Experimental Physiology

Raising the antioxidant levels within mouse muscle fibres does not affect contraction-induced injury

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A protocol of 75 lengthening contractions (LCP) administered to skeletal muscles of mice causes an initial force deficit owing to the mechanical disruption of sarcomeres and a reduction in calcium release from the sarcoplasmic reticulum. During the 3 days following the LCP, a 'sealing off process' and inflammatory response occurs. The reactive oxygen species (ROS) released by invading inflammatory cells produce a secondary force deficit that is more severe than the initial deficit. The timing of the infiltration of inflammatory cells and increase in force deficit relative to the sealing off process is not well documented. We tested the null hypothesis that following a lifetime of overexpression of the genes for the intracellular antioxidants manganese superoxide dismutase, copper zinc superoxide dismutase or catalase in transgenic mice, the force deficits 3 days following the administration of a 75 LCP to *in situ* extensor digitorum longus muscles are not different from those of wild-type mice. Following the LCP, the force deficits ranged from 39 to 59% for the muscles of transgenic mice that overexpressed the genes for intracellular antioxidants and were not different from the force deficit of 44% observed for muscles of wild-type mice. The results provide evidence that the ROS damage does not occur within the cytosol of the injured fibres. Apparently, the hypercontraction of sarcomeres and accumulation of vesicles seal off and protect the intact portions of damaged fibres, such that the ROS damage and repair occurs in the milieu of the necrotic segments that are continuous with the extracellular matrix.

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Immediately following a lengthening contraction protocol (LCP), the phenomenon of contraction-induced injury consists of an initial mechanical disruption of sarcomeres (Zerba *et al.* 1990; Brooks *et al.* 1995; Macpherson *et al.* 1996), an elevation in the resting levels of intracellular calcium concentration, and a reduction in calcium release from the sarcoplasmic reticulum during contractions (Balnave & Allen, 1995). Several hours to several days following the LCP, a 'sealing off process' (McCully & Faulkner, 1985; Friden & Lieber, 1998; Hamer *et al.* 2002) and a more severe secondary injury occur due to the invasion of inflammatory cells (Faulkner *et al.* 1989; van der Meulen *et al.* 1997; Pizza *et al.* 2002, 2005; Brickson *et al.* 2003). Recovery from a severe LCP is extended over several weeks (McCully & Faulkner, 1985; Faulkner *et al.* 1989; Brooks & Faulkner, 1990; van der Meulen *et al.* 1997). In contrast, when skeletal muscles of rodents are administered protocols of isometric (McCully & Faulkner, 1985; van der Meulen *et al.* 1997; Koh & Brooks, 2001) or shortening (McCully & Faulkner, 1985; Faulkner *et al.* 1989) contractions or passive lengthening (Faulkner *et al.* 1989; van der Meulen *et al.* 1997; Koh & Brooks, 2001), the force developed afterwards may be decreased owing to fatigue, but force recovers to control values rapidly and completely. The conclusion is that contraction-induced injury occurs after activities that include a predominance of lengthening contractions (McCully & Faulkner, 1985; Faulkner *et al.* 1989; van der Meulen *et al.* 1997).

The time course of the molecular events immediately following a focal injury to skeletal muscle fibres has been determined for fibres exposed to transection (Echeverria *et al.* 1987) or micropuncture (Carpenter & Karpati, 1989), but not for those exposed to a LCP. Within minutes of the initial injury produced by transection or micropuncture, an influx of calcium induces the hypercontraction of sarcomeres adjacent to the focal damage (Echeverria *et al.*

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1987; Carpenter & Karpati, 1989) and activates proteases (Jackson *et al.* 1984). The hypercontracted sarcomeres act as an initial barrier separating the viable region from the damaged segment of each injured fibre (Echeverria *et al.* 1987; Carpenter & Karpati, 1989). The hypercontracted myofibrils are replaced by the formation of a seal created by the fusion of vesicles within 1 h after transection (Echeverria *et al.* 1987) and within several hours after micropuncture (Carpenter & Karpati, 1989).

Although the sealing off process immediately following a LCP has not been well documented, the infiltration of inflammatory cells after contraction-induced injury has been investigated extensively (Zerba *et al.* 1990; Pizza *et al.* 2001, 2002, 2005). Several hours to several days following a LCP, the inflammatory cells, neutrophils and macrophages, infiltrate and release reactive oxygen species (ROS; McArdle *et al.* 1999; Pizza *et al.* 2001, 2002, 2005). The ROS degrade the mechanically damaged tissues by phagocytosis of cellular debris. For each necrotic segment, the freely permeable basement membrane remains intact but the plasma membrane is damaged, resulting in the segment becoming continuous with the extracellular matrix (ECM) and devoid of organelles (Lovering & De Deyne, 2004). During the process of degradation and phagocytosis, some previously uninjured tissues are degraded, with, at least in skeletal muscles of mice, a secondary injury of greater magnitude than the primary injury (McCully & Faulkner, 1985; Faulkner *et al.* 1989; Brickson *et al.* 2003; Pizza *et al.* 2005).

