Chronic AMPK activation evokes the slow, oxidative myogenic program and triggers beneficial adaptations in *mdx* mouse skeletal muscle

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A therapeutic approach for Duchenne muscular dystrophy (DMD) is to up-regulate utrophin in skeletal muscle in an effort to compensate for the lack of dystrophin. We previously hypothesized that promotion of the slow, oxidative myogenic program, which triggers utrophin up-regulation, can attenuate the dystrophic pathology in *mdx* animals. Since treatment of healthy mice with the AMP-activated protein kinase (AMPK) activator 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) enhances oxidative capacity and elicits a fast-to-slow fiber-type transition, we evaluated the effects of chronic AMPK stimulation on skeletal muscle phenotype and utrophin expression in *mdx* mice. Daily AICAR administration (500 mg/kg/day, 30 days) of 5–7-week-old *mdx* animals induced an elevation in mitochondrial cytochrome c oxidase enzyme activity, an increase in myosin heavy-chain type IIa-positive fibers and slower twitch contraction kinetics in the fast, glycolytic extensor digitorum longus muscle. Utrophin expression was significantly enhanced in response to AICAR, which occurred coincident with an elevated β-dystroglycan expression along the sarcolemma. These adaptations were associated with an increase in sarcolemmal structural integrity under basal conditions, as well as during damaging eccentric contractions *ex vivo*. Notably, peroxisome proliferator-activated receptor γ co-activator-1α (PGC-1α) and silent information regulator two ortholog 1 protein contents were significantly higher in muscle from *mdx* mice compared with wild-type littermates and AICAR further increased PGC-1α expression. Our data show that AICAR-evoked muscle plasticity results in beneficial phenotypic adaptations in *mdx* mice and suggest that the contextually novel application of this compound for muscular dystrophy warrants further study.

INTRODUCTION

Duchenne muscular dystrophy (DMD) is the most prevalent, genetically inherited neuromuscular disorder, affecting 1/3600–6000 male births (1,2). DMD is caused by mutations or deletions in the gene encoding dystrophin that prevent the synthesis of a full-length dystrophin protein. Multiple approaches to treat DMD are currently under investigation, including those aimed at the restoration of dystrophin (3–6) or the detrimental downstream effects of dystrophin deficiency (7–9). Other promising therapies aim to functionally compensate for dystrophin by increasing endogenous levels of utrophin at the sarcolemma of dystrophic muscle fibers (10,11).

Utophin may be able to serve as a surrogate for dystrophin in DMD muscle fibers because there is convincing evidence for functional redundancy between these two proteins (12,13). Utophin shares a high degree of sequence identity with dystrophin and also associates with members of the dystrophin-associated protein complex (DAPC) (12,14). One of the main differences between utrophin and dystrophin is their contrasting expression patterns. In healthy adult muscle fibers, dystrophin is expressed along the entire length of the sarcolemma, whereas utrophin expression is confined to the myotendinous and the neuromuscular junction (15,16). Therefore, a challenge for a utrophin-based therapy is to stimulate the expression of...
utrophin at appropriate levels along the entire sarcolemma of dystrophic myofibers, including synaptic and extrasynaptic compartments. Numerous studies employing transgenic animals have demonstrated that enhancing utrophin expression by moderate amounts (e.g. ~2-fold) can compensate for the lack of dystrophin and alleviate the muscle pathology (17–20).

In comparison to their fast-twitch, more glycolytic counterparts, slower, more oxidative muscles express significantly more utrophin in extrasynaptic regions of their constituent fibers (21,22). Slow, oxidative myofibers in both the dystrophin-negative mdx mouse model of muscular dystrophy and DMD patients show reduced damage in comparison to faster, more glycolytic muscle fibers (23,24). This suggests that the sarcolemmal expression of utrophin in slow, oxidative muscles protects against muscle damage. Based on our previous work (11,21,22,25–27), an overarching hypothesis in our laboratory has been that promotion of the slow, oxidative myogenic program can increase utrophin expression and mitigate the dystrophic phenotype.

Previous studies have identified three important elements involved in intracellular signal transduction and transcriptional control which are known to elicit the slow, oxidative myogenic program. These include (i) calcineurin (CN)/nuclear factor of activated T-cells (NFAT) signaling (28,29), (ii) peroxisome proliferator-activated receptor (PPAR) γ co-activator-1α (PGC-1α) action (30,31) and (iii) PPARγ transcriptional activity (32–34). In recent years, our laboratory (11,22,25–27) and others (35–37) have examined these molecular pathways within the context of DMD biology and shown, for example, that stimulation of the transcription factor PPARγ using the synthetic agonist GW501516 was able to evoke the slow myogenic program, including utrophin up-regulation, and rescue muscle function in mdx animals (11). Thus, utilization of GW501516, a therapeutically relevant molecule (38–40) introduces the possibility that other proteins which elicit the slow, oxidative, myogenic program are also potential therapeutic targets.

