J Appl Physiol 107: 1730–1735, 2009.
First published October 1, 2009; doi:10.1152/japplphysiol.91451.2008.

Relationship between Sirt1 expression and mitochondrial proteins during conditions of chronic muscle use and disuse

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Submitted 5 November 2008; accepted in final form 28 September 2009

Chabi B, Adhihetty PJ, O'Leary MF, Menzies KJ, Hood DA. Relationship between Sirt1 expression and mitochondrial proteins during conditions of chronic muscle use and disuse. *J Appl Physiol* 107: 1730–1735, 2009. First published October 1, 2009; doi:10.1152/japplphysiol.91451.2008.—Sirt1 is a NAD^+-dependent histone deacetylase that interacts with the regulatory protein of mitochondrial biogenesis PGC-1α and is sensitive to metabolic alterations. We assessed whether a strict relationship between the expression of Sirt1, mitochondrial proteins, and PGC-1α existed across tissues possessing a wide range of oxidative capabilities, as well as in skeletal muscle subject to chronic use (voluntary wheel running or electrical stimulation for 7 days, 10 Hz; 3 h/day) or disuse (denervation for up to 21 days) in which organelle biogenesis is altered. PGC-1α levels were not closely associated with the expression of Sirt1, measured using immunoblotting or via enzymatic deacetylase activity. The mitochondrial protein cytochrome c increased by 70–90% in soleus and plantaris muscles of running animals, whereas Sirt1 activity remained unchanged. In chronically stimulated muscle, cytochrome c was increased by 30% compared with nonstimulated muscle, whereas Sirt1 activity was increased modestly by 20–25%. In contrast, in denervated muscle, these markers of mitochondrial content were decreased by 30–50% compared with the control muscle, whereas Sirt1 activity was increased by 75–80%. Our data suggest that Sirt1 and PGC-1α expression are independently regulated and that, although Sirt1 activity may be involved in mitochondrial biogenesis, its expression is not closely correlated to changes in mitochondrial proteins during conditions of chronic muscle use and disuse.

mitochondrial biogenesis; muscle mass; exercise; chronic stimulation

CALORIC RESTRICTION IS KNOWN to promote longevity and delay the onset of age-related diseases in organisms from yeast to mammals (33). This happens concurrently with alterations in nutrient metabolism. The mechanisms involved in the lifespan extension during caloric restriction are unclear, but evidence shows that proteins of the Sir2 family may play a major role. In yeast *S. cerevisiae* where the protein was first identified, Sir2 overexpression increases organism lifespan (15). This happens through the silencing of specific DNA sequences by Sir2 histone deacetylase activity and is dependent on NAD^+ concentration (18). In mammals, expression and activity of the Sir2 homolog Sirt1 as well as NAD^+ concentrations have been shown to increase with caloric restriction and are related to the beneficial effects observed on lifespan and tissue metabolism. Sirt1 is able to deacetylate key proteins such as p53 or Bax, thus regulating the response to stress and apoptosis, and promoting longevity in caloric-restricted animals (5, 21, 32). In addition, Sirt1 acts on nutrient metabolism by deacetylating PPARγ repressors in white adipose tissue, as well as the coactivator PGC-1α in liver, favoring lipolysis (24) and gluconeogenesis (26), respectively.

Although exercise does not appear to increase lifespan (9–11), it contributes to longevity. Notably, exercise alters tissue metabolism, resulting in reduced fat accumulation and increased insulin sensitivity. This helps to delay the onset of age-related diseases (9). In skeletal muscle, exercise promotes mitochondrial biogenesis and alterations in energy metabolism through increases in PGC-1α expression (12, 13). Recent evidence has shown that alterations in metabolism and mitochondrial biogenesis are related to specific deacetylation of PGC-1α by Sirt1 in skeletal muscle (7, 17). Thus we hypothesized that Sirt1 expression might be regulated during exercise and that this may occur concurrently with changes in mitochondrial biogenesis and PGC-1α expression. Indeed, it has recently been suggested that Sirt1 expression may be more closely related to mitochondrial biogenesis than PGC-1α (29). Thus, to investigate this further, we characterized Sirt1 expression in tissues with a wide range of mitochondrial content and also assessed the level of Sirt1 in skeletal muscle subject to both chronic muscle use and disuse, conditions in which mitochondrial biogenesis is either up- or downregulated.

