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Chronic electrical stimulation drives mitochondrial biogenesis in skeletal muscle of a lizard, *Varanus exanthematicus*

Paul J. Schaeffer[1,*], Scott D. Nichols[2] and Stan L. Lindstedt[2,3]

[1]*Department of Zoology, Miami University, Oxford, OH 45056, USA,* [2]*Department of Biological Sciences, Northern Arizona University Flagstaff, AZ, USA and* [3]*Department of Physiology, University of Arizona School of Medicine, Tucson, AZ, USA*

*Author for correspondence (e-mail: schaefpj@muohio.edu)

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Summary

We investigated the capacity for phenotypic plasticity of skeletal muscle from *Varanus exanthematicus*, the savannah monitor lizard. Iliofibularis muscle from one leg of each lizard was electrically stimulated for 8 weeks. Both stimulated and contralateral control muscles were collected and processed for electron microscopy. We used stereological analysis of muscle cross-sections to quantify the volume densities of contractile elements, sarcoplasmic reticulum, mitochondria and intracellular lipids. We found that mitochondrial volume density was approximately fourfold higher in the stimulated muscle compared to controls, which were similar to previously reported values. Sarcoplasmic reticulum volume density was reduced by an amount similar to the increase in mitochondrial volume density while the volume density of contractile elements remained unchanged. Intracellular lipid accumulation was visibly apparent in many stimulated muscle sections but the volume density of lipids did not reach a significant difference. Although monitor lizards lack the highly developed aerobic metabolism of mammals, they appear to possess the capacity for muscle plasticity.

Key words: muscle plasticity, sarcoplasmic reticulum, intracellular lipid, exercise, activity.

Introduction

Vertebrate skeletal muscle is composed of a mosaic of fibers differing in contraction velocity, energy and oxygen requirements, and fatigability. These characteristics, once thought to be genetically fixed and inflexible properties of muscle, in fact respond adaptively to altered activity levels in humans and other mammals. Muscle phenotypic plasticity has been the focus of intense interest since first demonstrated in cats (Buller et al., 1960). In their groundbreaking report, Buller et al. demonstrated that the functional characteristics of muscle, rather than being genetically fixed, reflect the type of nervous input that the muscle receives (Buller et al., 1960). Later, Salmons and Vrbova developed the technique of using chronic electrical stimulation (CES). By eliminating normal neuronal input in exogenously activated muscle, they were able to critically differentiate between secreted neural factors and the pattern of activation. They found that stimulation of extensor digitorum longus and tibialis anterior muscle by implanted electrodes in both cats and rabbits led to characteristic fast-to-slow shifts in its metabolic and contractile properties (Salmons and Vrbova, 1969). Through the intervening 40 years, mammalian muscle is now considered among the most malleable tissues in the body, capable of dramatic changes in both structure and function in response to changes in the nature as well as magnitude of the demand placed on it.

Skeletal muscle phenotypic plasticity involves structural and functional changes in both the metabolic and contractile properties of skeletal muscle. Evidence of the capacity for muscle plasticity is provided by the dramatic transformations seen in skeletal muscle in response to strength and endurance training (Flück, 2006), disuse atrophy (Jackman and Kandarian, 2004), and CES (Pette and Vrbova, 1999; Pette, 2001). The 'fast-to-slow' transition in muscle type seen following CES leads to a muscle with a well characterized assemblage of proteins that are associated with slower contraction velocities as well as higher metabolic capacity and endurance (Booth and Baldwin, 1996). In other words, they possess the complete suite of characteristics common to slow fibers.