AMP-activated protein kinase (AMPK) is a key regulator of energy metabolism (41). AMPK is the downstream component of a kinase cascade that acts as a sensor of cellular energy levels, being activated by rising AMP coupled with falling ATP. This enzyme is involved in the fundamental regulation of energy balance at the whole body level by responding to environmental signals in peripheral tissues that modulate energy expenditure (42). Chronic pharmacological AMPK stimulation elicits phenotypic plasticity, including expression of the slow, oxidative myogenic program (43–48). 5-Aminoimidazole-4-carboxamide-1-β-d-ribofuranside (AICAR), a synthetic agonist of AMPK, was first shown by Winder and colleagues to stimulate AMPK activity in skeletal muscle (49) and be effective at evoking skeletal muscle plasticity via chronic exposure (50,51). Importantly, AICAR is currently being vetted in clinical trials for the treatment of type 2 diabetes (52–54). However, whether the habitual activation of AMPK can be of benefit to the dystrophic pathology in skeletal muscle is completely unknown. Thus, the purpose of this study was to investigate the effects of chronic AMPK activation on skeletal muscle phenotypic plasticity, function and utrophin expression in mdx animals. In line with our overarching hypothesis that promotion of the slow, oxidative myogenic program is beneficial for muscular dystrophy, we surmised that chronic AICAR-induced AMPK stimulation would promote the slow, oxidative phenotype, trigger utrophin up-regulation and mitigate the dystrophic pathology.

**RESULTS**

**AICAR-stimulated AMPK activation up-regulates utrophin A and PGC-1α expression in skeletal muscle cells**

We first assessed the effects of AICAR treatment on C2C12 cells. Myoblasts were treated for 48 h with 1 mM AICAR and cells were subsequently harvested and processed for Western blot and quantitative real-time polymerase chain reaction analyses. AICAR administration significantly increased the phosphorylation, as well as total AMPK content after acute vehicle (Veh) or AICAR treatment (48 h, 1 mM) of C2C12 muscle cells. mRNA expression of utrophin A and PGC-1α in C2C12 cells with AICAR administration. n = 3; *P < 0.05 versus Veh.

**Effect of chronic AICAR administration on tissue morphology**

Myofiber central nucleation and cross-sectional area (CSA) variability in mdx mice. Following our results in vitro showing that AMPK stimulation via AICAR induces utrophin and PGC-1α expression, we proceeded to treat adult dystrophin-null mdx mice with daily injections of AICAR at...
a concentration of 500 mg/kg body weight for a duration of 30 days. This treatment protocol has been shown to be effective at evoking skeletal muscle plasticity, such as mitochondrial bio-
genesis, TORC1 signaling and translational capacity, as well as GLUT 4 and PGC-1α expression, in other rodent models (44,45,55–59). Table 1 shows that chronic AICAR administration resulted in significant lipoatrophy and hepatic hypertrophy, noted off-target (i.e. non-skeletal muscle) side effects in rodents (51,60). Soleus and gastrocnemius muscle masses were reduced by a modest, but statistically significant 5–10% \( (P, 0.05) \) in response to chronic AMPK stimulation (Table 1).

We next quantified the degree of myofiber central nucleation and CSA in the slow, oxidative soleus muscle and the fast, glycolytic extensor digitorum longus (EDL) muscle. The quantity of centralized nuclei was similar between vehicle- and AICAR-treated mdx animals in both soleus and EDL muscles (Fig. 2A–C). In addition, there was no effect of chronic AICAR treatment on the median or mean CSAs of soleus and EDL myofibers (Fig. 3A and B).

### Chronic AMPK stimulation evokes the slow, oxidative muscle phenotype in mdx mice

Chronic AMPK activation evokes the slow, oxidative myogenic program (43,45,51,61,62). Thus, we analyzed markers of the slow, oxidative phenotype in mdx mice treated with AICAR. Cytochrome c oxidase (COX) activity, an established indicator of mitochondrial content and skeletal muscle oxidative capacity (63), was elevated by 1.2-fold \( (P < 0.05) \) in the fast, glycolytic EDL muscle (Fig. 4A and B). In contrast, COX activity was unchanged in the slower, more oxidative soleus muscle in

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<td>WT + Veh</td>
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<td>mdx + AICAR</td>
<td>4.0 ± 0.3</td>
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Values are the mean ± SEM (n = 9–14/group). EFW, epididymal fat weight; BW, body weight; LW, liver weight; HW, heart weight; SW, soleus weight; GW, gastrocnemius weight; TAW, tibialis anterior weight; EW, extensor digitorum longus; —, not measured.

\*\( P < 0.05 \) versus mdx + Veh.

\#\( P < 0.05 \) versus WT + Veh.
response to AICAR administration (Fig. 4A and C). Myosin heavy-chain (MHC) immunofluorescence was also utilized to identify any AICAR-induced shift in fiber-type composition. In response to chronic AMPK stimulation, the number of MHC IIa positive fibers in the EDL muscle was significantly increased by \( \approx 1.4 \)-fold (Fig. 5A and B). The quantity of MHC I fibers was unchanged in the EDL muscle (data not shown). The number of MHC I positive myofibers in the soleus muscle was also unaffected by AICAR treatment (Fig. 5A and C).

Next, we evaluated indices of muscle contractile function in an effort to determine whether the biochemical and molecular changes we observed above in response to chronic AICAR treatment were associated with appreciable physiological changes. Twitch contractile kinetics of the EDL muscle were assessed employing an ex vivo preparation, as described previously (64). Time to half-rise in force development and time to peak tension were significantly slower in AICAR-treated mdx mice when compared with vehicle-treated mdx animals (Table 2). Furthermore, the half relaxation time and the maximal rate of relaxation of the EDL muscle were also significantly slower after AICAR administration. These AICAR-induced adaptations resulted in an \( \approx 30\% \) longer twitch half-width \( (P < 0.05) \) as well (Table 2).

