The effects of chronic muscle use and disuse on cardiolipin metabolism

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Ostojić O, O’Leary MF, Singh K, Menzies KJ, Vainshtein A, Hood DA. The effects of chronic muscle use and disuse on cardiolipin metabolism. J Appl Physiol 114: 444–452, 2013. First published December 6, 2012; doi:10.1152/japplphysiol.01312.2012.—Cardiolipin (CL) is a phospholipid that maintains the integrity of mitochondrial membranes. We previously demonstrated that CL content increases with chronic muscle use, and decreases with denervation-induced disuse. To investigate the underlying mechanisms, we measured the mRNA expression of enzymes involved in CL biosynthesis and remodeling. CL is a phospholipid that maintains the integrity of mitochondrial membranes. We previously demonstrated that CL content increases with chronic muscle use, and decreases with denervation-induced disuse. To investigate the underlying mechanisms, we measured the mRNA expression of enzymes involved in CL biosynthesis and remodeling. For the first time, we evaluated the role of the transcriptional coactivator PPARγ coactivator-1α (PGC-1α) in regulating both CL synthesis and remodeling. These data suggest that chronic muscle use and disuse modify the expression of miRNAs encoding CL metabolism enzymes. Our data also illustrate, for the first time, that PPARγ coactivator-1α regulates the CL metabolism pathway in muscle.

THE MEMBRANES OF MITOCHONDRIA have a diverse structure consisting of many molecules including cholesterol, proteins, and phospholipids. The most abundant structures are the phospholipids, which further contribute to the diversity of membranes through their varied forms. Cardiolipin (CL) is a phospholipid exclusive to the mitochondria, with 90% of it localizing in the inner mitochondrial membrane (IMM), with the remaining 10% on the outer mitochondrial membrane (OMM) (14). CL exists as a dimer and is composed of four fatty acid (FA) chains which must be dynamically transferred and remodeled to ensure proper membrane configuration (19). The majority of these FA molecules are unsaturated, making them highly susceptible to peroxidation via reactive oxygen species (ROS). CL also binds to components of the electron transport chain (ETC), a major producer of ROS, and is therefore at even greater risk of peroxidation (20). When CL is peroxidized, proton leak is increased across the IMM, thus inhibiting energy production and leading to pathology (15).

A complex molecular pathway is involved in synthesizing and ensuring proper CL structure. Cardiolipin synthase (CLS) is a key enzyme involved in the de novo biosynthesis of immature CL, which occurs on the inner leaflet of the IMM (7). Another crucial enzyme involved in this pathway is CTP:PA cytidylytransferase (CDT), the rate-limiting step of this biosynthesis process (14). To function optimally, immature CL must have its FAs remodeled into a mature form. Two key enzymes involved in this are tafazzin (Taz) and acyl-CoA:lysocardiolipin acyltransferase-1 (ALCAT1). Taz remodels the FAs of CL to create a mature CL molecule (4, 5), while ALCAT1 converts the FAs back to the immature form (15). CL can also be transferred between the IMM and OMM by the membrane-embedded enzyme phospholipid scramblase-3 (Pls3c3) (22). This is critical, as the location of CL has been found to influence its cellular role (13). IMM CL that is bound to the ETC complex helps to stabilize and align them to ensure proper electron transfer (8), while CL that is located on the OMM aids in the assembly of import machinery and acts as a binding site for Bid, which triggers apoptosis (12, 13). OMM CL is also at risk of being cleaved and converted to phosphatic acid (PA) by mitochondrial phospholipase D (MitoPLD), which acts only on adjacent mitochondria (9). MitoPLD, therefore, is of particular importance, as it has the ability to mediate the amount of CL that resides on the OMM.