The study of muscle plasticity has been very active for more than 40 years and much knowledge has been gained regarding the nature of the changes that occur in response to altered use. Sufficient demonstration of phenotypic plasticity in mammalian skeletal muscle now exists to invite the question as to whether this is a unique evolved property of the mammals. Studies of avian muscle plasticity are less extensive; however, evidence suggests that plasticity is an avian feature as well. The mass of flight muscle relative to body mass increases both in preparation for migration (Piersma, 1998) as well as during moult, when relative lift is reduced (Lind and Jakobsson, 2001). The cellular phenotype of these hypertrophying muscles remains unknown. However, when muscle hypertrophy was induced by stretch overload, contractile properties slowed, suggesting a phenotypic shift (Alway, 1994). In addition to shifts in contractile properties, metabolic properties of avian muscle also appear to

be plastic. In ducklings, shivering induced by cold exposure resulted in increases in cytochrome oxidase activity (Barré et al., 1987) and increased reliance on fatty acid utilization by skeletal muscle (Bénistant et al., 1998). Studies of amphibian and reptilian muscle show that skeletal muscle properties correlate well with life history and behavior (Bonine et al., 2001), but there remains little information on muscle plasticity within an individual lifespan in these animals. There are considerable experimental challenges in altering muscle activity in amphibians and reptiles, not only due to motivation to exercise but also the limited capacity of the lungs and heart to supply sufficient oxygen to support increased aerobic activity. Endurance exercise training of *Amphibolurus nuchalis*, an Australian agamid lizard, failed to result in an adaptive response (Garland et al., 1987). Nonetheless, modest fast-to-slow shifts in myosin isoforms and ATPase activity were described following 'forced terrestrial stepping', a moderate exercise stimulus, in an Urodelan amphibian (Launay et al., 1998). Organismal limitations bypassed with short term, direct electrical stimulation of the gastrocnemius muscle in the Indian green frog *(Rana hexadactyla*) revealed increased mitochondrial protein content and cytochrome *c* oxidase activity (Moorthy et al., 1981). These data suggest that phenotypic plasticity of muscle, rather than a derived character reliant upon a high capacity oxygen delivery system, as found in endotherms, may be ancestral.

In the present study we investigate the effects of chronic electrical stimulation on skeletal muscle mitochondrial biogenesis in the Savannah monitor lizard *Varanus exanthematicus*. *V. exanthematicus* is a wide-ranging active predator with relatively high metabolic capacity and thus represents a species within the Squamata in which skeletal muscle may be likely to be capable of acclimative plasticity in response to alterations in activity level.

Materials and methods

Animals

Four Savannah monitor lizards *Varanus exanthematicus* (Bosc 1792) were purchased from wholesale distributors (Glades Herp Inc., Bushnell, FL, USA). Initially, the animals were housed collectively in a single 1.5 m×1.5 m pen with water provided *ad libitum* and fed a diet of dead mice once a week. Temperature was maintained around 28°C with the aid of infra-red heat lamps. Following electrode implantation, animals were maintained individually in 40-liter aquaria with all other conditions being the same.

Surgery

To implant the stimulating electrodes, animals were anesthetized with halothane and the iliofibularis muscle of the right hind limb was isolated. This muscle contains two distinct anatomical areas, which are unambiguously divided into 'white' and 'red' regions. Two 32-gauge, multi-stranded stainless steel wires (California Fine Wire, Grover Beach, CA, USA) were implanted into the white section of the muscle approximately one quarter muscle length from each end and secured using a small dab of tissue glue. The electrode wires were sutured to the skin at their point of exit near the pelvis and then ran to a 3 cm×3 cm 'backpack' plug attached to the lizard's back.

Stimulation protocol

Electrical stimulation was begun the day following electrode implantation by connecting the backpack plug with a Grass stimulator (Model 48, Grass Instruments, West Warwick, RI, USA). Stimulating voltage was set visually to be sufficient to cause a noticeable tremor of the hind leg. This was a voltage of 1–3 V for a duration of 2 ms. Stimulation frequency was initially 4 Hz (the maximum without tetany) for the first few days and was gradually increased over a period of 2 weeks until a maximum of 10 Hz was achieved. Stimulation at 10 Hz proceeded for 8 h per day, 5 days a week, for 6 weeks. During the 8-week period the voltage was adjusted daily to invoke the leg tremor. In general, the voltage was increased during the course of the experiment for each animal; the highest end-experiment voltage was 5 V.

