Length-tension relationships are altered in regenerating muscles of the rat after bupivacaine injection

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Plant, David R., Felice Beitzel, and Gordon S. Lynch. Length-tension relationships are altered in regenerating muscles of the rat after bupivacaine injection. J Appl Physiol 98: 1998–2003, 2005. First published February 17, 2005; doi:10.1152/japplphysiol.01381.2004.—Intra-muscular injection of bupivacaine causes complete degeneration of fibers in extensor digitorum longus (EDL) muscles of rats, followed by complete regeneration within 60 days. Previous studies have shown that regenerated EDL muscles are protected from contraction-induced injury 60 days after bupivacaine injection. It is possible that these regenerated muscles have altered length-tension relations because of fiber remodeling. We tested the hypothesis that length-tension relations are different in bupivacaine-injected and noninjected control muscles. EDL and soleus muscles of the right hindlimb of deeply anesthetized rats were injected with bupivacaine and then allowed to recover for 7, 14, 21, or 60 days (7D, 14D, 21D, 60D), and isometric contractile properties were assessed. Muscles of the contralateral limb were not injected and served as control. EDL muscles recovered from bupivacaine injection more rapidly than soleus muscles, with mass restored to control levels at 21D, and isometric tetanic force (P0) restored to control at 60D. In contrast, mass and P0 of injected soleus muscles was not restored to control even at 60D. In 7D EDL muscles, length-tension curves were shifted leftward compared with control, but in 21D and 60D EDL muscles length-tension curves were right shifted significantly (treatment × muscle length: P < 0.001). Although no clear shift in the position of the length-tension curve was observed in regenerating soleus muscles, force production was enhanced on the descending limb of the curve in 60D soleus muscles (treatment × relative muscle length: P < 0.01). The rightward shift in the length-tension curve of EDL muscles 60 days after bupivacaine injection is likely to contribute to the mechanism for their previously observed protection from contraction-induced injury.

muscle regeneration; optimum length; muscle function; contractile properties

At 60 days after bupivacaine injection, EDL muscles are less susceptible to contraction-induced injury than noninjected muscles (4). Devor and Faulkner (4) suggested that muscle damage occurs when weak sarcomeres are stretched onto the descending limb of the length-tension relationship. They proposed that regenerated muscles have greater homogeneity in the strength of sarcomeres and, therefore, fewer sarcomeres are stretched onto the descending limb of the length-tension relationship, protecting regenerating muscles from contraction injury (15, 16). Similar protection is also observed in the so-called “repeated-bout effect,” in which a single bout of eccentric exercise protects against damage from a subsequent bout (2, 13). The repeated-bout effect is associated with a rightward shift in the length-tension relationship, and conditioned muscles produce greater force (presumably by maintaining myofilament overlap) on the descending limb of the length-tension curve (14). A rightward shift in the length-tension curve is therefore a likely mechanism contributing to protection against contraction-induced injury observed in rat EDL muscles 60 days after bupivacaine injection (4).

The aim of this study was to measure length-tension curves of bupivacaine-injected rat EDL muscles, to determine whether there are temporal changes in length-tension relationships of regenerating muscles. We tested the hypothesis that length-tension curves of rat EDL muscles would be rightward shifted after bupivacaine injection. Whereas recovery from bupivacaine injection has been examined extensively in rat EDL muscles (which are predominantly comprised of fast-twitch fibers), far less is known about recovery from bupivacaine injection in rat soleus muscles (which are predominantly comprised of slow-twitch fibers). Because it is not known at what time point, if ever, normality of contractile properties of bupivacaine-injected rat soleus muscles is restored, we also examined the time course of recovery and length-tension relationships of rat soleus muscles after bupivacaine injection.

MATERIALS AND METHODS

Animals. All experiments were approved by the Animal Experimentation Ethics Committee of The University of Melbourne and were conducted in accordance with the Australian code of practice for the care and use of animals for scientific purposes (National Health and Medical Research Council). Adult (~10 mo old, n = 20) male Fischer 344 rats (460–500 g) used in these experiments were housed in standard cages and provided with food (rat chow) and water ad libitum.