MATERIALS AND METHODS

Animals. Experiments were conducted after approval by York University Animal Care Committee. Animals were treated in accordance with Canadian Council of Animal Care guidelines. Male Sprague-Dawley rats were used throughout, with initial body weight of 120–140 g for the voluntary running protocol ($n = 16$) and ~300–325 g for the in vivo chronic contractile activity and muscle denervation protocols. Animals were housed individually and given food and water ad libitum. At the end of the protocol, rats were anesthetized with pentobarbital sodium (60 mg/kg), and skeletal muscles (soleus and plantaris), heart, liver, and white adipose tissue (WAT) from the epididymal fat pad were harvested and quickly frozen.

Voluntary running. In this 8-wk protocol, rats were assigned randomly into two groups: a sedentary group (control) and a running group (runners) with free access to a rotating loaded wheel. Runners were exercised with no load for the first 2 wk and then loaded incrementally by 50 g (*week 3*), 100 g (*week 4*), and 200 g (*weeks 5–8*). At the end of the protocol, soleus (SOL) and plantaris (PL) muscles were harvested, weighed, and quickly frozen.

In vivo chronic contractile activity. The surgical procedure was followed as previously described (30). Briefly, animals were anesthetized and electrode wires were passed subcutaneously from the hindlimb to the back of the animal where they were secured to an external stimulator. After 1 wk of recovery, the tibialis anterior (TA) and extensor digitorum longus (EDL) muscles were chronically stimulated

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(10 Hz, 0.1 ms, 3 h/day) for 7 days. The contralateral limb was used as a nonstimulated internal control.

Muscle denervation protocol. To denervate the TA and EDL muscles, the peroneal nerve was exposed, and a 5-mm section was excised. The incision was closed with metal clips after administration of sterile ampicillin. Animals were randomly assigned to a denervation time of 7, 14, or 21 days. At the end of the protocol, muscle tissues were harvested and quickly frozen.

Protein extraction. Briefly, powdered frozen tissue (20–30 mg) was homogenized and diluted 10-fold in extraction buffer (20 mM HEPES, pH 7.4, 2 mM EGTA, 50 mM β-glycerophosphate, 1 mM dithiothreitol, 1 mM Na_3VO_4, 10% glycerol, 1% Triton X-100, 10 μM leupeptin, 5 μM pepstatin A, 3 mM benzamidine, 10 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride). Homogenates were rotated end-over-end for 1 h at 4°C, sonicated, and then centrifuged at 14,000 *g* for 10 min at 4°C. WAT extracts were delipidated by aspirating the supernatant before the protein determination. Protein concentrations were measured using the Bradford method.

Sirt1 activity. Sirt1 activity was assessed using a Sirt1 fluorometric assay kit (BIOMOL, Plymouth Meeting, PA) as described by the manufacturer. Briefly, 25 μg of total protein were incubated with Fluor de Lys-Sirt1 substrate (100 μM) and NAD (100 μM) at 37°C for 30 min. Total deacetylase activity was measured after the addition of developer reagent, and fluorescence was monitored for 60 min at 360 nm (excitation) and 460 nm (emission). Sirt1-specific activity was determined by subtracting total deacetylase activity from the remaining activity after Sirt1-specific inhibition with nicotinamide (50 mM).