Muscle samples

Following 6 weeks of muscle stimulation, the animals were sacrificed with a lethal dose of sodium pentobarbitol. Both the stimulated iliofibularis and its contralateral control muscle were immediately dissected out. Samples from the white region of the muscle were cut into small strips, fixed in 6.25% gluteraldehyde buffered with 0.1 mol l^{-1} sodium cacodylate, pH 7.4 and processed for electron microscopy as described (Schaeffer et al., 2003). Transverse sections were cut at a thickness of 60–100 nm, stained in 2% aqueous uranyl acetate for 20 min, alkaline lead citrate for 10 min and washed in 50% ethanol. Examination and photography of the grids was done using a JEOL 1200 transmission electron microscope.

Stereology

Quantitative analysis of intracellular volume densities was accomplished using published techniques (Weibel, 1979). Eleven resin blocks were made for each of the stimulated and control muscles. Five blocks for each condition were chosen randomly and sectioned. Four micrographs were taken from each grid at a magnification of 4000×. For stereological analysis, the micrographs were magnified 6.5× (final magnification 26 000×) and projected onto a 100-point grid with an Ausjena projection unit. Because half of each micrograph covered the grid, 200 points were counted for each micrograph for a total of 4000 points per sample using the Stepone stereology software (Wainschtein and Cruz-Orive, 1994). Volume densities of mitochondria, myofibrils, sarcoplasmic reticulum and lipid droplets were calculated following Weibel (Weibel, 1979).

Statistical analysis

In all cases, statistical comparisons were made for each cellular structure between electrically stimulated muscle and non-stimulated control muscle using a Student's *t*-test. The level of significance was set at $P<0.05$ in all cases. All data are presented as means ± standard error of the mean (s.e.m.) with $N=4$.

Results

During the stimulation protocol, the animals appeared to be in good health and fed normally. The iliofibularis muscle could be observed twitching under the skin, but the lizards appeared

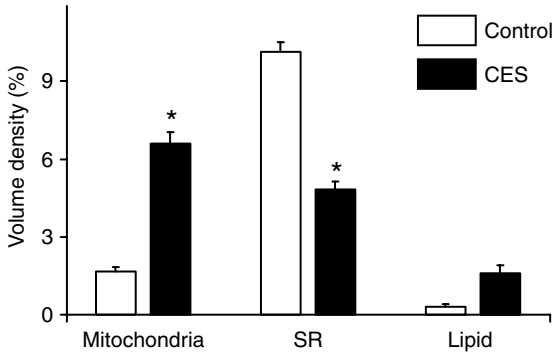

Fig. 1. Cellular volume occupied by mitochondria, sarcoplasmic reticulum (SR) and intracellular lipid droplets in the white portion of both chronic electrically stimulated (CES) and the contralateral control iliofibularis muscles of *Varanus exanthematicus*. The nearly fourfold increase in mitochondrial volume is similar to or greater than that exhibited by various mammalian species following a similar procedure. The lower volume density of the SR in CES muscle was similar in absolute magnitude to the increase in mitochondrial volume, suggesting minimal change in cell size, as there was no change in the proportion of the muscle cell occupied by myofibrils. *Significant difference from control at the $P<0.05$ level.

to tolerate this well. Varanids are normally very active lizards, however they were nearly completely inactive while in captivity.

