 nM) for the final 2 h. Phosphorylation and protein levels of IRS1 and IRS2 (A), PDK1 (B), Akt (C), p70S6K (D) and S6 (E)

were assessed by western blot. Representative blots and quantification are presented. Bars indicate mean $\pm$ s.e.m. * $P < 0.05$ vs the control group; # $P < 0.05$ vs the wortmannin treated group; $n = 6$.

HC toxin induces coordinated changes in the expression of metabolic genes

Multiple studies have shown that a coordinated change of metabolic gene expression occurs in skeletal muscle after exercise to enhance fuel substrate metabolism (Mahoney *et al.* 2005). To investigate if HC toxin reprograms metabolic gene expression in C2C12 myotubes, the mRNA levels of genes involved in glucose and lipid metabolism were assessed. Treatment with HC toxin increased the mRNA expression of glycolytic genes, *Pk* (82%) and *Ldh2b* (77%), but reduced the expression of *Pdk4* (38%), a profile that is associated with enhanced glycolysis (Fig. 3). HC toxin also elevated the mRNA expression of *Lpl* (159%), *Cpt1b* (78%), *Slc25a20* (112%), *Had* (87%) and citrate synthase (*Cs*, 75%), which are involved in lipid transport and metabolism. Consistently, the expression of the transcriptional coactivator *Pgc1a*, which regulates mitochondrial biogenesis and respiration (Finck & Kelly 2006), was increased (224%).

HC toxin modulates glucose uptake, cellular bioenergetics and FAO in myotubes

We next assessed whether these changes in HC toxin-induced cell signalling and gene expression are sufficient

to enhance glucose uptake. Myotubes were treated with HC toxin for 24 h to assess changes in glucose uptake. We found that HC toxin induced glucose uptake in a dose-dependent manner, with a maximal effect seen at 30 nM (Fig. 4A; white bars). Furthermore, stimulation with insulin had an additive effect, further increasing the rates of glucose uptake (Fig. 4A; grey bars). Consistent with the elevation of glucose uptake, HC toxin treatment increased basal and oligomycin-stimulated ECAR, suggesting an upregulation in glycolytic flux in response to the treatment (Fig. 4B). HC toxin treatment also increased basal OCR (50 and 100 nM for 24 or 48 h), providing evidence for an upregulation of mitochondrial respiration (Fig. 4C). In addition to an enhancement of glucose metabolism, HC toxin enhanced lipid metabolism. HC toxin (10 μ M) treatment elevated FAO in a time-dependent manner (Fig. 4D).

HC toxin-induced Akt activation is associated with the suppression of atrophy-related genes during nutrient deprivation

In addition to mTORC1, Akt can regulate FoxO transcription factors via direct phosphorylation (Manning & Cantley 2007). We found that HC toxin increased

Figure 3

HC toxin induces coordinated expression of metabolic genes. C2C12 myotubes were treated with HC toxin (100 nM) for 48 h. qPCR analysis of glycolytic genes: pyruvate kinase (*Pk*), lactate dehydrogenase 2b (*Ldh2b*) and pyruvate dehydrogenase kinase 4 (*Pdk4*); lipid metabolic genes: carnitine palmitoyltransferase 1b (*Cpt1b*), carnitine/acylcarnitine translocase (*Slc25a20*), lipoprotein lipase (*Lpl*), 3-hydroxyacyl-CoA dehydrogenase (*Had*) and citrate synthase (*Cs*) and peroxisome proliferator-activated receptor gamma coactivator 1a (*Pgc1a*). Bars indicate mean $\pm$ S.E.M.

* $P < 0.05$ vs the control group; $n = 6$.

p-FoxO3a (S253) in myotubes, consistent with the increase in p-Akt (S473) (Fig. 5A). The effects of HC toxin on Akt and FoxO phosphorylation were retained even under serum-starved conditions (Fig. 5A). Given that FoxO transcription factors mediate the expression of multiple muscle atrophy-related genes (also known as atrogenes) (Zhao *et al.* 2007), we next assessed whether HC toxin-induced FoxO3a phosphorylation is associated with the suppression of these genes. We found that HC toxin suppressed the expression of autophagy-related genes *Atg5*, *Ulk2*, *Bnip3*, *Bnip3L*, *Gabrarpl1*, *Lamp2a* and *Vps34* (Fig. 5B and C), as well as muscle-specific E3 ubiquitin ligases, *MurF1* and *atrogin-1* (Fig. 5D), which are involved in proteasomal proteolysis. We next tested whether this effect could be sustained under serum-starved conditions, as this is routinely used to induce atrophy and atroge expression in myotubes. Despite the absence of growth factor and the reduced nutrient supply, HC toxin was still able to prevent the induction of these atrogenes under serum-starved conditions (Fig. 5B, C and D).

