Lactate dehydrogenase expression at the onset of altered loading in rat soleus muscle

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THE LOADING DEMANDS PLACED on skeletal muscle can dramatically affect fiber cross-sectional area, phenotype, and metabolic profile (14, 30, 40). These adaptations are specific to the type of loading stimulus applied. Contractile and metabolic properties of skeletal muscle are linked during myofiber conversion, as evidenced by the fact that metabolic enzyme activity and gene expression are related to the new functional demands placed on that muscle. An overall shift in muscle phenotype will occur after an extended period of altered loading; however, a brief period of altered loading is also a powerful stimulus for changes to skeletal muscle that can regulate several processes associated with muscle remodeling. Functionally overloading the rat soleus muscle for 3 days alters gene expression for immune function, inflammation, and cell cycle regulation (9). Brief periods of muscle unloading initiate signaling that decreases muscle mass that includes a decrease in protein synthesis (26) and satellite cell proliferation (11).

Increasing or decreasing muscle loading for extended periods can reduce the soleus muscle’s ability to oxidize long-chain fatty acids (14, 41, 49) and is likely related to a reduced expression of 3-hydroxyacyl-CoA dehydrogenase (41), fatty acid transport proteins (9, 49), and fatty acid binding proteins (49). It has been shown that functional overload can decrease many markers related to glycogen metabolism in both slow and fast muscles (4, 35). Fourteen weeks of functional overload in the soleus muscle have been shown to decrease the activities of hexokinase, phosphofructokinase, and α-glycerophosphate dehydrogenase (35). In contrast, 14 days of hindlimb disuse shifts the soleus muscle’s substrate utilization profile toward a glycolytic fiber (14). Functionally overloaded the soleus muscle for 3 days alters the expression of metabolic genes such as pyruvate dehydrogenase-B, monocarboxylate transporter 1, and lactate dehydrogenase (LDH)-B (9).

LDH is a tetrameric enzyme that catalyzes the reversible NAD-dependent interconversion of pyruvate to lactate (18). Two independently regulated genes encode for two polypeptides that comprise the subunits in the LDH tetramer. The LDH-A polypeptide is highly expressed in glycolytic white skeletal muscle, whereas the LDH-B polypeptide is highly expressed in oxidative cardiac muscle (31). LDH activity is regulated by specific subunit combinations of LDH-A or -B gene products. LDH-A favors the complete and rapid conversion of pyruvate to lactate, whereas LDH-B favors the complete and rapid conversion of lactate to pyruvate. The intermediate isoforms LDH-A2B2 and LDH-A1B1 have reduced substrate affinity compared with LDH-A1 and LDH-B4 enzyme isoforms. The abundance of these different isoforms partly determines the activity of total LDH in various tissue types. The slow red soleus muscle’s LDH-B mRNA expression has been reported to be 17-fold higher than fast white muscle (8). Three weeks of hindlimb disuse have been shown to shift soleus muscle LDH isoenzyme pattern from an oxidative profile to a more anaerobic profile by decreasing the LDH-B...
subunit and increasing the LDH-A subunit (6). Similarly, total LDH enzymatic activity in the rat vastus medialis is also increased following exposure to 2 wk of microgravity (29). Furthermore, LDH-B mRNA is also decreased in the rat soleus muscle after 3 days of functional overload (9). LDH expression is affected by prolonged periods of altered loading, and these changes are consistent with fiber-type conversion. However, changes in muscle metabolism at the onset of altered loading may be due to physiological processes other than myofiber conversion.

Skeletal muscle LDH expression is regulated by many stimuli, including muscle activity, load bearing (40), estrogen levels (20, 23), and c-Myc expression (38). The LDH-A promoter contains a conserved E-box sequence (18), which interacts with basic helix-loop-helix (bHLH) transcription factors. The bHLH transcription factors include MyoD, myogenin, MRF4, c-Myc, and hypoxia inducible factor-1. The bHLH transcription factors are important in proliferation and differentiation of skeletal muscle. LDH-A expression can also be regulated by hypoxia inducible factor-1, which is expressed when there is low oxygen availability (34). Less information is known about the LDH-B promoter, but it does not appear to be regulated by mechanisms related to diminished oxygen availability (34). Although LDH expression and activity appear sensitive to long periods of altered muscle loading, specific LDH isoform expression and activity at the early onset of increased or decreased soleus muscle loading have not been described. After 3 days of either increased or decreased load, muscle fibers are initiating signaling programs related to muscle phenotype changes and muscle remodeling. These early time points of altered load represent the beginning of a dynamic state of remodeling, which occurs before a muscle has undergone extensive hypertrophy or atrophy.