Chronic electrical stimulation over a period of 6 weeks (a total of 240 h of 10 Hz stimulation) resulted in structural differences in a number of muscle properties in the 'white' portion of the iliofibularis muscle of these lizards. Perhaps most dramatic was a significantly higher mitochondrial volume density relative to the unstimulated contralateral control muscle ($P<0.05$; Fig. 1). This nearly fourfold greater volume of the muscle occupied by mitochondria (6.61±0.43% in stimulated *vs* 1.67±0.17% in control muscle) is evident in representative cross-sectional electron micrographs (Fig. 2). This proliferation in mitochondrial volume density occurred with no apparent change in the volume of the muscle fiber occupied by myofilaments (85.92±0.84% in stimulated *vs* 87.87±0.39% in control muscle; $P>0.05$). Instead, the higher mitochondrial volume was accompanied by a lower volume density of sarcoplasmic reticulum in the stimulated muscle than in controls ($P<0.05$; Fig. 1). The decrease in SR volume density from 10.14±0.36% to 4.83±0.32% was quantitatively nearly identical to the increase in mitochondrial volume. Additionally, although hardly any of the control micrographs demonstrated lipid accumulation (volume density of 0.31±0.10%), numerous sections from the stimulated muscle had a much higher volume of lipid (volume density of 1.60±0.32%), as shown in Fig. 2. Although stimulated muscle contained, on average, five times the lipid content of controls, the higher variability associated with rare structures and the low number of individuals examined led to insufficient power to identify a statistically significant difference ($P>0.05$; Fig. 1).

Discussion

Mammalian skeletal muscle is highly adaptable, capable of responding to both the nature and duration of muscle stimulation patterns, with shifts in both metabolic and contractile properties, throughout an animal's lifetime (Booth and Baldwin, 1996). Mitochondrial biogenesis is an integral component of the fast-to-slow fiber type switch to support increased activity (Hood et al., 2006). We began this study by questioning whether phenotypic plasticity of muscle mitochondria, intracellular lipid stores and excitation–contraction coupling is a unique (i.e. derived) feature of mammals, or alternatively is a 'primitive' or ancestral trait of vertebrate skeletal muscle. In other words, is vertebrate skeletal muscle inherently structurally responsive to activity level or is the observed phenotypic plasticity a property that evolved, perhaps subsequent to respiratory and cardiovascular systems capable of supplying oxygen to a large mass of aerobic muscle fibers? If the evolution of muscle plasticity required the prior evolution of endothermy or highly developed aerobic metabolism, then birds may exhibit this trait as well. This seems to be the case, based on data from chronic stretch overload (Alway, 1994), cold exposure (Barré et al., 1987; Bénistant et al., 1998) and treadmill exercise training (Brackenbury and Holloway, 1991).

In order to investigate this question we chose an experimental paradigm that was not intended to mimic, necessarily, the kinds of stimulation patterns found in nature. Rather we employed chronic electrical stimulation, as it circumvents any constraints on oxygen delivery that may limit the muscle's responsiveness. Using this method, a single muscle can be targeted and the need for a training regime eliminated. Thus, if the muscle were capable of demonstrating shifts in contractile and metabolic properties, CES should be the most powerful stimulus to induce that response. When this technique is employed with various mammalian

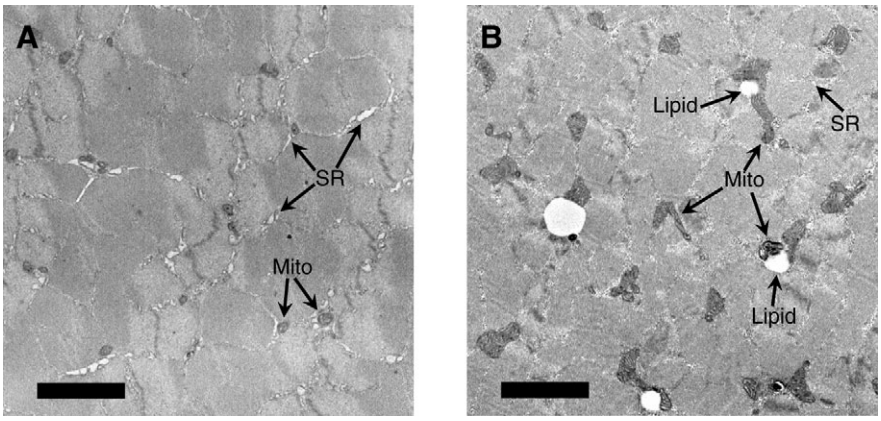

Fig. 2. Representative cross-sectional electron micrographs of control (A) and chronic electrically stimulated (B) iliofibularis muscles. The arrows indicate mitochondria (Mito), sarcoplasmic reticulum (SR) and lipid droplets. Note the increase in mitochondrial volume and associated decrease in sarcoplasmic reticulum. Scale bars, 2 μm.