NAD and NADH determination. NAD and NADH nucleotides were measured using a cycling assay as described (16). Frozen muscles tissues (15–20 mg) were acid extracted, neutralized, and used for NAD determination. Alkali extracts were obtained using potassium hydroxide buffer and neutralized to pH 7–7.6 with TEA for NADH determination. The concentration of nucleotides was measured photometrically at 340 nm using 10-μl samples. Values were detected within a linear range and are expressed as micromole per gram of wet muscle mass.

Immunoblotting. Proteins from tissue extracts were separated using 10–15% SDS-PAGE and were subsequently electroblotted onto nitrocellulose membranes. After transfer, membranes were blocked (1 h) with a 5% skim milk in 1× TBST (Tris-buffered saline Tween-20: 25 mM Tris·HCl, pH 7.5, 1 mM NaCl, and 0.1% Tween-20) solution. Blots were then incubated in blocking solution with antibody directed against PGC-1α (Calbiochem, 1:500 dilution), cytochrome *c* (1:750 dilution), Sirt1 (Sigma, 1:1,000), and GAPDH (Abcam, 1:20,000 dilution) overnight at 4°C. After three 5-min washes with TBST, blots were incubated at room temperature (1 h) with the appropriate secondary antibody coupled to horseradish peroxidase and washed again three times for 5 min each with TBST. Antibody-bound protein was revealed using the ECL method. Films were scanned and analyzed using SigmaGel software (Jandel Scientific, San Rafael, CA). All blots, with the exception of tissue comparisons, were corrected for loading using GAPDH. No loading correction was applied to tissue comparisons because of the variable expression of specific marker proteins among tissues.

Statistical analyses. Data are expressed as means ± SE. Differences between animals groups were assessed using a Student's *t*-test and a one-way ANOVA followed by Bonferroni post hoc test. Statistical differences were considered significant at $P < 0.05$.

RESULTS

Sirt1 characterization in rat tissues. The expression of PGC-1α and Sirt1 was measured in tissues of control animals possessing different mitochondrial contents (Fig. 1). Sirt1 expression was highest in liver and slow-twitch red muscle (Fig. 1*A*). In addition, Sirt1 enzymatic activity was highest in liver (Fig. 1*B*; $P < 0.01$ vs. other tissues) and exhibited a similar pattern among tissues to values obtained using immunoblotting. In contrast to Sirt1, PGC-1α immunoreactivity was the highest in muscle tissues (heart > soleus > plantaris) and the lowest in WAT and liver (Fig. 1*C*).

Sirt1 regulation by voluntary running. During the 8 wk of loaded, voluntary running, the average daily distance performed by the animals increased initially, then declined as the workload was increased. The average running distance reached a maximum of 5.8 km/day at *week 4*, but this distance progressively decreased as the final load (200 g) was applied during the last 3 wk (Fig. 2*A*). The work produced during the protocol, calculated as described previously (14), peaked at 15,900 $N \cdot m \cdot kg^{-1}$ at *week 4* and decreased to 4,500 $N \cdot m \cdot kg^{-1}$ by the end of the protocol (Fig. 2*B*). The running protocol produced a 14% increase ($P < 0.05$) in soleus muscle mass when expressed per gram of body weight. No significant effect on muscle mass was noted in the fast-twitch plantaris muscle.

To investigate whether Sirt1 was regulated under conditions of voluntary running, Sirt1 activity, as well as cytochrome c expression, were measured in both muscles. Cytochrome c protein was used as an indirect marker of mitochondrial content, as has been done previously (6, 31). Cytochrome c was