The purpose of the present study was to examine whether 3 days of either increased or decreased load would differentially regulate LDH expression in the rat soleus muscle. It was hypothesized that increased load would attenuate LDH-B expression and induce LDH-A expression to facilitate the increased energy demands associated with processes involved with the onset of altered loading. Animals were subjected to 3 days of either bilateral synergist ablation or hindlimb suspension.

METHODS

Animals. Male Sprague-Dawley rats (n = 33) were acquired from the Harlan rodent colony, housed individually at the University of South Carolina, fed ad libitum, and maintained on a 12:12-h light-dark photoperiod. Two independent experiments were performed. In the first experiment, rats were randomly assigned to 3 days of functional overload (n = 4) or control overload (n = 5). A subset of animals was functionally overloaded for 12 h (n = 5) or 24 h (n = 5). In the second experiment, rats were randomly assigned to 3 days of hindlimb suspension (n = 5) or control suspension (n = 5). The University of South Carolina Animal Care and Use Committee approved all procedures.

Skeletal muscle overload. The hindlimb soleus muscles were functionally overloaded for 3 days, and a subset of animals was functionally overloaded for 12 or 24 h by a modified bilateral surgical ablation method, as previously described (25). Body weights were obtained on the day of death. Briefly, rats were anesthetized with an intramuscular injection of ketamine hydrochloride (75 mg/kg body wt), xylazine (3 mg/kg body wt), and acepromazine (5 mg/kg body wt). In a sterile environment, the dorsal surface of the hindlimb was shaved and cleaned, and the gastrocnemius muscles were then exposed by a posterior longitudinal incision through the skin and biceps femoris muscle of each lower limb, and the distal two-thirds of heads of each gastrocnemius muscle were excised. Animals awakened within 1–2 h of surgery and returned to normal ambulation. There were no postoperative complications observed during the course of the study. The rats assigned to the control-overload group received a sham surgery that consisted of the same surgical procedure as the overload group, except the gastrocnemius was not removed. Soleus muscles were removed 12 and 24 h, as well as 3 days, following the initial surgery, frozen in liquid nitrogen, and stored at −80°C until further analysis.

Hindlimb suspension. Skeletal muscle disuse was induced by hindlimb suspension, as previously described (26). Rats were subjected to 3 days of hindlimb suspension. Body weights were obtained on the day of death. Briefly, unanesthetized animals’ tails were cleansed with alcohol, covered with a light coat of benzoin tincture, and dried until tacky. Strips of elastoplast (Biersdorf, Norwalk, CT) adhesive bandage were applied to the proximal two-thirds of all sides of the tail and looped through a swivel attachment mounted above the cage that was designed to allow 360° rotational movement, with only the forelimbs able to come into contact with the cage floor. The animals were weighed twice daily to ensure that tail blood flow was not compromised and that the hind feet were unable to contact the bottom or sides of the cages.

Crude protein extracts. Frozen soleus muscles were homogenized in sodium phosphate buffer (100 mM NaPO₄, pH 7.5), as previously described (32). Briefly, tissues were homogenized by using three 10-s pulses on ice with an Eberbach glass homogenizer. Homogenates were separated into soluble and insoluble fractions by centrifugation. The soluble fraction was assayed for LDH activity and LDH isoform expression. Total protein concentration was determined in the supernatant by the Bradford protein assay (Bio-Rad).

LDH activity assay. LDH activity was measured by using a Beckman Coulter DU-7 spectrophotometer (Fullerton, CA). LDH-A activity was determined by measuring the increase in absorbancy at 340 nm when NADH is reduced to NAD in the presence of pyruvate, as described by Powers et al. (32). LDH-B activity was determined by measuring the increase in absorbancy at 340 nm when NAD is oxidized to NADH in the presence of lactate. One unit of enzymatic activity is defined as the amount of enzyme that catalyzed the oxidation and reduction of 1 μmol of NADH/NAD per minute at 25°C.

LDH isoform expression assay. The cellular composition of LDH isoforms was evaluated, as described previously (23, 24). LDH isoforms were fractionated by running 5 μg of total soluble protein on a 1% agarose gel. The protein was separated for 60 min at 100 V using LDH isolt (Sigma) as an internal positive control. Bands that represented the five isoforms were revealed colorimetrically (Sigma Chemical procedure 705), and the gels were fixed with 5% acetic acid. The LDH isoform bands were scanned and quantified with an imaging densitometer (Scion Image, Frederick, MD). LDH isoforms were expressed as a percentage of total LDH.