Bupivacaine injection. All rats were deeply anesthetized with pentobarbital sodium (Nembutal, Rhone Merieux, Pinkenba, QLD, Australia; 60 mg/kg body mass) via intraperitoneal injection, such that...
they were unresponsive to tactile stimuli. The right EDL and soleus muscle of each rat were surgically exposed and injected intramuscularly with 0.5% bupivacaine hydrochloride (bupivacaine) (Marcain, Astra, North Ryde, NSW, Australia). Several injection sites in the distal, proximal, and midbelly regions of each muscle were used to ensure degeneration of all fibers (7). The EDL and soleus muscles of the left hindlimb served as noninjected controls. These muscles were not injected with saline vehicle, because previous studies have shown that this does not affect the morphological, structural, or functional characteristics of skeletal muscles (1, 7, 20). After the intramuscular injections, the skin incision was closed with Michel clips (Aesculap, Tutlingen, Germany). After the surgical procedure, rats were randomly assigned to testing at 7, 14, 21, or 60 days after bupivacaine injection (7D, 14D, 21D, or 60D; n = 5 rats in each group).

**Contractile properties.** After the specified duration of recovery, rats were anesthetized deeply with pentobarbital sodium (60 mg/kg body mass ip), and the EDL and soleus muscles from the left (untreated) and right (bupivacaine injected) hindlimb were surgically excised and mounted between the lever arm of a servomotor (isometric mode) and an immovable bracket and secured at each tendon with braided silk suture (3–0, Pearsalls Sutures, Somerset, UK) in a custom-built Plexiglas filled with oxygenated Krebs-Ringer solution. Isometric contractile properties were assessed in vitro at 25°C by standard procedures that we have reported in detail elsewhere (1, 21). Initially, single pulses were delivered to the muscle to generate isometric twitch contractions, and the muscle lengthened in small increments until maximum twitch force was reached. For the remainder of the experiments, tetanic pulses were delivered to each muscle (EDL = 350-ms train duration and soleus = 1,200-ms duration).

A stimulation frequency-force relationship was established for each muscle (over a range of frequencies) to determine relative restoration of force production after bupivacaine injection. A length-tension relationship was then constructed for each muscle by delivering a stimulation frequency that generated peak isometric tetanic force at a range of different muscle lengths (25.0–40.0 mm for EDL muscles and 23.0–34.0 mm for soleus muscles), in 0.5-mm increments and with 2 min of rest between each stimulation, to prevent fatigue. Optimal muscle length for contraction (L0) was deemed as the muscle length at which maximal isometric tetanic force was generated (i.e., peak of length-tension curve) and was measured by digital Vernier calipers with the aid of a dissecting microscope. Length-tension curves were plotted for each muscle and described as absolute muscle length vs. absolute tetanic tension, absolute muscle length vs. relative tension, and relative length vs. relative tension.

After functional measurements, the muscles were carefully blotted on filter paper, trimmed of tendons and nonmuscle tissue, and weighed. Muscle CSA was calculated from muscle mass, optimum muscle fiber length (L0) based on previously determined Lr-to-L0, ratios of 0.44 for EDL muscles and 0.71 for soleus muscles, and also muscle density (1.06 g/cm3) (3, 12) to calculate muscle-specific force (tetanic force/muscle CSA).

**Statistical analyses.** Individual variables were compared using two-way ANOVA, with factors being bupivacaine and time followed by Fisher’s least significant difference post hoc multiple-comparison procedure. Evidence for a shift in the length-tension curve was assessed with a treatment (bupivacaine vs. control) × length repeated-measures ANOVA for isometric tetanic force at each time point after bupivacaine injection. Significance was set at P < 0.05. All values are expressed as means ± SE.

**RESULTS**

**Morphological properties.** Bupivacaine injection caused characteristic degeneration and subsequent regeneration in both EDL and soleus muscles, with the mass of EDL and soleus muscles only 50 ± 3 and 54 ± 6% of control at 7D (Fig. 1). Mass of EDL muscles was restored to control levels at 21D, and exceeded control by 27 ± 5% at 60D (P < 0.05, Fig. 1A). In contrast, the mass of the regenerating soleus muscles was not restored to control levels over the course of experiment and reached only 81 ± 2% that of control at 60D (Fig. 1B).