The synthesis and remodeling of CL is crucial for the integrity of the mitochondrial membrane and overall functioning of the organelle. We have previously shown that chronic contractile activity (CCA), a form of muscle use, elicits a significant increase in CL content (21). In contrast, denervation, a form of muscle disuse, significantly decreases CL concentration within muscle (23). The mechanisms underlying these changes have yet to be examined. Thus, the purpose of this study is to examine the adaptations of the enzymes of the CL metabolism pathway during changes in mitochondrial biogenesis. We set out to analyze the expression of the six aforementioned enzymes in response to conditions of chronic muscle use and disuse, and the effect of aging. Further, since many mitochondrial proteins are regulated by the transcriptional coactivator PPARγ coactivator-1α (PGC-1α), we also evaluated the role of this protein in the regulation of CL metabolism enzymes. We hypothesized that the gene expressions of the enzymes involved in CL biosynthesis and remodeling would increase with CCA and decrease with denervation and PGC-1α knockout conditions. We also predicted that the expression of enzymes that reduce CL content or function would be downregulated with CCA and increased with denervation and PGC-1α knockout conditions. The combined effects of these changes would result in increased CL content with CCA, and decreased...
content in denervated and PCG-1α knockout rodents. This could contribute to the expansion and degradation of mitochondrial membranes as alterations in mitochondrial content occur.

METHODS

Animals. Male Sprague-Dawley (SD) rats were purchased at 6 mo of age (Charles River, St. Constant, QC, Canada). Male Fischer 344 X Brown Norway (F344XBN) F1 hybrid rats (National Institute on Aging, Bethesda, MD) were divided into one of two groups depending on age: 6 mo (young) or 33 mo old (aged). One set of young and aged rats was designated for our CCA protocol (n = 10 for both young and aged), while a separate set was used for denervation (n = 8 for both young and aged). The generation of PGC-1α knockout mice used was as previously described (16), and are bred in our facility. Offspring were genotyped by crude DNA extraction from ear clippings. DNA was combined with DNA Tag polymerase (Jumpstart REDtag Ready Mix PCR Reaction Mix; Sigma, Oakville, ON, Canada) and primers specific for wild-type or knockout genes, and were detected using traditional PCR methods. Mice were used at 10 mo of age. All animals were housed individually under a 12:12-h light-dark cycle in a temperature controlled room (20–21°C) and were given food and water ad libitum. Animal usage in this study followed protocols that were approved by the York University Animal Care Committee, in accordance with the Canadian Council on Animal Care.

In vivo stimulation protocol. Portable stimulation devices were unilaterally implanted into the animals as previously described (21). In brief, rats were anesthetized with a ketamine-xylazine cocktail (0.2 ml/100 g body wt). Electodes (Medwire, Leico Industries, New York, NY) located unilaterally at either side of the common peroneal nerve caused palpable contractions in the tibialis anterior (TA) and extensor digitorum longus (EDL) muscles. All implantations were followed by a week-long recovery period, upon which stimulation began. The CCA protocol lasted for 3 h a day, for 7 days, with 21-h recovery periods in between. The stimulation frequency was 10 Hz. Stimulation units were externalized for the SD rats (21), whereas the F344XBN rats had silicone-encased stimulators inserted into the intraperitoneal cavity and sutured to the musculature of the abdomen (17). All other surgical procedures and stimulation protocols were identical between the two rat strains. The rats were then anesthetized and the stimulated TA and EDL muscles were removed. The nonstimulated, contralateral muscles served as internal controls. Upon removal, one-half of the TA muscles were used immediately for mitochondrial isolation. The remaining half of the TA, as well as the EDL muscles, was frozen in liquid nitrogen and pulverized into a fine powder for subsequent experiments. The animals were killed by removal of the heart.

In vivo denervation protocol. Male, 6-mo-old, SD rats underwent unilateral common peroneal denervation as previously described (11). Briefly, rats were anesthetized with a ketamine-xylazine mix (0.2 ml/100 g body wt). The common peroneal nerve was exposed and a 0.5 cm long segment was removed. After 7 days, both EDL muscles were removed and the nonnerve, contralateral muscles were used as internal controls. The tissues were then frozen in liquid nitrogen and then pulverized into a fine powder.

Cytochrome c oxidase activity. Cytochrome c oxidase (COX) activity was used as a marker of mitochondrial biogenesis. Pulverized whole muscle homogenates were prepared and sonicated for 10 s on ice at a power output of 20–30%. A buffered test solution was also prepared, containing fully reduced horse heart cytochrome c (C-2506; Sigma, Oakville, ON, Canada). With the use of a multipipette, 250 μl of test solution were added to 20 μl of whole muscle homogenate in a 96-well plate. The enzyme activities of the homogenates were analyzed by measuring the maximal oxidation rate of cytochrome c.