**Utrophin protein expression is enhanced in fast, glycolytic muscles of AICAR-treated mdx mice**

We next assessed the protein content and localization of the dystrophin homolog utrophin in fast, glycolytic and slow, oxidative skeletal muscles in mdx mice. Employing Western blot analysis, the data show that utrophin content in the tibialis anterior (TA) muscle of mdx animals was augmented by 2.1-fold \( (P < 0.05) \) by chronic AICAR administration (Fig. 6A). Immunofluorescence was also used to identify the compartmentalization of utrophin within the myofibers. In the EDL muscle, utrophin expression was enhanced along the extra-synaptic regions of the sarcolemma in agonist-treated mdx mice (Fig. 6B, left). Quantitative analysis of the fluorescence intensity in serial sections revealed a significant 1.25-fold increase in the AICAR-treated animals compared with the vehicle-treated mice (Fig. 6C, left). In contrast, in the soleus muscle, there was no qualitative (Fig. 6B, right) or quantitative (Fig. 6C, right) indication of an AICAR effect.

In addition, we evaluated the localization of \( \beta \)-dystroglycan \( (\beta \text{-DG}) \), a component of the DAPC, in order to examine whether chronic AMPK stimulation caused the reassembly of the DAPC along the myofiber membrane. Similar to the utrophin immunofluorescence data (Fig. 6), AICAR treatment...
augmented β-DG expression along the sarcolemma (Fig. 7A, left) resulting in a 2.2-fold increase in fluorescence intensity in the EDL muscle (Fig. 7B, left). AICAR had no effect on β-DG expression in the soleus muscle of mdx mice (Fig. 7A and B, right). Moreover, the total number of neuromuscular junctions was not affected by chronic AMPK stimulation (1.1-fold higher in AICAR-treated, compared with vehicle-treated animals, \( P > 0.05 \), data not shown).

**AICAR administration enhances sarcolemmal integrity in fast, glycolytic skeletal muscle**

Next, to further quantify the degree of phenotypic improvement in AICAR-treated mdx animals, EDL myofiber structural integrity during repetitive electrically stimulated eccentric contractions (ECs) ex vivo was determined, similar to previous studies (11,18,65). During the EC protocol, EDL muscles were bathed in 0.1% procion orange (PO) solution. This dye penetrates into the cytosol of myocytes whose sarcolemma has been disrupted, allowing for determination of the extent of cell membrane damage caused by the ECs. In control, non-stimulated mdx EDL muscles, the level of PO intracellular fluorescence was similar between vehicle- and AICAR-treated animals (Fig. 8A). Conversely, subsequent to the EC procedure the PO fluorescence intensity in EDL myofibers from AICAR-treated animals was much lower compared with vehicle-treated mdx mice (Fig. 8A). These data were quantified and reveal that PO infiltration was reduced by 33.1 ± 2.4% \( (P < 0.05) \) in response to chronic AMPK stimulation (Fig. 8B). Maximal tetanic tension was also recorded during this intense EC protocol. Force output decreased to 60.4 ± 3.3, 33.3 ± 2.3 and 27.2 ± 3.1% of initial tension in vehicle-treated WT, vehicle-treated mdx and AICAR-treated mdx animals, respectively, after the 10th and final EC (Fig. 8C). Chronic AMPK stimulation in mdx mice had no effect on force maintenance.

In an effort to gain a more comprehensive understanding of the effect of chronic AICAR treatment on the structural integrity of the sarcolemma, we employed immunoglobulin M (IgM)
immunofluorescence on serial cryosections of the fast, glycolytic EDL and the slower, more oxidative soleus muscles of mdx animals. Serum proteins, such as IgM, are normally extracellular, but increased fragility of the sarcolemma results in their intramyocellular accumulation (11,66). Qualitative examination of the IgM-stained EDL muscle

![Figure 6. Urophin protein expression and localization in fast, glycolytic and slow, oxidative muscles of AICAR-treated mdx mice. (A) Representative Western blots of utrophin and GAPDH, representative Ponceau S stain and graphical summary of utrophin protein content in TA muscle extracts isolated from vehicle-treated wild-type mice (WT + Veh), vehicle-treated mdx mice (mdx + Veh) and AICAR-treated mdx animals (mdx + AICAR); n = 4–10. (B) Typical micrographs of utrophin immunostaining in EDL (left side) and soleus muscle (right side) cryosections from mdx + Veh and mdx + AICAR mice. α-BTX-positive staining, indicative of neuromuscular junction localization, is shown beneath the corresponding utrophin micrographs. (C) Graphical summaries of utrophin fluorescence. n = 6–7; *P < 0.05 versus mdx + Veh.
cryosections indicated that chronic AICAR administration to \textit{mdx} mice attenuated serum protein penetration into the myocytes (Fig. 9A). Indeed, quantitative analysis revealed that influx of IgM into EDL myofibers was reduced by 55\% (\(P\), 0.05) in AICAR-treated \textit{mdx} mice compared with vehicle-treated littermates (Fig. 9B). In contrast, chronic AMPK stimulation had no effect on IgM fluorescence intensity observed in soleus myofibers (Fig. 9A and C). Analysis of serum creatine kinase activity revealed no effects of AICAR treatment in \textit{mdx} animals (data not shown).