species, a profound alteration of skeletal muscle phenotype is observed (Pette and Vrbova, 1999). One example of CES in non-mammalian species is provided by the experiments of Moorthy et al. (Moorthy et al., 1981). Although the time course of their experiments was very short, upregulation of mitochondrial protein content and cytochrome *c* oxidase activity in stimulated frog muscle supports the proposal that muscle plasticity is an ancestral trait of tetrapods.

Our control values for mitochondrial volume density in un-stimulated muscle are similar to those reported for hindlimb muscle of Cuban iguanas (2.94%; *Cyclura nubila*) (Conley et al., 1989) and from the 'white' portion of the iliofibularis muscle from *Agama pallida* (3.2%) (Abu-Ghalyun, 1995), *Dipsosaurus dorsalis* (3.8%) (Gleeson et al., 1984) and *Varanus exanthematicus* (2%) (Mutungi, 1990). Thus even though the animals in this study were relatively sedentary, the inactivity imposed by laboratory housing did not appear to have an appreciable effect on the ultrastructure of their white muscle. In these species, the 'red' portion of the muscle, containing more oxidative fiber types, was reported to possess considerably higher mitochondrial volume density (7–12%), higher than we found in the electrically stimulated iliofibularis in the present study (Gleeson et al., 1984; Mutungi, 1990; Abu-Ghalyun, 1995). In the present study, the lizards were very inactive, perhaps explaining why the control iliofibularis muscle had slightly lower mitochondrial volume density than previously reported, although this difference is small. As inactivity favors a 'fast' phenotype, this had to be overcome for CES to drive the observed fast-to-slow skeletal muscle transition, demonstrating the strength of the intervention. Additionally, in these studies we used the iliofibularis muscle from the contralateral limb as a control muscle. It is possible that circulating factors released from the stimulated limb could influence our control muscle. However, we saw no evidence that this occurred and indeed, if this is an important variable, it would serve only to reduce the magnitude of the response, making our conclusions more robust.

The results of this study suggest that skeletal muscle mitochondria, SR structure and lipid stores all respond to chronic electrical stimulation, even in the Savannah monitor lizard, a vertebrate with limited aerobic capacity (Wang and Hicks, 2004), and in much the same way as they do in mammalian species. Higher mitochondrial volume as well as lower sarcoplasmic reticulum volume reflects a shift toward slower, more oxidative fibers. Our data thus support the idea that, in addition to sharing common mechanisms of contraction and metabolism across vertebrate taxa, another common feature of adult vertebrate skeletal muscle that may have been set early in evolution is phenotypic plasticity.

In general the varanids have relatively more highly developed respiratory and cardiovascular systems compared to other reptiles; they could be capable of the levels of activity necessary to demonstrate this phenotypic plasticity. One may wonder about the adaptive value of a trait in animals that appear unlikely to exploit it. However, across evolutionary time scales, the capacity of muscle to respond to altered activity demands may be a critical first step towards adaptive change, leading to the wide variation in muscle structure and function such as exists in the Iguanidae (Bonine et al., 2001). Given that muscle function is a critical component of the ability of animals to forage, escape predation and seek mates, and is thus likely linked with fitness (Irschick and Garland, 2001), muscle plasticity may have played a critical role in the evolutionary diversification of many tetrapod lineages (Losos et al., 2000). Apparently an inherent property of vertebrate muscle is the ability to acclimate to current use patterns.

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