Table 1. PCR primers and probes

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Alternative names are in parentheses. CDS-1, CTP:PA-cytidylyltransferase-1; ALCAT1, acyl-CoA:lysocardiolipin acyltransferase-1; CLS, cardiolipin synthase; Taz, tafazzin; MitoPLD, mitochondrial phospholipase D; Plscr3, phospholipid scramblase 3; bp, base pairs.
This was done via the absorbance change at 550 nm at 30°C in a Synergy HT (Bio-tek Instruments, Winooski, VT) microplate reader. For each sample, the COX activity measurement was calculated as an average of three trials.

In vitro RNA isolation and reverse transcription. Total RNA was isolated from frozen, whole muscle EDL powders. Tissue powder (~90 mg) was added to 1 ml of TRizol® reagent (Invitrogen, Carlsbad, CA) and homogenized for 30 s at a power output of 30%. Upon 5 min of incubation at room temperature, 200 µl chloroform were added and tissues were shaken for 15 s. After another 3 min of room temperature incubation, samples were centrifuged at 4°C at 16,000 g for 15 min. Next, the upper aqueous phase of the sample was transferred into a new tube along with 500 µl of isopropanol, shaken for 15 s, and left overnight at −20°C to precipitate. Samples were then centrifuged at 4°C at 16,000 g for 10 min. The resultant supernatant was discarded, and the pellet was washed with 700 µl of 75% ethanol. After a centrifugation (10 min, 4°C), the supernatant was discarded and the pellet was resuspended in 100 µl of sterile water. The concentration and purity of the RNA were measured at 260 nm and 280 nm, respectively (Ultrospec 2100 Pro; Biochrom, Cambridge, United Kingdom). The quality of the RNA was determined by observing the 28S and 18S bands on a 1% formaldehyde-agarose gel. Following the manufacturer’s recommendations, SuperScript® III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) was used to reverse transcribe 1.5 µg of total RNA into cDNA.
**Real-time PCR.** Using sequences from GenBank, primers were designed with Primer 3 v. 0.4.0 software (Massachusetts Institute of Technology, Cambridge, MA) for genes of interest: CDS-1, CLS, Taz, ALCAT1, MitoPLD, and Plscr3 (Table 1). Primer specificity was confirmed by OligoAnalyzer 3.1 (Integrated DNA Technologies, Toronto, ON, Canada). mRNA expression was measured with SYBR® Green chemistry (PerfeCTa SYBR® Green SuperMix; ROX, Quanta BioSciences, Gaithersburg, MD). Each well contained: SYBR® Green SuperMix, forward and reverse primers (20 µM), sterile water, and 10 ng of cDNA. In cases where functioning primers could not be designed, 1.25 µl of TaqMan probes was used along with 12.5 µl TaqMan Universal Master Mix (4304437; Life Technologies, Carlsbad, CA; Table 1) with 10 ng of cDNA and 7.25 µl of water per well. The detection of all real-time PCR amplification took place in a 96-well plate using a StepOnePlus® Real-Time PCR System (Applied Biosystems, Foster City, CA). The final reaction volume of each well was 25 µl. Samples were run in duplicates to ensure accuracy. The PCR program consisted of an initial holding stage (95°C for 10 min), followed by 40 amplification cycles (60°C for 1 min, 95°C for 15 s), and was completed with a final melting stage (95°C for 15 s, 60°C for 1 min, 95°C for 15 s). Nonspecific amplification and primer dimers were controlled for by the analysis of melt curves generated by the instrument for SYBR® Green analyses. Negative control wells contained water in place of cDNA.