Chronic AMPK activation alters the protein expression of phenotypic modifiers in the skeletal muscle of \textit{mdx} mice

Skeletal muscle plasticity is mediated in part by the content and activity of factors effecting muscle gene expression, including transcription factors, transcriptional co-activators and co-repressors and histone modifying proteins (27,31,37,67–70). Therefore, we investigated the content of the potent phenotypic modifiers PGC-1\(\alpha\), receptor-interacting protein 140 (RIP140) and silent information regulator two ortholog 1 (SIRT1) in the TA muscle of \textit{mdx} animals subsequent to AICAR treatment. The level of the transcriptional co-activator PGC-1\(\alpha\) was 1.4-fold greater (\(P\), 0.05) in \textit{mdx} animals, compared with wild-type (WT) mice (Fig. 10A). AICAR treatment augmented PGC-1\(\alpha\) protein content by \(-\)45\% (\(P\), 0.05) relative to vehicle-treated \textit{mdx} mice. The expression of the transcriptional co-repressor RIP140 was 1.3-fold greater in \textit{mdx} mouse muscle relative to WT mice (\(P\), 0.07, Fig. 10B). RIP140 content was 35\% lower (\(P\), 0.05) after chronic AICAR treatment in \textit{mdx} mice compared with vehicle-treated \textit{mdx} animals. The abundance of the histone de-acetylase SIRT1 was significantly greater in \textit{mdx} mouse muscle by 2.6-fold over WT animals (Fig. 10C). There was no difference in SIRT1 expression in response to chronic AMPK activation in \textit{mdx} mice.

Intracellular compartmentalization of PGC-1\(\alpha\) and RIP140 in dystrophic muscle

We next sought to investigate whether the AICAR-elicited changes in PGC-1\(\alpha\) and RIP140 contents were associated with alterations in the intracellular localization of these phenotypic modifiers. Immunofluorescence analysis was employed to identify the myonuclei, along with the expression of PGC-1\(\alpha\) and RIP140. The overall fluorescence intensity of PGC-1\(\alpha\) staining appeared to be greater in the muscles from AICAR-treated compared with vehicle-treated \textit{mdx} animals (Fig. 11A, center panels), which supports the Western blot results discussed above (Fig. 10A and B). Qualitative analysis revealed that the nuclear localization of PGC-1\(\alpha\) was similar between vehicle- and AICAR-treated groups (Fig. 11A, right panels). The overall intensity of RIP140 immunofluorescence appeared to be lower in the muscles from AICAR-treated compared with vehicle-treated mice (Fig. 11B, center panels), which also supports the Western blot results (Fig. 10A and B).
C). Qualitative analysis revealed that the extent of nuclear localization of RIP140 was not different between vehicle- and AICAR-treated groups (Fig. 11B, right panels).

**Effect of chronic AMPK stimulation on downstream transcriptional targets of PGC-1α in the skeletal muscle of mdx mice**

Finally, in an effort to more comprehensively examine the role of PGC-1α in the AMPK-mediated phenotypic plasticity of dystrophic muscle, we investigated the content of additional downstream transcriptional targets of PGC-1α. The level of cytochrome c was 40% lower ($P < 0.05$) in vehicle-treated mdx animals compared with WT mice (Fig. 12A and B). AICAR treatment augmented cytochrome c content by $\sim 20\%$ ($P = 0.09$) relative to vehicle-treated mdx mice. The expression of COX subunit IV (COX IV) was lower in vehicle-treated mdx mouse muscle relative to WT mice ($225\%$, $P = 0.07$, Fig. 12A and C). COX IV content was 20% higher ($P = 0.14$) after chronic AICAR treatment in mdx mice compared with vehicle-treated mdx animals, which reflected the change observed in COX activity (Fig. 4B). Thus, in addition to the AICAR-evoked increases in utrophin and PGC-1α expression (Figs 6 and 10B), a clear trend for an increase in the expression of alternative PGC-1α transcriptional targets was observed after chronic AICAR administration.

**DISCUSSION**

The current investigation demonstrates that chronic AMPK activation in skeletal muscle elicits a significant phenotypic shift from fast, glycolytic to slow, oxidative myofibers and is accompanied by augmented utrophin expression and reduced sarcolemmal fragility in mdx mice. We first...
hypothesized (21) and subsequently demonstrated (11,22,25–27, see also 36,37) that promotion of the slow, oxidative myogenic program, which includes triggering of utrophin up-regulation, could attenuate disease pathology while eliciting appreciable functional adaptations in dystrophic muscle. The results here are the first to demonstrate that chronic AMPK activation by AICAR administration evokes an improved muscle phenotype in mdx mice. Since AICAR is currently being tested in human subjects as a therapy for type 2 diabetes (52–54), our current findings suggest that chronic AMPK stimulation may represent a viable therapeutic target in dystrophic muscle and thus necessitates continued scientific pursuit.