Discussion

Activation of AMPK signalling in human and rodent skeletal muscles is a hallmark of physical exercise (Richter & Ruderman 2009). The activation of AMPK during

periods of energy stress, such as exercise, induces glucose uptake and lipid oxidation to restore the skeletal muscle energy balance (Long & Zierath 2006). Here, we show that HC toxin can activate AMPK signalling in cultured myotubes, which demonstrates its potential to induce exercise-like adaptations in skeletal muscle. In addition, we observed an enhancement of Akt phosphorylation in C2C12 myotubes in response to HC toxin, an adaptation commonly observed in skeletal muscle in response to exercise and *in situ* muscle contraction (Taniguchi *et al.* 2006). Other HDAC inhibitors, including trichostatin A (TSA), sodium valproate and butyrate, have been reported to activate Akt in human neuroblastoma cells (De Sarno *et al.* 2002), suggesting that numerous HDAC inhibitors may share a similar pharmacological capacity to increase Akt activity. However, the molecular mechanism underlying Akt activation by HDAC inhibitors was largely unknown, and thus, here we sought to characterize the effect of HC toxin on the upstream components of the Akt signalling pathway. We found that HC toxin increases IRS1 protein content (Fig. 2A), which is a critical upstream adaptor protein that regulates insulin sensitivity in both humans and rodents (Henriksen 2002). Previous studies have demonstrated that genetic ablation of IRS1 in skeletal muscle results in insulin resistance and impaired insulin-stimulated glucose uptake (Yamauchi *et al.* 1996). Given that insulin-resistant individuals present with significantly lower expression of skeletal muscle IRS1 (Kovacs *et al.* 2003), this HC toxin-induced signalling network is, conceivably, an attractive means to restore insulin signalling.

IRS1 is subjected to extensive post-translational modifications, and several serine phosphorylation sites modulate its function. We found that HC toxin increased S318 phosphorylation, an early phosphorylation event that potentiates insulin signalling (Weigert *et al.* 2008). The phosphorylation of S612, which is mediated by S6K in response to insulin stimulation (Shah & Hunter 2006), was also intact in HC toxin-treated myotubes. Both of these phosphorylation events were PI3K-dependent, as wortmannin greatly diminished the activity. Downstream of IRS1, we found a consistent elevation in PDK1, which is responsible for the initial activating phosphorylation of Akt in HC toxin-treated myotubes (Manning & Cantley 2007). We further showed that this elevation in Akt phosphorylation in treated myotubes is wortmannin-sensitive (Fig. 2), supporting the notion that HC toxin increased IRS1–Akt signalling. Thus, HC toxin-enhanced IRS–Akt signalling involves the phosphorylation of these signalling components in a PI3K-dependent manner.

Figure 4

HC toxin enhances glucose uptake, glycolysis, mitochondrial respiration and fatty acid oxidation. (A) C2C12 myotubes were incubated with HC toxin for 24 h, followed by insulin stimulation (100 nM), and glucose uptake was assessed. * $P < 0.05$, ** $P < 0.01$ vs the control group; # $P < 0.05$, ### $P < 0.01$ vs the insulin-treated group. (B) ECARs were measured in control and HC toxin-treated (50 nM, 48 h) myotubes. Oligomycin (1 μ M), FCCP (0.7 μ M) and antimycin (1 μ M)/rotenone (1 μ M) were sequentially added in the

process. Error bars indicate s.e.m.; $n = 3$. (C) Basal OCR was measured in control and HC toxin-treated myotubes (50 or 100 nM for 24 or 48 h). Bars indicate mean $\pm$ s.e.m. * $P < 0.05$ vs the control group; $n = 5$. (D) C2C12 myotubes incubated with HC toxin (10 μ M) for 0, 4, 8 and 16 h, and fatty acid oxidation was determined. Bars indicate mean $\pm$ s.e.m. * $P < 0.05$ vs the control group; $n = 4-5$.

The biological significance of HC toxin-induced IRS–Akt signalling is further supported by a corresponding increase in the phosphorylation of p70S6K and S6, two critical downstream targets of the Akt–mTORC1 axis.

Exercise is known to induce adaptations in cellular signalling and gene expression to enhance substrate metabolism in skeletal muscle (Mahoney *et al.* 2005, Egan & Zierath 2013). Given that Akt plays a critical role in the regulation of glucose metabolism, we assessed whether the activation of Akt in HC toxin-treated myotubes altered the expression of certain metabolism-related genes. We found that HC toxin increased the expression of *Pk* and *Ldh2b*, whereas *Pdk4* expression was reduced – an expression profile that is associated with enhanced glucose utilisation (Fig. 3) (Sugden & Holness 2003). Consistently, HC toxin-treated myotubes displayed an enhanced basal as well as insulin-stimulated glucose uptake, concomitant with a higher rate of glycolysis, as indicated by ECAR (Fig. 4). Our results corroborate the findings of a previous study that showed that skeletal muscle-specific Akt1 transgene expression is sufficient to

induce a glycolytic gene program and glucose metabolism in obese mice (Izumiya *et al.* 2008).