To identify the site, timing and underlying causes of the events associated with the secondary ROS-induced injury, a 75 LCP was administered to the extensor digitorum longus (EDL) muscles of mice. We tested the null hypothesis that the force deficit at 3 days after the LCP is not affected by a lifetime of overexpression of genes for intracellular antioxidants. The transgenic mice overexpressed a single gene or a combination of genes for manganese superoxide dismutase (MnSOD), copper zinc superoxide dismutase (CuZnSOD) or catalase (Raineri *et al.* 2001; Chen *et al.* 2003, 2004). Superoxide dismutase catalyses the dismutation of superoxide anion to hydrogen peroxide (Chance *et al.* 1979). Copper zinc superoxide dismutase is present in the cytosol and the intermembrane space of mitochondria (Sturtz *et al.* 2001; Okado-Matsumoto & Fridovich, 2001), whereas MnSOD is located within the mitochondrial matrix, a potential source of superoxide anions (McArdle *et al.* 2004*b*). Catalase, located in peroxisomes, cellular organelles capable of generating hydrogen peroxide (Chen *et al.* 2004), converts hydrogen peroxide to oxygen and water (Chance *et al.* 1979). Since hydrogen peroxide (Chen *et al.* 2004) and, to a lesser extent, superoxide anions (Han *et al.* 2003) are able to cross membranes into the cytosol, the overexpression of their corresponding antioxidants, catalase, CuZnSOD and MnSOD, has the potential to reduce the concentration of ROS in the cytosol. If the infiltration of inflammatory cells occurs after the completion of the sealing off process, the ROS damage would be limited to the site of initial injury that is continuous with the ECM. Under these circumstances, an increase in the levels of intracellular antioxidants would not affect the deficit in force 3 days after the LCP, and the null hypothesis would be supported.

Methods

Measurements of enzyme activities and Western blot analyses were performed to confirm the overexpression of catalase, CuZnSOD and MnSOD. To test the null hypothesis, the maximum isometric tetanic force (P_o) was measured for *in situ* EDL muscles of mice before and 10 min and 3 days after a 75 LCP to assess the magnitude of the force deficit after the LCP. Following the measurement of P_o at 3 days, histological sections of a sample of muscles were stained with Haematoxylin and Eosin to determine whether the fibre damage 3 days following the LCP was consistent with previous reports (Zerba *et al.* 1990; Koh *et al.* 2003). All procedures were approved by the University of Michigan Committee on the Use and Care of Animals and were in accordance with the guidelines of the United States Public Health Service, National Institutes of Health Publication no. 85-23.

Mice

Male wild-type (WT) and transgenic mice were obtained from the University of Texas Health Science Center at San Antonio. Four groups of the transgenic mice had hemizygous overexpression of the genes for catalase (*CAT*), CuZnSOD (*SOD1*) and MnSOD (*SOD2*) in different combinations: *Tg(SOD1)*, *Tg(SOD2)*, *Tg(SOD1,SOD2)* and *Tg(SOD1,CAT)* mice. The WT mice were littermates of the *Tg(SOD1,SOD2)* mice. In a fifth group of transgenic mice, the *Tg(CAT)* mice, the overexpression of the *CAT* gene was homozygous. The experiment was performed on mice 4–15 months of age with seven mice per group. With the exception of a marginal negative correlation between contralateral control muscle masses and age that indicated a ∼10% reduction in mass over the range of ages tested, all of the data were independent of age. The composition of each of the groups in terms of age was consistent among the six groups. The mean age for each group ranged from 7 to 10 months with standard deviations of 1–4 months. Consequently, age was not an influential factor when comparing data for the groups of transgenic and WT mice.

The *Tg(SOD1)* and *Tg(CAT)* mice were generated on a C57BL/6 background using large fragments of human genomic DNA (> 60 kb) containing the gene and the

5′- and 3′-flanking sequences (Chen *et al.* 2003, 2004). The *Tg(SOD2)* transgenic mice were generated on a C57BL/6 background using 13 kb of endogenous mouse DNA containing the gene and 2 kb of the flanking sequence (Raineri *et al.* 2001). The *Tg(SOD1,SOD2)* and *Tg(SOD1,CAT)* mice were produced by breeding the appropriate transgenic mice that overexpressed each gene. The flanking regions of the transgenes contained the regulatory sequences required for a tissue-specific expression pattern not different from the endogenous genes. The transgenic mice were bred and raised in a barrier facility at the University of Texas Health Science Center at San Antonio. The mice were shipped to the University of Michigan, where they were housed in a barrier facility in the Unit for Laboratory Animal Medicine prior to the administration of the LCP and remained in a separate barrier facility after the LCP.