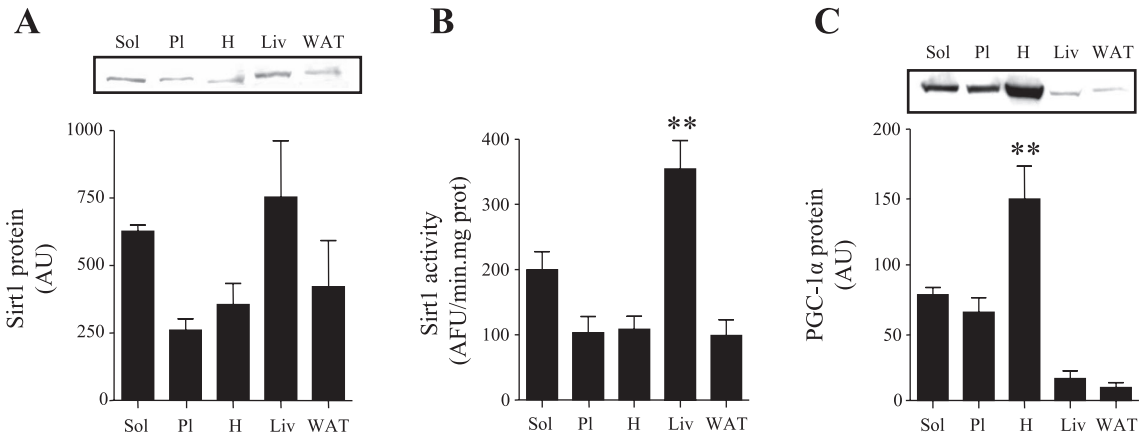

Fig. 1. Basal expression and activity of Sirt1 and PGC-1α protein expression in rat tissues. Western blot analysis and representative blots of Sirt1 (*A*) and PGC-1α (*C*) immunoreactivity in tissues. The only visible immunoreactive band in these gels ran at >100 kDa. *B*: Sirt1 activity quantified by fluorometric assay in tissue extracts. Values are means ± SE; $n = 5$ for each tissue. Sol, soleus; Pl, plantaris; H, heart; Liv, liver; WAT, white adipose tissue. **Significant difference vs. other tissues ($P < 0.01$).

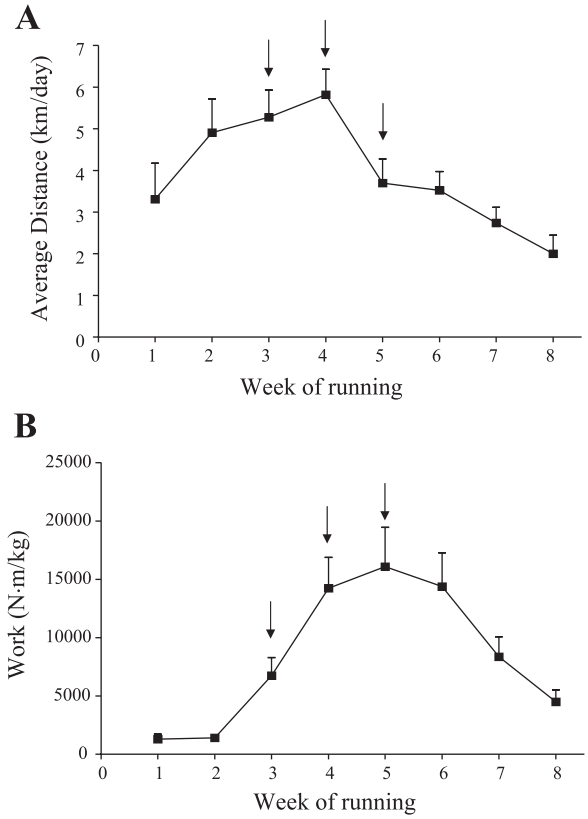

Fig. 2. Average daily distance (*A*) and work (*B*) for each week of the voluntary running protocol. Runners were exercised with no load for the first 2 wk and then loaded incrementally by 50 g (*week 3*), 100 g (*week 4*), and 200 g (*weeks 5–8*), as indicated by the arrows. Values are means ± SE; $n = 8$.

significantly increased by 1.9- and 1.7-fold in soleus and plantaris, respectively (Fig. 3, *A* and *C*; $P < 0.01$ vs. control). In contrast, Sirt1 protein content and enzymatic activity were unaltered by running in both muscles (Fig. 3, *B*, *D*, and *E*).