**Real-time PCR quantification.** First, the threshold cycle (CT) number of the endogenous reference gene was subtracted from the CT number of the target gene \( \Delta CT = CT(\text{target}) - CT(\text{reference}) \). Next, the \( \Delta CT \) value of the control tissue was subtracted from the \( \Delta CT \) value of the...
experimental tissue (ΔΔCT = ΔCT(experimental) − ΔCT(control)]. Results were reported as fold-changes using the ΔΔCT method, calculated as 2−ΔΔCT. Different endogenous control genes were tested and selected based on the highest P value computed in a t-test comparing control and experimental muscles. The CT values of selected control genes were averaged using RT² Profiler PCR Array Data Analysis software (http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php; SABiosciences, QIAGEN, Toronto, ON, Canada). For CCA, myelocytomatosis oncogene (Myc) and ribosomal protein Rps12 were chosen, while primers detecting glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin were used as endogenous controls for the denervated samples. Rps12 was used as an endogenous control for the aged CCA samples, while Rps12 and β-actin were averaged for aged denervated samples. For PGC-1α mouse tissues, β2-microglobulin, β-actin and GAPDH were averaged (Table 1).

Subsarcolemmal mitochondrial isolation. TA muscles were excised and tissues were immediately submerged in 10–20 ml buffer. The TA muscles were then patted dry, cut away from any excess connective tissue or fat, minced, and homogenized. The mitochondrial subsarcolemmal (SS) isolation procedure has been previously described (10). The mitochondria were suspended in resuspension medium and then were used in flow cytometry. The amount of mitochondrial protein was determined using the Bradford protein assay.

Mitochondrial cardiolipin content. Flow cytometry was used to measure CL content in isolated SS mitochondria using a four-color FACSCalibur flow cytometer equipped with a 488-nm argon laser (Becton Dickinson, San Jose, CA). Mitochondrial CL content was measured using the fluorescent probe 10-N-nonyl-3,6-bis(dimethylamino) acridine orange as previously described (6). Measurements were made on taken from 50 μg of SS mitochondria isolated from rat or mouse TA muscles. Data were collected from the forward-scatter and side-scatter light detectors. The flow cytometry data were obtained following a minimum of 20,000 gated events.

Statistical analyses. To compare between control vs. stimulated and control vs. denervated muscles, paired t-tests were performed, while wild-type vs. knockout muscles were compared using unpaired t-tests. A split-plot ANOVA calculation was used to analyze results from the CCA and denervated tissues within the aging muscles, A split-plot ANOVA calculation was used to analyze results from the CCA and denervated tissues within the aging muscles, with respect to CL metabolism enzymes, CDS-1 expression increased significantly by 240% in young, and by 88% in aged animals following CCA, while CLS remained unaltered (P < 0.05; Fig. 3B). This reflects a change in organelle composition with CCA, since CL was measured in the mitochondrial fraction. With respect to CL metabolism enzymes, CDS-1 expression increased significantly by 240% in young, and by 88% in aged animals following CCA, while CLS remained unaltered (P < 0.05; Fig. 3, C and D). With respect to the CL remodeling enzymes, Taz mRNA levels were not altered by CCA, or aging. The mRNA period of denervation decreases CL content by 40% (23), thus matching the observed change in COX activity. Following denervation, the mRNA expression of CDS-1, CLS, ALCAT1, and Plscr3 all increased by 280%, 58%, 290% and 59%, respectively (P < 0.05; Fig. 2C), while the expression of Taz and MitoPLD remained unaltered.

**Chronic muscle use and aging.** In aged animals, muscle COX activity was diminished by 15–20%. CCA resulted in a 50% increase in COX activity in young animals, but only in a 40% augmentation in the aged animals (P < 0.05; Fig. 3A). Mitochondrial CL content was increased by 26% with CCA (P < 0.05), but only in young animals, as this response was eliminated with age (Fig. 3B). This reflects a change in organelle composition with CCA, since CL was measured in the mitochondrial fraction. With respect to CL metabolism enzymes, CDS-1 expression increased significantly by 240% in young, and by 88% in aged animals following CCA, while CLS remained unaltered (P < 0.05; Fig. 3A, C and D). With respect to the CL remodeling enzymes, Taz mRNA levels were not altered by CCA, or aging. The mRNA

**RESULTS**

Chronic muscle use. We elicited the effects of chronic muscle use by employing the CCA model. COX activity was used as a marker of mitochondrial content. CCA resulted in a 40% increase (P < 0.05) in COX activity per gram of muscle compared with the contralateral control muscles (Fig. 1A). Previous work in our laboratory has shown that 7 days of CCA increases CL content by 48% (21), reflecting changes in mitochondrial content, since the levels closely paralleled COX activity, as well as possible changes in organelle composition. We also measured the mRNA expression of CDS-1, CLS, Taz, ALCAT1, MitoPLD, and Plscr3. With CCA, the mRNA levels of CDS-1 and Plscr3 increased significantly by 128% and 90%, respectively. In contrast, CCA led to decreases in ALCAT1 and MitoPLD mRNA levels of 32% and 40%, respectively (P < 0.05; Fig. 1C).