Measurement of maximum isometric force

For the measurement of contractile properties and administration of the LCP, mice were anaesthetized with an intraperitoneal injection of 1.3% Avertin®, Sigma, St. Louis, MO, USA (222,-tribromoethanol, 0.015 ml (g body wt)$^{-1}$. Additional intraperitoneal injections of 0.1 ml were administered as required to maintain the depth of anaesthesia. The tendon of the right EDL muscle was exposed by making a small incision at the ankle. The mouse was placed on a platform maintained at 37°C. The hindlimb was secured by pinning the knee and taping the foot to the platform. The tendon was tied with 4–0 suture to the lever arm of a servomotor (Cambridge Technology model 305). Throughout the experiment, the tendon was left intact (McCully & Faulkner, 1985; Faulkner *et al.* 1989). The servomotor was used to control the position and measure the force developed by the muscle. Position and force were monitored using a computer program and an oscilloscope. Stainless-steel needle electrodes were inserted transcutaneously on either side of the peroneal nerve to activate the EDL muscle. The stimulation voltage and subsequently the length of the muscle were adjusted until the maximum isometric twitch force (P_t) was developed. This length was defined as optimal muscle length (L_o) and was measured based on anatomical markers. The muscle fibre length (L_f) was estimated as the product of L_o and the L_f:L_o ratio of 0.45 (Brooks & Faulkner, 1988). The frequency of stimulation was adjusted until a plateau in the isometric tetanic force indicated that P_o was achieved.

Lengthening contraction protocol

After measuring P_o, the EDL muscle was administered a protocol of 75 lengthening contractions. The first 100 ms of each lengthening contraction consisted of an isometric contraction. The maximally activated muscle was then stretched at a velocity of 1 L_f s^{-1} from L_o to $L_o + 0.20L_f$. The stimulation was stopped and the quiescent muscle was returned to L_o at the same velocity. The lengthening contractions were repeated at a rate of 0.25 Hz. Ten minutes after the injury protocol, the isometric tetanic force was measured at the same settings that produced the preinjury P_o. The incision to expose the tendon was sutured closed, and the mouse was allowed to recover from the anaesthesia. At 3 days, the mouse was anaesthetized, and P_o was determined as described above for the injured and contralateral control EDL muscles. For the muscle exposed to the LCP, force deficit was defined as the difference between pre- and postinjury P_o expressed as a percentage of preinjury P_o. Following the measurements of force, the muscle was immediately removed, and the anaesthetized mouse was killed by cervical dislocation. The injured and contralateral muscles were blotted dry and weighed. The cross-sectional area (CSA) was estimated as the muscle mass (in mg) divided by the product of L_f (in mm) and 1.06 mg mm^{-3} (Brooks & Faulkner, 1988). Specific P_o (in kN m^{-2}) was calculated as the P_o (in mN) divided by the CSA (in mm^2). Each muscle was cut into three approximately equal parts. The distal and proximal parts of each muscle were immediately frozen in liquid nitrogen and stored at −80°C for homogenization. The middle portion was covered with tissue freezing medium (TFM™) and kept frozen at −80°C for histology.

Homogenization

The muscle tissue was minced into fine pieces and homogenized (10:1 w/v) in ice-cold 20 mM Tris buffer (pH 7.4) with 0.05% Triton X-100 and proteinase inhibitor (Calbiochem, La Jolla, CA, USA). Minced muscle samples were homogenized using a ground glass homogenizer (Glas-Col, Terre Haute, IN, USA) at 4°C. Homogenized samples were frozen in liquid nitrogen and thawed in water three times, and then centrifuged at 14 000*g* at 4°C for 10 min to obtain the supernatant as muscle homogenates.

Muscle CuZnSOD and MnSOD activities

The activities of CuZnSOD and MnSOD were measured in EDL muscle homogenates using native gels as previously described (Van Remmen *et al.* 1999). Extracts containing 30 μg of protein were separated on a 10% polyacrylamide gel at 100 V in a cold room (4°C) for 4 h. Then the gel was stained in a solution containing nitroblue tetrazolium (1 ml), riboflavin (1 ml) and TEMED (N,N,N′,N′-tetramethyl-Ethylenediamine, Sigma, St. Louis, MO, USA) (50 μl) in 20 ml of potassium phosphate buffer for 15 min in darkness. The riboflavin was activated to oxidize an electron donor, TEMED. The gel image was scanned and recorded using an Alpha Imager™ 3400 (Alpha Innotech, San Leandro, CA, USA) and analysed using ImageQuant

Software (Sunnyvale, CA, USA) to quantify the intensity of the regions representing the activities.