Sirt 1 regulation by chronic contractile activity. Since we have previously shown that 7 days of chronic contractile activity is a potent activator of mitochondrial biogenesis (13, 19), we used this experimental paradigm to further investigate the potential association between Sirt1 and the regulation of mitochondrial proteins (Fig. 4). Using this protocol, the expression of the important mitochondrial regulatory protein PGC-1α was elevated by 1.5-fold (13), and this occurred coincident with a similar 1.5-fold increase in cytochrome c expression (Fig. 4*A*). Chronic contractile activity also resulted in a modest but significant 20–25% increase in Sirt1 activity; however, a change in Sirt1 protein content as evaluated by immunoblotting was not detectable (Fig. 4*B*, *top*). We also wished to identify whether chronic contractile activity produced a change in the levels of the regulatory coenzymes NAD and NADH. NAD levels tended to be higher in the chronically stimulated muscles; however, this did not lead to a statistically significant alteration in the NAD-to-NADH ratio (Fig. 4*C*).

Sirt 1 regulation by muscle denervation. To contrast with the exercise and contractile activity-induced mitochondrial biogenesis response, muscle denervation was employed to decrease muscle mass and to reduce mitochondrial content. Following 7, 14, and 21 days of denervation-induced muscle atrophy, a significant 30% reduction in cytochrome c was evident across all time points (Fig. 5*A*). The denervation-induced reduction in this mitochondrial protein occurred coincident with 45–55% decrements in both muscle mass and in the important mitochondrial regulator, PGC-1, as previously reported (1). In contrast to mitochondrial protein and PGC-1α expression, Sirt1 activity (Fig. 5*B*) was markedly increased by two- to fourfold in denervated skeletal muscle between 7 and 21 days of denervation. This coincided closely with Sirt1 protein levels assessed by immunoblotting (Fig. 5*B*, *top*).

DISCUSSION

Previous studies have shown that the NAD-dependent histone deacetylase Sirt1 is involved in the regulation of tissue metabolism. Among its targets, the coactivator PGC-1α has been shown to be specifically deacetylated by Sirt1, leading to its activation (26) or inhibition (23), depending on the tissue studied or the cell type. In muscle, the deacetylation and