Total RNA isolation and Northern blot analysis. RNA was extracted with TRIzol reagent (Life Technologies, Grand Island, NY), as previously described (21). Briefly, powdered muscle was homogenized in TRIzol. Total RNA was isolated, DNase was treated, and its concentration and purity were determined by UV spectrophotometry. RNA with a 260-to-280-nm ratio of ≥1.6 was used for Northern blotting. Northern blot analysis was performed, as previously described (26). Total RNA (15–20 μg) was fractionated on a denaturing 1% agarose gel (1× MOPS, 6.7% formaldehyde) and transferred to a positively charged nylon membrane (Amer sham) by capillary action. The integrity and concentration of the RNA were confirmed by visual inspection of ethidium bromide-stained 18S and 28S rRNAs. All probes for Northern blot analysis were made by random priming, as
previously reported (26). Membranes were visualized by autoradiography and then quantified by densitometry scanning (Scion Image). An integrated optical density was obtained, which was used to calculate integrated optical density mRNA per microgram of total RNA. LDH mRNA abundance was normalized to 18S rRNA.

Data analysis. Results were reported as means ± SE. Data were analyzed by using a one-way ANOVA and Student’s t-test. Statistical significance was determined if $P \leq 0.05$.

RESULTS

Body mass and muscle characteristics. Body weights were attained on the day of death. Body weights of control and functionally overloaded rats were not different (167 ± 3 vs. 163 ± 1 g; $P < 0.2$). There was no effect of overload on soleus muscle weights ($P < 0.07$) or soleus-to-body weight ratio ($P < 0.06$). Hindlimb suspension decreased rat body weight 22% ($179 \pm 4$ vs. $140 \pm 6$ g; $P < 0.0004$). Soleus muscle weight decreased by 29% ($P < 0.0002$) after 3 days of suspension. However, there was no effect of hindlimb suspension on muscle weight-to-body weight ratio (Table 1).

LDH activity following 3 days of functional overload. Muscle LDH-A activity did not change ($P < 0.6$) after 3 days of functional overload (Fig. 1A). There was an 80% decrease ($P < 0.01$) in LDH-B activity after 3 days of functional overload (Fig. 1A).

Expression of LDH mRNA following 3 days of functional overload. LDH-A mRNA increased 372% ($P < 0.05$) after 3 days of functional overload in the soleus (Fig. 1B). In contrast, LDH-B mRNA decreased by 33% ($P < 0.05$) after 3 days of functional overload (Fig. 1B). Further analysis demonstrated that LDH-B mRNA was decreased by 37% ($P < 0.01$) and 43% ($P < 0.002$) after only 12 and 24 h of functional overload, respectively (Fig. 1C).

LDH protein expression following 3 days of functional overload. LDH-A and LDH-B transcripts encode two different polypeptides. The two polypeptides can be arranged into five possible LDH tetramers known as LDH-A$_4$, LDH-A$_3$B, LDH-A$_2$B$_2$, LDH-AB$_3$, and LDH-B$_4$. LDH isoforms LDH-A$_4$ and LDH-A$_3$B increased by 203% ($P < 0.02$) and 157% ($P < 0.05$), respectively, following functional overload (Fig. 2). In contrast, LDH-B$_4$ decreased by 74% ($P < 0.008$) in functionally overloaded soleus muscle (Fig. 2). There were no observed changes in the other LDH isoforms.

LDH activity following 3 days of hindlimb suspension. LDH-A activity increased by 234% ($P < 0.05$) after 3 days of hindlimb suspension (Fig. 3A), whereas LDH-B activity was not changed ($P < 0.8$) by 3 days of hindlimb suspension (Fig. 3A).