Chronic AMPK stimulation evokes a robust fast, glycolytic to slow, oxidative myofiber program transition in a variety of animal models (43–46,51,55–57,59,61,62). Here, the data show that the phenotype-shifting effects of AICAR can be extended to include mdx mice, a well-established model for DMD. In particular, COX activity, a marker of mitochondrial content, was elevated in AICAR-treated mice. This is important because augmented mitochondrial biogenesis in skeletal muscle is associated with increased fatigue resistance (63), reduced apoptotic susceptibility (71) and decreased metabolic abnormalities (72). Moreover, recent evidence suggests that improved mitochondrial function is beneficial in mitigating the dystrophic phenotype in skeletal muscle (73,74). Furthermore, AICAR-induced alterations in MHC expression in the EDL muscle toward slower isoforms were consistent with changes in physiological function of the EDL muscle ex vivo. Thus, chronic pharmacological AMPK stimulation, similar to PPARδ activation (11), elicits the slow, oxidative myogenic program in mdx mice, which is known to provide a greater degree of protection against the dystrophic pathology in animal models of DMD, as well as in DMD patients (11,23,24).

Earlier work from our laboratory has established that the level of utrophin is expressed to a significantly greater extent in slow, oxidative myofibers versus their faster, more glycolytic counterparts (21,22). In healthy skeletal muscle, dystrophin protein is normally found along the entire length of the sarcolemma, whereas utrophin is compartmentalized to the myotendinous and neuromuscular junctions. An important finding in the current study is that the AICAR-elicited shift in muscle phenotype was associated with an increase in utrophin content by ≈2-fold in the fast, glycolytic EDL muscle. Our immunofluorescence analysis further revealed that AICAR treatment extended utrophin expression beyond the neuromuscular junction along the length of the sarcolemma. Previous studies have noted that even modest elevations in utrophin expression (e.g. ≈2-fold) in mdx animals can result in dramatic improvements in the dystrophic phenotype (11,18,25). Moreover, sarcolemmal β-DG expression in EDL muscle was rescued in response to AICAR treatment, which suggests that a reassembly of the DAPC occurred. Earlier investigations have shown that AMPK activation can substantially affect the protein profile of the cell membrane in skeletal muscle and other tissue types (75–77), and Budinger et al. (78) recently demonstrated a link between dystroglycan and AMPK in mediating mechanotransduction within epithelial alveolar type II cells. These AICAR-induced adaptations in the sarcolemma of fast, glycolytic muscles were no doubt responsible for the improvement in sarcolemmal integrity, evidenced by significant reductions in PO dye infiltration during ECs ex vivo, as well as in IgM localization within the myofibers under basal ambulatory conditions. As the efficacy of chronic AMPK stimulation for

![Figure 10. Effect of chronic AMPK stimulation on the protein expression of phenotypic modifiers in the skeletal muscle of mdx mice.](https://academic.oup.com/hmg/article-abstract/20/17/3478/2527260/Downloaded-24-January-2019)
muscular dystrophy continues to be tested, it will also be important to address its effects on other critically affected muscles, such as the diaphragm.

It is reasonable to suggest that the AICAR-evoked shift toward the slow, oxidative myogenic program, including utrophin up-regulation, was due in part to the increase in PGC-1α content. PGC-1α has been shown to be a master regulator of phenotypic plasticity, including the neuromuscular junction program and utrophin expression (27,37). Interestingly, our data demonstrate a similar pattern of utrophin and PGC-1α expression in WT, mdx and AICAR-treated mdx animals. To our knowledge, this study represents the first account of endogenous PGC-1α protein levels in mdx animals. Therefore, we posit that both PGC-1α and utrophin are up-regulated in mdx animals in an effort to compensate for the myriad abnormalities present within dystrophic skeletal muscles, including primarily the lack of dystrophin. Furthermore, we hypothesize that the elevated levels of utrophin in mdx mice may be driven by a PGC-1α-mediated co-activation of the transcription factors PPARδ and GABP (11,27,37), although further research is necessary to substantiate this assertion.

In recent years, a number of investigations have documented the dynamic intracellular compartmentalization of PGC-1α in skeletal muscle (79–81), as well as the importance of its nuclear and/or mitochondrial localization to its function as a transcriptional co-activator (79,81). Our data demonstrate that after 30 days of AICAR injections, global myocellular PGC-1α content was significantly elevated in dystrophic muscle; however, there appeared to be no effect on the nuclear abundance of the co-activator. This finding was not surprising, considering that translocation of PGC-1α from the cytosolic compartment to the nucleus seems to account for a relatively transient, early phase of the adaptive response (79–81). It is important to note that these studies employed an acute bout of exercise as a stimulus to evoke the sub-cellular flux of PGC-1α, a stimulus that also involves the robust activation of AMPK (31,41). Thus, we hypothesize that a more pronounced intracellular shuttling of PGC-1α to the nucleus occurred early in the time course of chronic AICAR administration and was responsible, in part, for driving the initial phenotypic adaptations. Nonetheless, the overall elevation of PGC-1α content by chronic AMPK stimulation was associated with the augmented expression of numerous downstream transcriptional targets, including utrophin (27,37), cytochrome c and COX IV (31,79), as well as PGC-1α itself in a positive autoregulatory fashion (82). These data clearly indicate that PGC-1α plays an important role in mediating the phenotype-shifting effects of chronic AMPK stimulation in muscular dystrophy.