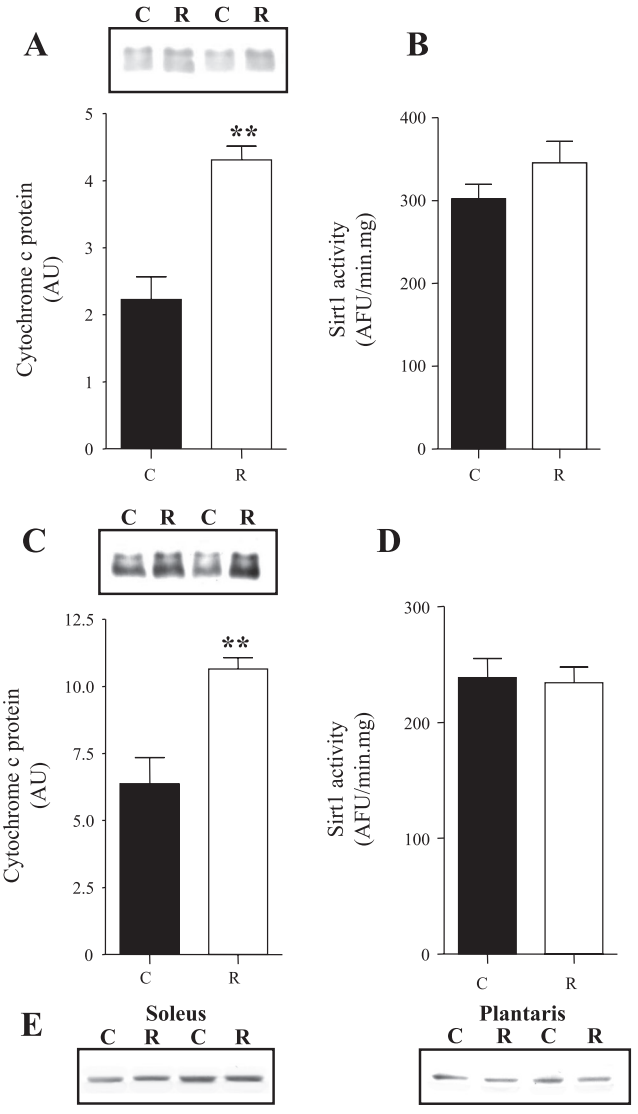

Fig. 3. Effect of voluntary running on cytochrome c (*A* and *C*) protein and Sirt1 activity (*B* and *D*) in soleus (*top*) and plantaris muscles (*bottom*) from controls (C) and voluntary wheel running (R) animals. *E*: representative blots of Sirt1 protein content in soleus and plantaris. Values are means ± SE; $n = 4$ for each group. **Significant difference vs. controls ($P < 0.01$).

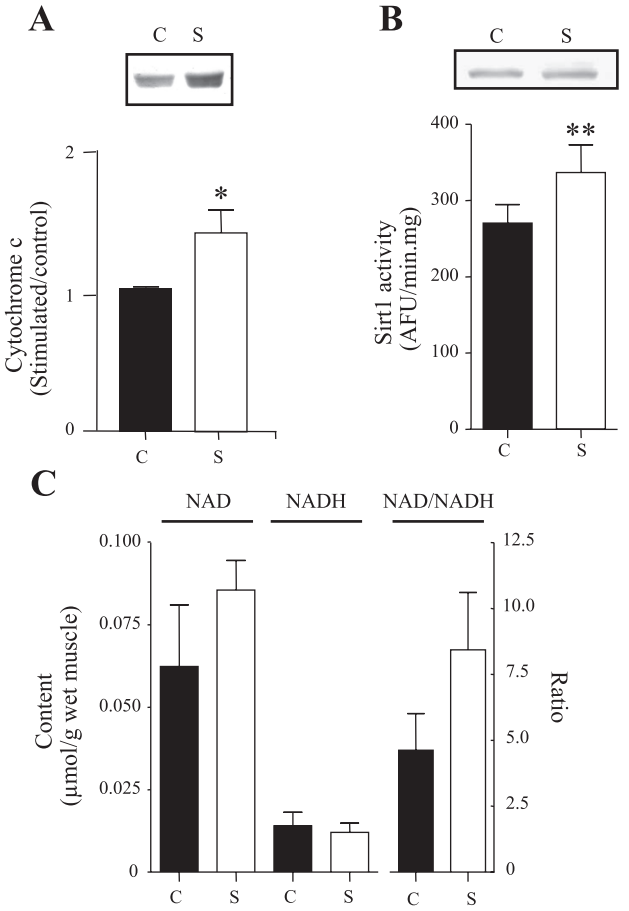

Fig. 4. Effect of chronic contractile activity on cytochrome c expression, Sirt1 activity, and NAD-to-NADH ratio. *A*: Western blot analysis and representative blots of cytochrome c protein content in EDL muscle from control (C) and 7-day chronically stimulated (S) muscle. *B*: Sirt1 immunoreactivity (*top*) and activity quantified by fluorometric assessment in control (C) and chronic contractile activity (S) muscles extracts. *C*: NAD and NADH content, and the ratio of NAD to NADH in control and stimulated muscle. Values are means ± SE; $n = 8–9$ for each group. Significant difference vs. controls: $^{*}P < 0.05$; $^{**}P < 0.01$.

translocation of PGC-1 has been shown to be associated with mitochondrial biogenesis (7, 17, 34). We have previously demonstrated the close relationship between PGC-1α protein levels and mitochondrial content under a variety of induced and steady-state conditions (4, 13). Thus we hypothesized that Sirt1 expression would be proportional to mitochondrial content in a variety of tissues under steady-state conditions. Furthermore, we expected that, in skeletal muscle during conditions of use (i.e., endurance training) and disuse (i.e., muscle denervation), PGC-1α and Sirt1 expression and activity would be directly correlated with the resultant increase or decrease in mitochondrial biogenesis, respectively.