Table 1. Soleus muscle weight and muscle weight relative to body weight in overloaded and suspended rats

<table>
<thead>
<tr>
<th>Variable</th>
<th>Con-OV</th>
<th>OV</th>
<th>Con-Sus</th>
<th>Sus</th>
</tr>
</thead>
<tbody>
<tr>
<td>$n$</td>
<td>5</td>
<td>4</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Soleus muscle mass, mg</td>
<td>60 ± 3</td>
<td>69 ± 7</td>
<td>67 ± 3</td>
<td>48 ± 2*</td>
</tr>
<tr>
<td>Muscle mass-to-body mass ratio, mg/g</td>
<td>0.36 ± 0.02</td>
<td>0.42 ± 0.02</td>
<td>0.38 ± 0.02</td>
<td>0.34 ± 0.02</td>
</tr>
</tbody>
</table>

Values are means ± SE; $n$, no. of rats. OV, overload group; Con-OV, control-overload group; Sus, suspension group; Con-Sus, control-suspension group. Functional overload was accomplished by bilateral surgical ablation. Skeletal muscle disuse was induced in the hindlimb muscles by hindlimb suspension. *Significantly different from treatment-matched control animals, $P < 0.05$.

Expression of LDH mRNA following 3 days of hindlimb suspension. No changes in LDH-A ($P < 0.8$) or LDH-B ($P < 0.8$) mRNA abundances were detected after 3 days of hindlimb suspension (Fig. 3B).
LDH protein expression following 3 days of hindlimb suspension. The effect of hindlimb suspension on LDH protein expression was also examined. Disuse induced a 199% increase ($P < 0.01$) in LDH-A$_4$ protein expression and a 90% decrease ($P < 0.01$) in LDH-B$_4$ (Fig. 4). There were no observed changes in the other LDH isoforms.

DISCUSSION

Skeletal muscle’s metabolic profile can be altered by a host of physiological conditions, including the loading demands placed on the muscle. The effect of extended periods of increased or decreased mechanical load on skeletal muscle’s metabolic profile has been well described (3, 29, 43, 46). To our knowledge, this is the first study to demonstrate rapid changes in specific rat soleus LDH enzyme isoform expression patterns at the onset of altered loading. The primary finding of this study is that altered loading of the soleus muscle induced a differential LDH response with respect to enzymatic activity, mRNA, and protein isoform expression. These changes in LDH regulation were independent of changes in the muscle-to-body weight ratios and may represent skeletal muscle’s earliest response to stressors related to altered loading.

Increasing the mechanical demands placed on skeletal muscle both induces and represses the expression of a large number of genes (9). Disuse appears to have a different affect on LDH expression compared with an overload stimulus. In the present study, disuse decreased soleus muscle mass by 28%, and chronic muscle disuse is associated with reduced muscle power and increased fatigability (14). Disuse is linked with an increase in protein degradation and a decrease in protein synthesis (16). Phosphorylation of p70s6k is decreased by 52% after 3 days of hindlimb suspension (25), which is indicative of a decrease in the initiation phase of protein translation. Skeletal muscle disuse also induces increases in proteins involved with sarcoplasmic reticulum intracellular calcium flux (17). Disuse is also linked to a decrease in myofiber nuclei (27), and

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**Fig. 2.** A: representative zymogram of LDH isoform expression in 3-day functionally overloaded soleus muscle. Stds, standards. B: effect of 3 days of overload on LDH isoenzyme expression. LDH isoforms are expressed as a percentage of total LDH. Values are means ± SE. *Significantly different from control group, $P \leq 0.05$.

**Fig. 3.** A: effect of 3 days of suspension on LDH activity in the soleus muscle. B: effect of 3 days of hindlimb suspension on LDH mRNA abundance in the soleus muscle. LDH mRNA abundance was corrected for with 18S rRNA. Sus, hindlimb-suspension group; Con-Sus, control-suspension group. Values are means ± SE. *Significantly different from control group, $P \leq 0.05$. 

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apoptosis has been implicated for this elimination of myonuclei (2). The loss of skeletal muscle capillaries during disuse can lead to a hypoxic state (7). Hypoxia favors an increase in lactate production, which has been shown to stimulate apoptosis (42). In the presence of ATP, apoptosis is favored over necrosis. The increase in LDH-A activity and protein expression is indicative of an increase in the glycolytic capacity of the soleus muscle, which could be responsible, in part, for the increase in apoptosis associated with disuse. LDH may not be sensitive to shifts in metabolic demand due to decreased loading as an adaptation to prevent the loss of functional capacity.