Chronic muscle disuse. To contrast with chronic muscle use, we employed denervation, a model of muscle disuse. Seven days of denervation lead to a significant 33% decrease in COX activity (P < 0.05; Fig. 2A). Previous work has shown that a similar

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**Fig. 5.** Effects of denervation and age on mitochondrial content and mRNA expression in skeletal muscle. A: average COX activity in young and aged, control and denervated muscles (n = 8). mRNA levels of cardiolipin de novo biosynthesis enzymes CDS-1 (B) and CLS (C) in control (open bars) and denervated (solid bars) muscles of 6-mo and 36-mo old rats (n = 6 for each group). Values are means ± SE; *P < 0.05 vs. control; **P < 0.05 vs. main effect of aging.
levels of ALCAT1 and MitoPLD both decreased by 30% as a result of CCA in young animals, while Plscr3 increased by 178%. These three effects were all attenuated with age ($P < 0.05$; Fig. 4, B, C, and D).

**Chronic muscle disuse and aging.** A separate set of aged animals was used to investigate the combined effect of aging and chronic muscle disuse produced by denervation. In these animals, a 30% difference in COX activity was observed between muscles of young and old animals. Denervation resulted in a 50% and 34% decrease in mitochondrial content in both the young and aged muscles, respectively ($P < 0.05$; Fig. 5A). CDS-1 mRNA expression increased by 100% following denervation in young animals only ($P < 0.05$; Fig. 5B), while CLS increased by 56% and 38% in the young and aged animals, respectively ($P < 0.05$; Fig. 5C). When we measured CL remodeling enzyme expression, there was no effect of denervation or aging on Taz (Fig. 6A) or MitoPLD (Fig. 6C) mRNA levels. ALCAT1, however, increased with denervation by 208%. While ALCAT1 did not change with aging in control muscle, it decreased by 67% in aged, denervated muscle (Fig. 6B). For Plscr3, we observed 33% and 20% increases following denervation in young and aged animals, respectively ($P < 0.05$; Fig. 6D).

**PGC-1$\alpha$ knockout conditions.** To investigate a role for the transcriptional coactivator PGC-1$\alpha$ in the regulation of CL metabolism, we used PGC-1$\alpha$ knockout animals. Muscle from

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### Fig. 6. mRNA levels of CL remodeling and outer membrane enzymes Taz (A), ALCAT1 (B), MitoPLD (C), and Plscr3 (D) in control (open bars) and denervated (solid bars) 6-mo and 36-mo old rats. Values are means ± SE; $n = 6$ for each group. *$P < 0.05$ vs. control; **$P < 0.05$ vs. main effect of aging.

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### Fig. 7. Effects of PGC-1$\alpha$ knockout on mitochondrial content, mitochondrial CL levels, and mRNA expression in skeletal muscle. A: average COX activity of PGC-1$\alpha$ knockout (KO) and wild-type (WT) mice ($n = 4$). B: CL content in isolated subsarcolemmal mitochondria of wild-type and PGC-1$\alpha$ knockout muscles ($n = 6$). C: mRNA levels of CDS-1, CLS, Taz, ALCAT1, MitoPLD, and Plscr3 in muscles of wild-type and PGC-1$\alpha$ knockout mice ($n = 7$). Graph represents fold changes relative to wild-type mice. Values are means ± SE; *$P < 0.05$ vs. control or wild type.
the animals exhibited a 42% decrease in COX activity relative to wild-type animals, reflecting a decline in organelle content per gram of muscle. CL content in the SS mitochondrial fraction remained unaltered (P < 0.05; Fig. 7, A and B). The lack of PGC-1α resulted in a significant decrease in CDS-1 (61%), whereas ALCAT1 significantly increased by 97% (P < 0.05; Fig. 7C).