Our data do not support these hypotheses. We found little evidence for a coordinated expression between Sirt1 and PGC-1α in tissues under steady-state conditions. This was apparent in heart and plantaris muscles, which exhibit different oxidative capacities (13) yet have the same Sirt1 activity. Alternatively, liver, and to a lesser extent adipose tissue, displayed higher levels of Sirt1 activity, despite having low PGC-1α protein content. Although these results in liver and adipose tissue are in accordance with the reported role of Sirt1 in the activation of lipolysis and gluconeogenesis (24, 26), our data do not indicate that the Sirt1 expression pattern is related to the oxidative capacity of a given tissue. While this manuscript was in review, Gurd et al. (8) published similar findings comparing muscle types ranging from low (white gastrocnemius) to high (heart) oxidative capacities.

Endurance training and in vivo chronic contractile activity are paradigms that dramatically increase mitochondrial biogenesis, mediated in part through the elevation in PGC-1α expression and activation (13, 34). As expected, under these experimental conditions, we observed substantial increases in cytochrome c, a typical marker of mitochondrial biogenesis. However, Sirt1 protein content was not induced by voluntary wheel running and was only modestly increased by chronic contractile activity. In addition, this small increase was not

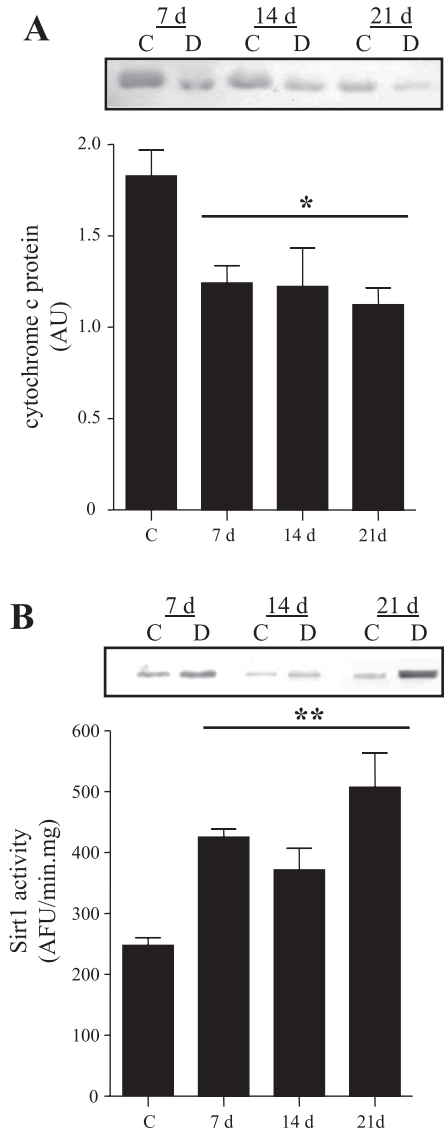

Fig. 5. Effect of denervation on cytochrome c protein expression and Sirt1 activity. *A*: Western blot analysis and representative blots of cytochrome c protein content in EDL muscle from control (C) and denervated (D) muscle. *B*: Western blot of Sirt1 and Sirt1 activity in control (C) and denervated muscle. Values are means ± SE; $n = 4–6$ for each group. 7d, 7 days; 14d, 14 days; 21d, 21 days of denervation. Significant difference vs. controls: $^{*}P < 0.05$; $^{**}P < 0.01$.

accompanied by a significant change in the NAD-to-NADH ratio in the chronically stimulated muscle samples. Thus we cannot conclude that cellular environment favored an increase in Sirt1 activity as a result of the chronic contractile activity treatment. However, this conclusion requires verification using a direct measure of Sirt1 target protein deacetylaction. It also appears likely that the NAD-to-NADH ratio will be susceptible to change under more acute conditions of contractile activity in which the redox status of the muscle is altered by an elevated metabolic rate. Since Sirt1 activity is NAD-dependent, we expect that Sirt1 activity will be altered under acute exercise conditions.