This study is also the first to document that SIRT1 content is up-regulated in dystrophic muscle. SIRT1 is a member of a potent phenotype-shifting signaling cascade comprised, in part, of AMPK and PGC-1α (69,70). Similar to utrophin and PGC-1α, elevated SIRT1 levels in dystrophic muscle suggest a compensatory adaptation in an attempt to minimize the extent of the pathology. Since a number of SIRT1 agonists such as resveratrol, SRT1720 and SRT501 are currently in various stages of the discovery pipeline (83–86), including clinical trials aimed at investigating their therapeutic potential in the management of type 2 diabetes and obesity (79), further studies will undoubtedly examine the possibility of SIRT1 as a therapeutic target in muscular dystrophy.
The transcriptional co-repressor RIP140 is also a powerful phenotypic modifier in skeletal muscle (67). Seth et al. (67) demonstrated that RIP140 transgenic mice have reduced mitochondrial enzyme activity and a blunted expression of a number of genes indicative of the slow, oxidative phenotype, whereas RIP140 null animals display an induction of the slow, oxidative myogenic program. Chronic AICAR treatment evoked the slow, oxidative phenotype in mdx mice concurrent with a reduction in RIP140 protein expression. This finding is consistent with the recent study by Williams et al. (46) which showed that chronic AMPK activation via β-guanidinopropionic acid administration significantly down-regulated RIP140 expression in the triceps muscle of rodents. Since RIP140 appears to promote the fast, glycolytic myogenic program and/or repress the slow, oxidative phenotype, this factor may represent a novel regulator of gene expression in dystrophic muscle. Our data demonstrated that the intracellular localization of RIP140 did not appear to be affected by chronic AICAR treatment, which does preclude the possibility that the early adaptive phenotypic responses were influenced by subcellular or subnuclear RIP140 spatial dynamics. Interestingly, we found high negative correlations between the AICAR-evoked change in (i) RIP140 and utrophin expression (Pearson’s \( r = -0.72 \), coefficient of determination \( r^2 = 0.52 \), \( n = 5 \)), as well as (ii) RIP140 and β-DG expression (\( r = -0.86 \), \( r^2 = 0.74 \), \( n = 4 \); data not shown). However, these correlations were not statistically significant, which is due primarily to a lack of statistical power (i.e. low \( n \), and possibly low effect size). Whether the utrophin promoter is a target of RIP140 trans-repression in muscle cells, as medium-chain acylcoenzyme A dehydrogenase and heart-type fatty acid binding protein (67), is an intriguing question that we are currently pursuing. Thus, the current study also highlights the crucial roles that PGC-1α, SIRT1 and RIP140 play as mediators of gene regulation in dystrophic muscle thereby indicating that these molecules represent important targets for modulating the dystrophic phenotype.

We recently demonstrated that beneficial phenotypic plasticity could be elicited in mdx animals via the chronic administration of a contextually novel small molecule agonist of PPARδ, GW501516 (11). First, we observed that GW501516 treatment induced utrophin expression in cultured cells via a transcriptional mechanism involving a PPRE located within the utrophin promoter. In vivo and in agreement with our hypothesis, we showed that treatment of mdx mice with GW501516 resulted in a significant increase in utrophin expression as well as in a clear attenuation of the dystrophic phenotype. These findings are important as they demonstrate that progression of DMD can be mitigated using a therapeutically relevant molecule (38–40). This compound therefore represents a promising potential therapeutic in DMD patients that our laboratory is currently investigating. It has also been previously demonstrated that another clinically relevant compound, green tea extract (GTE), provides structural and functional benefits to dystrophic skeletal muscle (87,88). Of note, many of the phenotypic improvements in response to chronic GTE treatment were observed in fast, glycolytic muscles (i.e. TA and EDL) only, similar to the results of the current study. Whether the GW501516- and AICAR-evoked benefits in the dystrophic pathology are due, in part, to enhanced anti-oxidant defense, which represents one possible mechanism driving the beneficial GTE effects (87,88), is an intriguing question that requires further study.

Interestingly, GW501516 and AICAR treatments possess some commonalities, such as enhanced utrophin expression, phenotype-shifting capability and augmented sarcolemmal integrity. Thus, multiple redundant pharmacologically sensitive AMPK and PPARδ signaling pathways enable phenotypic plasticity in dystrophic skeletal muscle. In support of this theory, it has been demonstrated that a number of identical skeletal muscle genes are up-regulated in response to both AMPK and PPARδ agonist treatment in healthy animals (89). Moreover, there is evidence in AD293 cells that AMPK and PPARδ physically interact to form a transcriptional complex which enhances PPARδ transcriptional activity, and this is increased in the presence of PGC-1α (89). Although AMPK and PPARδ represent potential pharmatherapeutic targets to attenuate the dystrophic pathology,
many questions regarding the molecular mechanisms driving agonist-evoked phenotypic adaptations remain to be answered.

Chronic AMPK and PPARδ activation also result in agonist-specific effects. For example, habitual AMPK stimulation induced phenotypic plasticity only in the fast, glycolytic EDL and TA muscles, whereas chronic PPARδ activation in mdx mice elicited adaptations in both fast EDL and slow soleus muscles (11). Furthermore, GW501516 administration improved force maintenance during repeated ECs, whereas AICAR did not. Narkar et al. (89) have noted that while pharmacological AMPK and PPARδ activation share some common targets, many genes are specifically modulated by one or the other, not both. These compound-specific effects are not surprising since AMPK and PPARδ signaling are differentially regulated and have unique functions (34,90–92).