HC toxin also modulated the expression of genes involved in lipid metabolism. The expression of genes critical for the uptake (*Lpl*) and transport of lipid-derived fatty-acyl CoA into the mitochondria (*Cpt1b* and *Slc25a20*) were consistently elevated (Fig. 3). *Had* (a β -oxidation enzyme) and *Cs* (a TCA cycle enzyme) were also upregulated. Consistently, we observed an elevation in FAO in response to HC toxin treatment. HC toxin also upregulated the mRNA levels of the mitochondrial biogenesis regulator, *Pgc1 α* . Consistently, we found that HC toxin increased OCR in cultured myotubes (Fig. 4), providing evidence for an enhanced oxidative capacity in the treated myotubes. Interestingly, several gene expression changes induced by HC toxin parallels those observed in exercise. Indeed, multiple human studies have shown that *Lpl* and *Cs* mRNA levels are upregulated in skeletal muscle after exercise (Siu *et al.* 2003, Vissing *et al.* 2005). Additionally, chronic exercise increases *Cpt1b* (Tunstall *et al.* 2002), *Slc25a20* (Lammers *et al.* 2012) and

Figure 5

HC toxin suppresses the expression of atrophy-related genes during nutrient deprivation. (A) C2C12 myotubes were incubated with HC toxin (100 nM) for 48 h with or without serum-starvation for the last 6 h of treatment. Phosphorylation and protein levels of Akt and FoxO3a were assessed by western blot. Representative blots and quantification are

presented. (B, C, and D) C2C12 myotubes were incubated with HC toxin (100 nM) for 48 h with or without serum starvation for the final 8 h. Expression of (B and C) autophagy and (D) ubiquitin-proteasome related genes were assessed by qPCR. Bars indicate mean $\pm$ S.E.M. * $P < 0.05$ vs the control group; # $P < 0.05$ vs the serum starvation group; $n = 6$.

Pgc1a (Baar *et al.* 2002). Thus, the pharmacological effects of HC toxin are sufficient to enhance gene expression and metabolism induced by exercise.

The Akt–FoxO axis regulates the transcription of genes involved in autophagy and the ubiquitin-proteasomal pathway (collectively termed atrogenes) (Zhao *et al.* 2007); these genes are associated with muscle atrophy/wasting during prolonged periods of inactivity and in disease states such as diabetes and cancer cachexia (Glover & Phillips 2010). Akt phosphorylates and induces the cytoplasmic

localisation of FoxO3a, which suppresses the expression of atrogenes. Given that Akt is activated in response to treatment with HC toxin, we asked whether this activation is sufficient to suppress the expression of atrogenes. We show that HC toxin-induced Akt activation causes FoxO3a phosphorylation and an associated suppression of atro-gene expression (Fig. 5). This suppression was retained under serum-starved conditions, which is routinely used to induce atrophy in myotubes. Our findings corroborate those of several studies that have demonstrated

the suppression of various atrogenes following HDAC inhibition. The inhibition of class I and II HDACs by the pan-HDAC inhibitor, TSA, suppresses the induction of *MuRF-1* and *atrogin-1* by dexamethasone or nutrient deprivation in C2C12 myotubes (Bricceno *et al.* 2012, Beharry *et al.* 2014). Similarly, the class I HDAC inhibitor, MGCD0103, suppresses the expression of numerous autophagy genes (*Vps34*, *Gabapap*) and several *Atg* proteins in CLL cells (El-Khoury *et al.* 2014). Additionally, fasting-induced autophagy gene expression (*Atg5* and *Gabapap1*) is attenuated in the skeletal muscle of HDAC1/2 knockout mice (Moresi *et al.* 2012). Overall, our results demonstrate that HC toxin is able to suppress atrogenes expression, which is associated with reduced skeletal muscle atrophy.

While Akt activation in skeletal muscle exerts multiple beneficial effects as discussed earlier, activation of Akt in the liver leads to many undesired effects. This was previously demonstrated in a study in which expression of constitutively active Akt in mouse liver resulted in elevated serum cholesterol and triglyceride levels, as well as hepatic triglyceride accumulation and hepatomegaly (Ono *et al.* 2003). Thus, we evaluated whether HC toxin could induce activation of Akt in multiple liver cell lines. Our data provided evidence that HC toxin treatment did not activate IRS1–Akt signalling in HepG2, H4IIE, Huh7 and Huh6 cells. (Supplementary Figure 1, see section on supplementary data given at the end of this article). This suggested that HC toxin-induced Akt activation may be limited to terminally differentiated myotubes and not actively proliferating hepatoma cells.

Studies on exercise-induced cellular signalling and gene signatures have shed light on the potential mechanisms that mediate the medical benefits of exercise in obesity, type 2 diabetes and muscle atrophy (Richter & Ruderman 2009, Glover & Phillips 2010, Egan & Zierath 2013). Here, we provide evidence that HC toxin (an HDAC inhibitor) induces cellular signalling and metabolic modifications that are associated with exercise adaptations. Long-term treatment of HC toxin in cultured myotubes induced IRS1–Akt signalling and affected the expression of metabolic genes involved in substrate utilisation and genes linked with muscle atrophy. Our findings suggest that HDAC inhibitors may have therapeutic potential in recapitulating the physiological adaptations of exercise through skeletal muscle metabolic reprogramming. Given that HDAC inhibitors are widely pursued cancer therapeutics, the field of diabetes research may leverage on the wealth of information and compound libraries from oncological studies.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JME-15-0140>.

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.

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