Relative gene expression levels in rats and mice. We also sought to use a comparative approach to analyze the relative mRNA expression levels of CL metabolism enzymes in rat and mouse muscle. Of the CL de novo biosynthesis enzymes, CDS-1 had the lowest level of expression in both the rat and mouse. This is consistent with CDS-1 as the rate-limiting enzyme in the CL synthesis pathway. Of the remodeling enzymes, the expression of Taz and ALCAT1 was similar in the rat; however, Taz was significantly higher than ALCAT1 in mouse. When we compared the expression levels of the OMM enzymes MitoPLD and Plscr3, we found Plscr3 to be significantly higher than MitoPLD in both species (Fig. 8, A and B; P < 0.05).

**DISCUSSION**

The objective of this study was to investigate the underlying basis for previously discovered findings that CL concentration increases with muscle use and decreases with muscle disuse. To do this, we used similar conditions of muscle use and disuse, as previously reported, and measured COX activity to confirm that we were successful at altering mitochondrial content. We next set out to compare the CL content in muscle under these conditions, with the expression of the enzymes involved in the CL metabolism pathway. We chose to examine the mRNA levels of these enzymes 1) due to the lack of suitable antibodies and 2) to provide a comprehensive analysis of the trends in gene expression within the pathway, as produced by stimuli which alter mitochondrial biogenesis.

We analyzed enzymes of the CL de novo biosynthesis, remodeling, and outer mitochondrial membrane pathways following CCA, denervation, and another form of muscle disuse, aging. Our expectation was that the expression of the biosynthesis enzymes CDS-1 and CLS would increase with CCA and decrease with denervation, thus paralleling mitochondrial and CL content. We found that CCA induced an increase in CDS-1, while CLS remained unaltered. Thus, the increase in CL content measured with muscle use is accompanied by the upregulation of certain, but not all, CL biosynthesis genes. Since CDS-1 is the rate-limiting enzyme in CL biosynthesis (14), the augmented expression of CDS-1 correlates well with the increase of CL in CCA-treated muscle. Our comparative approach to analyze the relative mRNA levels of these enzymes confirmed the probable rate-limiting function of CDS-1, since CDS-1 mRNA levels were among the lowest of the genes measured in both rat and mouse muscle. However, we were surprised to find that denervation also elicited increases in the mRNAs encoding these biosynthesis enzymes. Our observations are suggestive of a secondary, compensatory response which occurs during chronic muscle disuse to restore the lost organelle CL content during this condition (23).

Taz and ALCAT1 are enzymes responsible for the remodeling of CL FAs. Taz has been shown to transfer linoleic FAs onto CL, thus creating mature, functional tetrailinoleic CL. A loss of function mutation in the Taz gene in humans has been shown to lead to Barth syndrome, which is characterized by numerous myopathies (3). In contrast, ALCAT1 has been shown to add polyunsaturated FAs onto CL, creating an immature form of the molecule which is elevated during pathological conditions (15). Based on this, we hypothesized that Taz and ALCAT1 expression would change in opposite directions with chronic muscle use, and also with muscle disuse conditions. Surprisingly, Taz was completely unresponsive to the treatments. However, ALCAT1 decreased with CCA, and became elevated following denervation. This suggests that chronic muscle use not only elevates CL levels, but also serves to induce a more functional form of the phospholipid. Interestingly, the higher level of Taz relative to ALCAT1 in the mouse, compared with the rat, suggests that CL in mouse muscle may have a greater ratio of functional to nonfunctional CL than in rat muscle.

MitoPLD and Plscr3 both interact with CL on the outer mitochondrial membrane. Plscr3 serves to transport CL from the inner to the outer mitochondrial membrane, thereby dispersing CL within the organelle. MitoPLD, on the other hand, cleaves outer membrane CL into PA, thereby reducing total CL content. We hypothesized that CCA would induce Plscr3 to help redistribute the increase in CL content, while MitoPLD would decline to facilitate this process. We found that CCA elicited this response in both of these enzymes, supporting our hypothesis. To our surprise, denervation elicited a similar effect. We speculate that this alteration may serve a very...
different purpose during chronic muscle disuse. The increase in outer mitochondrial membrane CL may act as a binding site for tBid, which triggers cytochrome c release and leads to mitochondrially mediated apoptosis. Thus, the effect may be a contributing factor to the previously reported increased apoptotic susceptibility following denervation (1). Our comparative analyses of mRNA levels showed the existence of high ratios of Plscr3 relative to MitoPLD in both mouse and rat. This suggests that the need for CL transport to the outer membrane is greater than that for CL cleavage under steady-state conditions.