In summary, this study demonstrates that AMPK stimulation, via daily AICAR administration, induced the slow, oxidative myogenic program, including triggering utrophin up-regulation, in mdx mouse muscle. Pharmacological AMPK activation also enhanced sarcolemmal structural integrity during damaging muscle contractions in fast, glycolytic skeletal muscle, the phenotype more susceptible to the dystrophic pathology (23,24). Thus, our original hypothesis that promotion of the slow, oxidative myogenic program elicits utrophin up-regulation and attenuates the dystrophic pathology (21) is methodically being substantiated by examining numerous phenotype-shifting signaling networks, including CN/NFAT (22,25,26,36), PGC-1α (27,37), PPARδ (11) and now AMPK. Moreover, the current study, as well as our recent work (11), highlights the potential for the novel use of pharmacological activators of AMPK and PPARδ as components of a rational drug treatment for DMD patients. The fact that these agonists are currently in clinical trials for other pathologies (38–40,52–54) may help accelerate these compounds through the discovery pipeline for novel and effective DMD treatment.

MATERIALS AND METHODS

Muscle cell culture treatment

C2C12 muscle cells were cultured as described previously (11). Briefly, cells were maintained in DMEM containing 10% fetal bovine serum and 1% antibiotic/antimycotic (Invitrogen, Burlington, Canada). When cells reached ≏30% confluence, they were switched to DMEM containing 5% heat-inactivated horse serum and 1% antibiotic/antimycotic and treated for 48 h with either vehicle (sterile water) or AICAR (1 mM) to activate AMPK (93).

mRNA analysis

Total RNA was isolated from cells using TRizol reagent (Invitrogen, Carlsbad, USA) and treated with DNase I (Fermentas, Burlington, Canada) to eliminate possible DNA contamination. Endogenous mRNAs were measured by real-time quantitative reverse transcription-polymerase chain reaction (Agilent Tech, Mississauga, Canada), and the delta CT method was used to quantify the expression of utrophin A and PGC-1α relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as described previously (11).

In vivo AICAR treatment

Experiments were conducted after approval by the University of Ottawa Animal Care Committee in accordance with Canadian Council of Animal Care guidelines. AICAR (TRC, Toronto, Canada) was dissolved in sterile saline at 50 mg/ml immediately prior to daily treatments (59). Five-to-seven-week-old mdx mice (C57BL/10ScSn-Dmd<sup>mdx15Qj</sup>, Jackson Laboratory, Bar Harbor, USA) were treated at 500 mg/kg/day with AICAR or vehicle daily by subcutaneous interscapular injections for 30 days. This AICAR dosage and method of delivery was based on previous studies (44,45,55–58). Mice were euthanized and tissues were harvested, weighed and immediately frozen in liquid nitrogen, or in melting isopentane cooled with liquid nitrogen for histological and immunofluorescence analyses.

Assessment of muscle fiber central nucleation and size

Cross-sections (10 μm) of EDL and soleus muscles were stained with hematoxylin and eosin, dehydrated through a series of alcohol solutions, cleared with xylene and mounted using Permount (Fisher Scientific, Ottawa, Canada). The extent of central nucleation was determined by counting the number of muscle fibers and those with centralized myonuclei from four 10× cross-sectional views of myofibers from the midbelly of the muscle, facilitated by Northern Eclipse Software (NES; Empix Imaging, Mississauga, Canada). CSAs for each individual fiber were also measured using NES.

COX activity

Mitochondrial COX enzyme activity was determined as previously described (94) with modifications. Briefly, muscle cross-sections were incubated in staining solution [0.1% diamobenzidine (Vector Labs, Burlington, Canada), 0.1% cytochrome c, 0.04% catalase] for 3 h at 37°C. Slides were washed and fixed with formal calcium, followed by sequential dehydration steps and clearing with xylene. High-resolution 10× views of the muscle sections were digitally enlarged to facilitate counting, and NES and ImageJ software (NIH, Bethesda, USA) were employed to quantify staining intensity.

MHC immunofluorescence

Immunofluorescent fiber-type analysis was performed using undiluted A4-840 antibody to detect MHC I or undiluted SC-71 to detect MHC Ila (Developmental Studies Hybridoma Bank, University of Iowa, USA). Cross-sections (10 μm) were double-stained with a polyclonal anti-laminin antibody at 1:1000 dilution (Sigma-Aldrich, St Louis, USA) to permit identification of individual fiber boundaries and detection of extracellular matrix surrounding the sarcolemma. Secondary detection was performed using appropriate FITC-conjugated anti-mouse antibodies and/or a TRITC-conjugated anti-rabbit antibody. High-resolution 5–10× views of the cryosections were digitally enlarged to facilitate counting, and NES and ImageJ were used to quantify MHC staining.
Utrophin and β-DG immunofluorescence localization

For immunofluorescence analysis of utrophin and β-DG proteins, images were acquired at 20× magnification on a Zeiss Axiophot-2 microscope. Muscle cryosections were stained with monoclonal utrophin (Novocastra NCL-DRP2, Newcastle upon Tyne, UK) or β-DG antibodies (Novocastra NCL-b-DG) along with Alexa 488 conjugated α-bungarotoxin (BTX; Jackson ImmunoResearch, West Grove, USA). Immunostaining was performed on the EDL or soleus muscles at the same time for all experimental groups in order to minimize variability in background fluorescence intensity. For each animal, four fields of view were analyzed per cross-section obtained from the midbelly of the EDL and soleus muscles. Quantification of the fluorescence intensity was performed using NES and ImageJ (NIH).