A significant number of early changes in gene expression induced by functional overload are associated with muscle regeneration and growth processes. However, gene expression analysis of 3-day functionally overloaded soleus muscle using microarrays also identified many differentially expressed genes associated with muscle metabolism (9). Specifically, LDH-B gene expression was dramatically repressed in response to 3 days of functional overload (9). The present study corroborates this observation by demonstrating that this rapid decrease in LDH-B mRNA is present after only 12 h of functional overload. However, LDH-A and LDH-B mRNA levels were not changed at the onset of skeletal muscle disuse. With the decrease in LDH-B and the increase in LDH-A, the shift to glycolytic metabolism in the soleus following functional overload would allow energy to be produced rapidly. Changes in muscle loading also alters the muscle’s overall glycolytic capacity, which can be regulated pretranslationally (19, 28). LDH enzymatic activity was also altered at the onset of disuse. LDH-A activity increased by 234% following 3 days of disuse in the soleus muscle. Muscle functional changes brought on by extended periods of disuse are preceded by a shift in the muscle’s metabolic capacity. In the present study, LDH-A and LDH-B mRNA abundance in overloaded muscle changed concomitantly with LDH protein isoform changes. However, following disuse, protein expression of LDH-A4 increased and LDH-B4 decreased without changes in mRNA abundance. Thus it appears, at least initially, that muscle disuse regulates LDH through translational and posttranslational mechanisms. LDH-B expression and activity are often associated with mature fiber metabolic capacity related to terminal differentiation of satellite cells.

The regulation of LDH expression by muscle loading appears complex. The present data demonstrate that unloading and loading differentially regulate LDH expression. Increased loading induces gene expression related to structural damage, myofiber growth, satellite cell activation, and immune cell infiltration (9, 15). Satellite cell proliferation is induced over the first several days of functional overload (1, 15). Because increased satellite cell proliferation requires energy, it is possible that increased LDH-A mRNA is indicative of overload-induced satellite cell proliferation. Proliferating myoblasts have high LDH activity compared with quiescent satellite cells (5). LDH-A contains a conserved consensus E-box sequence in its promoter region (18, 38), which can bind myogenic regulatory transcription factors. This family of proteins includes MyoD, Myf-5, myogenin, and MRF4. Myogenic regulatory factors may regulate LDH-A expression in proliferating myoblasts. Both oxidative and glycolytic metabolism are necessary to supply ATP for regulatory and biosynthetic events occurring during myogenesis (5, 12). Decreased loading demands on skeletal muscle have been shown to decrease mitotic activity, as well as suppress satellite cell proliferation (11, 36). Although LDH-A and LDH-B mRNA levels were not altered at the onset of skeletal muscle disuse, LDH enzymatic activity did change. Increased LDH-A4 and decreased LDH-B4 isoforms were the only alterations in isoform expression observed with decreased loading. These changes are in agreement with a metabolic profile shift away from a slow-type muscle.

LDH-A has been shown to be involved in cell cycle regulation in different cell types (22, 37, 38). For example, elevated LDH-A expression contributes to unchecked proliferation of human cancers (38). Additionally, LDH-A expression in rat fibroblasts is a marker of neoplastic transformation (10). Thus LDH-A overexpression may confer a neoplastic growth advantage through its enzymatic function (31, 38), because tumor cells maintain a high-glycolytic rate, even under aerobic conditions (47). The LDH-A mRNA induction can be regulated by
transcription factors such as c-Myc, an oncogene whose deregulation is prevalent in various cancers (22, 38). c-Myc participates in the regulation of cell proliferation, differentiation, and apoptosis (10, 22). Following 3 h and 3 days of functional overload, c-Myc mRNA levels increased (9, 48), and the LDH-A gene is a direct transcriptional target of c-Myc (22). Protooncogene c-Myc overexpression activates LDH-A promoter activity (18). The upregulation of LDH-A mRNA may also be attributed to increased mRNA stability, because LDH-A mRNA is characterized by a relatively short half-life (14, 45). LDH-A mRNA stability has been shown to be regulated through PKA and PKC pathway activation (39, 44). PKC is increased by 126% in the soleus muscle after 4 days of functional overload (33). PKC activity is thought to inhibit differentiation and stimulate proliferation in myogenic satellite cells (13). LDH-B expression does not appear to be regulated by these pathways (34).

In summary, altered LDH expression is an early cellular response to meet functional demands brought on by altered loading. Several lines of evidence, including enzymatic activity, enzyme isoform protein profile, and mRNA expression, point to a shift toward a less aerobic soleus metabolic profile when subjected to brief periods of increased or decreased loading. These facts support a long-held hypothesis that cellular regulation during muscle disuse is not simply the repression of growth signals. Increasing or decreasing the loading state of muscle activates independent cellular regulatory signaling pathways. The present data indicate that increases in LDH-A and decreases in LDH-B isoform expression are among the early gene responses to altered muscle loading.

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