**Contractile properties.** Isometric tetanic force of 7D, 14D, and 21D EDL muscles was lower than control at all frequencies tested (Fig. 2A), but not different from control values at 60D (P < 0.05). In contrast, tetanic force of the regenerating soleus muscles was lower than control at all frequencies tested at each time point after bupivacaine injection (P < 0.05; Fig. 2B). Specific force (sPo, peak tetanic force/muscle CSA) of regenerating EDL muscles was also lower than control at 7D and 14D, indicating intrinsic weakness (Fig. 3A). At 21 D days after bupivacaine injection, however, sPo of EDL muscles was not different from control, but at 60D postinjection was lower than control, because of the muscle hypertrophy that was observed at this time point (P < 0.05; Table 1). The time taken to reach peak twitch force and the one-half

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**Fig. 1.** Mass of extensor digitorum longus (EDL; A) and soleus (B) muscles at 7, 14, 21, and 60 days (7D, 14D, 21D, 60D) after bupivacaine injection (solid bars) compared with noninjected control (open bars). Mass of regenerating EDL muscles was restored to control at 21D and exceeded control at 60D. In contrast, mass of soleus muscles was less than control at all time points after bupivacaine injury. *P < 0.05 different from control.
relaxation time was longer in 7D EDL muscles ($P < 0.05$; Table 1), indicating a slowing of the rate of contraction. Twitch force of regenerating soleus muscles was also lower than control at 7D, 14D, 21D, and 60D ($P < 0.05$; Table 1), and time taken to reach peak twitch force and one-half relaxation time were faster in 7D soleus muscles compared with control ($P < 0.05$; Table 1).

Length-tension relationship. Values from the uninjured control EDL and soleus muscles at each time point after bupivacaine injection were combined and designated as control length-tension curves (EDL $n = 20$, soleus $n = 20$; Fig. 4, A and B). When plotted as absolute length vs. relative force (Fig. 4C), peak force of control EDL muscles was generated at a length of $31.6 \pm 0.4$ mm ($L_o$). The length-tension curve of 7D EDL muscles was shifted leftward compared with control, with peak force generated at $L_o = 29.7 \pm 0.9$ mm ($P < 0.05$ compared with control; Table 1). The treatment $\times$ muscle length interaction ($P < 0.001$) demonstrated that 7D EDL muscles generated lower force than control at muscle lengths of 27.0–29.5 mm, but higher force than control at muscle lengths of 34.0–36.5 mm (Fig. 4C). 60D EDL muscles produced force of ~98% peak at 34.0 mm, whereas control muscles only generated ~70% peak force at the same length, indicating a clear rightward shift of the length-tension curve. Length-tension curves for control soleus muscles indicated that peak force was generated at a length of $28.5 \pm 0.3$ mm ($L_o$). Unlike bupivacaine-injected EDL muscles, there was no clear shift in the position of the length-tension curves of soleus muscles at any time point after bupivacaine injection (Fig. 4D). The treatment $\times$ muscle length interaction ($P < 0.001$), however, indicated that 60D soleus muscles produced forces 10–15% greater than control at muscle lengths of 30.0–32.0 mm.

Fig. 2. Stimulation frequency-force relationships for EDL (A) and soleus (B) muscles at 7D, 14D, 21D, and 60D after bupivacaine injection (dashed lines) compared with uninjured control (combined values from noninjected muscles; solid line). Isometric force was restored progressively over the time course of muscle regeneration.

![Graph](image1.png)

![Graph](image2.png)

![Graph](image3.png)

![Graph](image4.png)

Fig. 3. Specific force (force per cross-sectional area) of EDL (A) and soleus (B) muscles at 7D, 14D, 21D, and 60D after bupivacaine injection (solid bars) compared with noninjected control (open bars). Specific force of regenerating EDL muscles was restored to control at 21D but was lower than control at 60D, because of the increased muscle mass. Specific force of soleus muscles was restored to control at 60D, despite lower isometric tetanic force and mass. *$P < 0.05$ different from control.
When length-tension curves were plotted as relative muscle length vs. relative tetanic force, the treatment × relative muscle length interaction \((P < 0.001)\) demonstrated that in 7D, 14D, and 21D EDL muscles the ascending limb of the length-tension curve was flatter than control, with force enhanced at muscle lengths of 84.5–92.0% \(L_o\), but not different from control on the descending limb of the curve (Fig. 4E). In 7D soleus muscles, force was enhanced at muscle lengths of 84.5–92.0% \(L_o\), but in 21D and 60D soleus muscles, force was enhanced at muscle lengths of 82.0–84.0% \(L_o\) (Fig. 4F).