When we analyzed the CL levels within mitochondria of young and aged animals, we found no difference between the two age groups. This finding was in parallel with the lack of difference exhibited for the measured mRNAs. In general, the response of aging animals to either CCA or denervation was attenuated compared with the responses observed in young animals. Notably, both the protective CCA-induced and the detrimental denervation-induced changes of ALCAT1 were lost with age. CCA-induced changes of MitoPLD and Plscr3 were also both reduced with aging. These findings are consistent with the observation that aging muscle can adapt to variations in chronic muscle use or disuse but that the response is attenuated compared with that in young muscle (18).

In an effort to elucidate the transcriptional underpinnings behind our observations, we examined the possible role of PGC-1α. PGC-1α is an established nuclear coactivator, often referred to as the master regulator of mitochondrial biogenesis because its activation or overexpression promotes the transcription of a wide variety of nuclear gene products that are destined for the mitochondria. However, its function is currently unknown regarding its regulation of the transcripts of genes encoding enzymes of CL. PGC-1α has been shown to be elevated by CCA (2), while chronic muscle disuse via denervation downregulates PGC-1α levels (1). Thus we used PGC-1α knockout animals to determine whether this transcriptional coactivator is involved in mediating the mRNA changes observed. We found that CDS-1 was suppressed by the absence of PGC-1α, suggesting that the coactivator directly regulates CL expression of this rate-limiting enzyme and, therefore, the flux through the CL biosynthesis pathway. Indeed, inspection of the CDS-1 promoter reveals binding sites for MyoD, USF-1, NF-κB, PPAR-γ, CREB, and MEF-2, all transcription factors through which PGC-1α may be acting. Considerable further work is required to verify the possible functional nature of these binding sites within the CDS-1 promoter region. With respect to the remodeling enzymes, ALCAT1 levels were increased in the absence of PGC-1α. This suggests that PGC-1α normally suppresses ALCAT1, thereby exhibiting a protective role on the fatty acid composition of CL. In contrast, none of the outer membrane CL remodeling genes was affected by the absence of PGC-1α. Thus PGC-1α has a selective role in regulating the transcription of CL metabolism enzymes.

This study provides the first detailed analysis of the expression of CL synthesis and remodeling enzymes in skeletal muscle. Our results set the stage for further exploration of this complex network of mitochondrial membrane regulators, particularly when suitable antibodies become available. Both protein level measurements and the localization of these enzymes will be crucial for the expansion of our knowledge regarding this molecular pathway. All CL biosynthetic enzymes are nuclear-encoded proteins. Thus they rely on the mitochondrial import machinery to be transported into the mitochondria to manufacture or modify CL. It would, therefore, be of great interest to analyze the trafficking, localization, and possible posttranslational modifications of many of these enzymes following chronic muscle use or disuse, or in diseases of phospholipid metabolism. Our data suggest that the transcriptional activation of certain genes responsible for CL synthesis is upregulated during muscle use, perhaps to produce more CL, and also during muscle disuse as part of a possible compensatory mechanism. Many of these observed effects are lost with aging. Finally, it was notable that some CL metabolism genes are regulated by PGC-1α, documenting a role for this coactivator in mitochondrial phospholipid synthesis, alongside its well-established function in the regulation of typical nuclear genes encoding mitochondrial proteins.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

D.A.H. conception and design of research; O.O., M.F.N.O., K.S., K.J.M., and A.V. performed experiments; D.A.H. and O.O. analyzed data; D.A.H. and O.O. interpreted results of experiments; O.O. prepared figures; O.O. drafted manuscript; D.A.H. edited and revised manuscript; D.A.H. approved final version of manuscript.

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