Intracellular PGC-1α and RIP140 localization

For immunofluorescence analysis of PGC-1α and RIP140 proteins, images were acquired at 40× magnification on a Zeiss Axiophot-2 microscope. Muscle cryosections were stained with polyclonal PGC-1α (Santa Cruz Biotechnology sc-13067, Santa Cruz, USA) or RIP140 antibodies (Abcam ab42126, Cambridge, USA) in VECTASHIELD mounting medium with DAPI (VECTOR Labs H-1200, Burlingame, USA). Immunostaining was performed on the EDL at the same time for all experimental groups in order to minimize variability in background fluorescence intensity. For each animal, four fields of view were analyzed per cross-section obtained from the midbelly of the EDL muscle.

Western blotting

Frozen TA muscle sections were ground to a powder with a porcelain mortar and pestle on dry ice. Muscle samples were suspended in a modified RIPA buffer (Sigma-Aldrich) supplemented with cOmplete Mini Protease Inhibitor Cocktail and PhosSTOP (Roche, Laval, Canada), mechanically homogenized, repeatedly freeze-thawed and spun. The protein concentrations of the supernates were determined by the Bradford method (95) using bovine serum albumin as the standard. Proteins extracted from the muscle homogenates were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (6–8% polyacrylamide) and subsequently electroblotted to nitrocellulose membranes (Bio-Rad, Mississauga, Canada). After transfer, membranes were stained with Ponceau S (Sigma-Aldrich) to confirm equal loading with a 700 ms train duration of supramaximal 10 V, 0.3 ms square pulse applied at 2 min intervals. For each EC, force was elicited every 100 s for the determination of contractile kinetics. Following equilibration, the EDL muscle was subjected to a set of five maximal twitch contractions (10 V, 0.3 ms square pulse) every 100 s for the determination of contractile kinetics. Next, an EC protocol was employed consisting of 10 ECs applied at 2 min intervals. For each EC, force was elicited with a 700 ms train duration of supramaximal 10 V, 0.3 ms square pulses at 200 Hz (model S88, Grass Technologies, West Warwick, USA) with a 10% lengthening at a velocity of 0.5 Le/s applied during the last 200 ms. Force was recorded using a Keithley data acquisition board (model KPCI-3104, Cleveland, USA) at a sample rate of 5 KHz throughout the experiment. Following the contraction protocol, cross-sections from the midbelly of EDL muscles were examined at 10× magnification for intracellular green fluorescence, indicative of PO dye infiltration into the core of the muscle. The fibers around the periphery of the EDL section were omitted from this analysis due to the potential for myofiber damage artifact caused by the muscle excision process.

Immunofluorescent assessment of sarcolemmal damage

Sarcolemmal integrity was assessed by evaluating the level of cytoplasmic staining within muscle fibers for the normally membrane impermeable IgM. Muscle cross-sections were incubated for 1 h with a fluorescein-conjugated IgM anti-mouse secondary antibody (Sigma-Aldrich). Sections were viewed using fluorescent microscopy to identify injured fibers. The fluorescence intensity from within the muscle fibers was then determined from four 20× cross-sectional views of myofibers from the midbelly of the EDL and soleus muscles. The fibers lining the periphery of the muscle section were not included in this analysis due to the possibility of experimenter-induced myofiber damage during the muscle excision and harvesting process.

Ex vivo EC protocol

The EC protocol was performed on the EDL muscles from Veh-treated WT, mdx and AICAR-treated mdx mice as described recently (11). Briefly, one tendon was attached to a Cambridge ergometer (model 300, Aurora Scientific, Aurora, Canada), whereas the other was affixed to a metal pin, and muscles were continuously superfused by physiological saline solution containing 0.1% PO dye (Sigma-Aldrich) at 15 ml/min and maintained at 25°C. Prior to the ECs, muscle length was adjusted to give maximum force output, and subsequently muscles were allowed to equilibrate for 30 min. Following equilibration, the EDL muscle was subjected to a set of five maximal twitch contractions (10 V, 0.3 ms square pulse) every 100 s for the determination of contractile kinetics. Next, an EC protocol was employed consisting of 10 ECs applied at 2 min intervals. For each EC, force was elicited with a 700 ms train duration of supramaximal 10 V, 0.3 ms square pulses at 200 Hz (model S88, Grass Technologies, West Warwick, USA) with a 10% lengthening at a velocity of 0.5 Le/s applied during the last 200 ms. Force was recorded using a Keithley data acquisition board (model KPCI-3104, Cleveland, USA) at a sample rate of 5 KHz throughout the experiment. Following the contraction protocol, cross-sections from the midbelly of EDL muscles were examined at 10× magnification for intracellular green fluorescence, indicative of PO dye infiltration into the core of the muscle. The fibers around the periphery of the EDL section were omitted from this analysis due to the potential for myofiber damage artifact caused by the muscle excision process.
Statistical analysis
The data were analyzed using paired and unpaired Student’s t-tests and analysis of variance (ANOVA) procedures, as appropriate (StatPlus, Vancouver, Canada). Bonferroni’s post hoc test was used to test significant differences revealed by the ANOVA. Statistically significant distinctions between groups represented in the graphs depicted as fold differences are computed using the raw data sets prior to conversion to the fold difference values. Significance was accepted at \( P < 0.05 \).

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