**DISCUSSION**

The most important finding of this study was that length-tension relationships of rat EDL and soleus muscles are altered after bupivacaine injection. At 7 days after bupivacaine injection, when the muscle is undergoing regeneration, EDL muscles generate forces of 11% of (noninjected) control and have length-tension curves shifted leftward compared with control. In contrast, 60 days after bupivacaine injection, EDL muscles are fully regenerated (mass and force production restored) and

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**Table 1.** Twitch contraction, time-to-peak twitch, one-half relaxation time, and optimum length of EDL and soleus muscles 7D, 14D, 21D, and 60D post bupivacaine injection compared with noninjected control muscles

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 5)</th>
<th>Bupivacaine (n = 5)</th>
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<tr>
<td><strong>EDL</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(P_t, \text{mN})</td>
<td>1082 ± 104</td>
<td>165 ± 21*</td>
</tr>
<tr>
<td>(TPT, \text{ms})</td>
<td>25.8 ± 1.2</td>
<td>35.2 ± 2.0*</td>
</tr>
<tr>
<td>(\frac{1}{2}\text{RT, ms})</td>
<td>20.9 ± 0.4</td>
<td>22.1 ± 1.1</td>
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<tr>
<td>(L_o, \text{mm})</td>
<td>32.2 ± 0.4</td>
<td>31.1 ± 0.8</td>
</tr>
<tr>
<td>(\Delta L_o, %)</td>
<td>-8 ± 3*</td>
<td>-3 ± 3</td>
</tr>
<tr>
<td><strong>Soleus</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(P_t, \text{mN})</td>
<td>272 ± 29</td>
<td>95 ± 14*</td>
</tr>
<tr>
<td>(TPT, \text{ms})</td>
<td>77.6 ± 2.1</td>
<td>63.1 ± 1.8*</td>
</tr>
<tr>
<td>(\frac{1}{2}\text{RT, ms})</td>
<td>127.4 ± 15.1</td>
<td>126.8 ± 6.1</td>
</tr>
<tr>
<td>(L_o, \text{mm})</td>
<td>28.1 ± 0.4</td>
<td>27.5 ± 0.5</td>
</tr>
<tr>
<td>(\Delta L_o, %)</td>
<td>-3 ± 2</td>
<td>1 ± 4</td>
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Values are means ± SE. \(P_t\), peak twitch force; \(TPT\), time to peak twitch; \(\frac{1}{2}\text{RT}\), one-half relaxation time of the twitch; \(L_o\), optimum muscle length (determined from peak of length-tetanic tension curve); \(\Delta L_o\), bupivacaine − control (%). *\(P < 0.05\) different from control (2-way ANOVA with Fisher’s least significant difference test).
have length-tension curves shifted rightward compared with control. In contrast, soleus muscles do not fully regenerate within 60 days after bupivacaine injection and do not have significant shifts in the length-tension curve during regeneration. Nonetheless, subtle changes in the steepness of the length-tension relationship are observed in soleus muscles after bupivacaine injection.

Previous studies have shown that EDL muscles are protected against contraction-induced injury 60 days after bupivacaine injection (4). Muscles are susceptible to injury when they are operating on the descending limb of the length-tension relationship. Indeed, strength loss after an initial bout of eccentric exercise is related to the ability to generate force on the descending limb of the length-tension curve (14). Data from the present study indicate that EDL muscles 60 days after bupivacaine injection generate higher force than noninjected muscles on the descending limb of the length-tension curve. Thus the ability to maintain myofilament overlap on the descending limb of the length-tension curve is a likely mechanism providing protection against contraction-induced injury observed in rat EDL muscles 60 days after bupivacaine injection (4).

Although similar protection from contraction-induced injury is observed in eccentrically conditioned muscles that also have rightward shifted length-tension curves (2, 13), the best test of this hypothesis would be to determine the correlation between the relative protection from contraction-induced injury and the relative rightward shift of the length-tension curve in the same muscle 60 days after bupivacaine injection. However, this is not possible in these isolated muscle experiments in which the determination of the length-tension relationship would impact on measurement of the same muscle’s susceptibility to contraction-induced injury.