Our observation of a lack of Sirt1 inducibility compared with the results of Suwa et al. (29) may be related to the employment of different exercise intensities. These authors employed a program of enforced treadmill training, which is of a higher intensity than voluntary wheel running, despite our use of a progressively increasing wheel load. Alternatively, it is possible that Sirt1 was induced early in our voluntary running protocol (i.e., before 8 wk) when the work performed was at its peak and when the rate of mitochondrial biogenesis might be expected to be at its highest. Thus our tissue sampling time could have missed the early adaptive response. Certainly, the very modest increase in Sirt1 enzymatic activity after only 7 days of chronic contractile activity is consistent with this. In the chronic stimulation model, the constant workload used may impose a more consistent and strong stimulus to maintain a high rate of organelle synthesis throughout the short 7-day protocol. Thus we speculate that the induction of Sirt1 follows a different time course than that of mitochondrial proteins and that Sirt1 expression may be more closely related to the rate of mitochondrial biogenesis rather than the steady-state content of mitochondria, as measured here. Certainly, the sensitivity of Sirt1 adaptations to alterations in chronic muscle contractions is different from that of mitochondrial proteins, which adapt more readily to both locomotion and chronic electrical stimulation. This is further reinforced by the recent data of Gurd et al. (8). Thus several activation pathways involving Sirt1, as well as other proteins, likely coexist for mitochondrial biogenesis and PGC-1α activation, dependent on exercise intensity (20) and duration.

A further dissociation between the level of Sirt1 and mitochondrial content was provided by our muscle disuse experiment. Interestingly, we found that denervation provoked a stronger induction of Sirt1 expression and activity despite a reduced mitochondrial content. This increase in Sirt1 expression was inversely related to the ~50% reduction in muscle mass evident during denervation. We propose that the increase in Sirt1 may be involved in denervation-induced phenotypic adaptations in two ways. First, denervation-induced atrophy involves the activation of an ubiquitin-proteasome pathway that is regulated by transcription factors such as NF-κB and FoxO. Stimulation of the NF-κB pathway leads to the increased expression of the E3 ubiquitin ligase MuRF1 (3), whereas the upregulation of FoxO3a expression with denervation induces atrogin-1, another E3 ubiquitin ligase (27, 28). Notably, Sirt1 deacetylates and inactivates both NF-κB and FoxO3a (2, 22, 35). Thus Sirt1 induction may serve as a compensatory protective mechanism against a massive increase in protein degradation during denervation. Second, it is also known that satellite cells proliferate in response to denervation (36). An important stimulator of this process is Sirt1, via its modulation of cell cycle regulators such as p21 and p27 (25). This effect of Sirt1 on satellite cell activation may compliment its role in protein degradation and serve as a second mechanism designed to attenuate the loss of muscle mass associated with denervation. Thus it is evident that, in addition to its effects on metabolism, Sirt1 may have profound effects related to the regulation of muscle mass, particularly during conditions of muscle disuse. Future work involving the tissue-specific knockout or overexpression of Sirt1 should help to further resolve the role of this protein in skeletal muscle.

ACKNOWLEDGMENTS

We are grateful to Radhika Sharma for technical assistance during this study.

GRANTS

This research was supported by a Canadian Institutes of Health Research (CIHR) Institute of Aging grant to D. A. Hood. K. J. Menzies is a recipient of a CIHR doctoral award from the Institute of Aging. D. A. Hood is the holder of a Canada Research Chair in Cell Physiology.

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