The rightward shift in the length-tension curve of EDL muscles could be attributed to the longitudinal addition of sarcomeres (15). It is well established that an increase in muscle fiber length is mediated by sarcomeres added serially at the ends of the fibers (24). Therefore, bupivacaine-injured EDL muscles could have a greater number of sarcomeres in series than uninjured muscles. In contrast, no hypertrophy or longitudinal growth was observed in bupivacaine-injured soleus muscles, and so serial addition of sarcomeres is unlikely. Confirmation of alterations in sarcomere number would be possible with light and electron microscopy of the bupivacaine-injected muscles to distinguish between increased sarcomere number and alterations in sarcomere length. Further experiments are needed to determine whether there are alterations in sarcomere numbers and sarcomere lengths along the length of fibers from bupivacaine-injected muscles, although accurate quantification of these parameters would be difficult. Insight into understanding how a muscle’s susceptibility to contraction-induced injury is related to its length-tension relationship would also be gained by performing a lengthening-contraction protocol on regenerating soleus muscles. Although it would be unlikely that these muscles would be protected from contraction-induced injury, because there was no shift in the length-tension relationship, these experiments warrant further investigation.

Not only were changes in the position of the length-tension curves observed in bupivacaine-injected EDL muscles, subtle changes in the slope of the ascending and/or descending limb of the curve were also observed in both EDL and soleus muscles. Changes in the slope of the length-tension curve were also not uniform across the curve, with a flattening of the slope of the ascending limb of the length-tension curve observed in EDL muscles 7, 14, and 21 days after bupivacaine injection, but no change in the shape of the descending limb of these curves (Fig. 4E). This indicates that the 7-, 14-, and 21-day postbupivacaine-injected EDL muscles were able to generate greater myofilament overlap than control muscles at the same relative muscle length. In contrast, the steepness of the relative length vs. relative force curve is greater in 60-day postbupivacaine-injected soleus muscles than control, indicating reduced myofilament overlap at the same relative muscle length (Fig. 4F). The effect of these changes in the shape of the length-tension curve on relative susceptibility to contraction-induced injury has yet to be determined.

A shortcoming of these experiments is that the results do not differentiate between the mechanical aspects of loading and of regeneration. During recovery from bupivacaine injection, rats were able to walk freely about their cage, with normal mobility. Because EDL and soleus muscles have different functions during walking, they are subject to different stimuli during regeneration, which might also affect their regenerative capacity. Limb casting after bupivacaine injection might provide further insight into the relative role of mechanical loading during regeneration, although these experiments would be complicated by the resulting muscle atrophy.

Our finding of a return to control values for EDL muscle mass at 21 days postinjection, followed by accelerated muscle growth until 60 days postinjection, is consistent with previous reports (19). The magnitude of hypertrophy in 60-day postbupivacaine-injected EDL muscles observed in our study (27% increase in muscle mass) was less than observed by Rosenblatt (58% increase in muscle mass) (19), but this is likely explained by the different ages of the rats used in the these studies. Soleus muscles responded differently to the EDL muscles after bupivacaine injection, with incomplete restoration of mass and force even at 60 days after bupivacaine injection.

During the early stages of muscle regeneration (7 days after bupivacaine injection), we observed alterations in the time course of contraction, with EDL muscles displaying slowed contraction and soleus muscles exhibiting faster contraction. Our findings are similar to reports from Rosenblatt (19), who demonstrated a slowing of contraction in EDL muscles at 8 days postinjection. The altered contractile speed at 7 days postinjection is likely explained by the presence of “immature” fibers in the regenerating muscles (8).

The findings support the hypothesis that length-tension curves are rightward shifted in regenerated EDL muscles 60 days after bupivacaine injection. Unlike EDL muscles, soleus muscles regenerate more slowly after bupivacaine injection, and their length-tension curves are not left or right shifted relative to control. Nonetheless, subtle changes in the length-tension relationships of soleus muscles are observed after bupivacaine injection. We conclude that the rightward shift in the length-tension curve of EDL muscles 60 days after bupivacaine injection may contribute to the mechanism that protects newly regenerated EDL muscles from contraction-induced injury.
REFERENCES