Western blot analysis to determine catalase protein levels

The measurement of catalase activity would have been advisable, but there was less than 100 μg of protein, the minimum required for each sample. Therefore, the levels of catalase protein in the EDL muscle were determined by Western blot analysis which required only 20–50 μg of protein (Chen *et al.* 2004). Protein (50 μg) samples were boiled for 5 min and loaded into the wells of the 4–20% SDS-PAGE premade gel and eletrophoresed at 100 V using a Bio-Rad Protein III gel box. The gel was then transferred to a nitrocellulose membrane at 20 V for 45 min using Trans-Blot SD semidry transfer cell (Bio-Rad, Hercules, CA, USA). Membranes were blocked in 5% fat-free milk in TBS (Tris Buffered Saline) with 0.1% Tween-20 (TBST) for 2 h. Following blocking, membranes were incubated in TBST (4°C) overnight with a catalase antibody (1:5000; Research Diagnostics, Minneapolis, MN, USA). Following three washings using TBST, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) in TBST at room temperature for 90 min. The immunoblots were detected with an enhanced chemiluminescence (ECL) system (Amersham, Piscataway, NJ, USA), and then visualized and scanned on Typhoon 9400 (Amersham) immediately. Quantification of immunoblots was performed with ImageQuant Software.

Histology

For the estimation of fibre damage, six mice were selected from all of the mice studied and their contralateral control and injured muscles analysed using a modified method of that previously described (Faulkner *et al.* 1989). Each frozen middle portion of muscle was cryo-sectioned at a 10 μm thickness from the mid-belly of the muscle and then stained with Haematoxylin and Eosin. A single section per muscle was studied using an image analysis software package (Bioquant Imaging System, Nashville, TN, USA). The area of each of the sections, excluding any torn regions of the outer edge that resulted from the cryo-sectioning, was determined, and all of the intact fibres within each of these areas were counted. Intact fibres were defined as those with uniform staining of the cytosol and without infiltration of inflammatory cells. For the measurement of the mean area for an individual intact fibre, ~30% of the fibres were selected and analysed. Each cross-section was viewed at ×45 magnification with approximately 20 frames required to observe the entire section. An image was acquired, and the fibre areas analysed for every third frame using the Bioquant system. The percentage of the section occupied by intact fibres was determined by multiplying the mean cross-sectional area of individual fibres by the number of intact fibres and dividing by the area of the entire section. For each mouse, the difference between these percentages for the injured and contralateral control muscle was defined as the extent of fibre damage.

Statistical analyses

Values are expressed as means ± 1 S.E.M. The data for the activities of CuZnSOD did not pass the Shapiro-Wilk test for normality and therefore were analysed with the Kruskal–Wallis test. All of the other data were distributed normally. Data that passed the homogeneity of variance tests were analysed by a one-way analysis of variance and, in the event of a significant F statistic, group means were compared by the Bonferroni correction for multiple comparisons. For data with unequal variances, the Welch test for the equality of means was performed and, when significance was observed, group means were compared by Dunnett's T3 tests. The level of significance for each statistical test was set *a priori* at $P = 0.05$.

Results

For body masses, the only differences among the groups were between *Tg(SOD2)* and WT mice and between *Tg(CAT,SOD1)* and *Tg(SOD2)* mice (Table 1). The mean body mass of *Tg(SOD2)* mice was 10% less than that of WT mice and 11% less than that of *Tg(CAT,SOD1)* mice (Table 1). For the muscles of transgenic mice, exposure to the LCP did not alter the activities of CuZnSOD and MnSOD or the protein levels of catalase (Fig. 1*A–C*). Compared with WT levels, CuZnSOD activity levels of *Tg(SOD1)*, *Tg(CAT,SOD1)* and *Tg(SOD1,SOD2)* mice, MnSOD activities of *Tg(SOD2)* and *Tg(SOD,SOD2)* mice, and catalase protein levels of *Tg(CAT)* and *Tg(CAT,SOD1)* mice were twofold greater (Fig. 1*A–C*).

Despite the overexpression of antioxidants for the transgenic mice, no differences were observed among the muscles of transgenic and WT mice for the forces before the LCP or the values of mass or force deficit 10 min or 3 days afterwards (Table 1 and Fig. 2). For the pooled group of transgenic and WT mice 3 days following the LCP, the transverse sections of contralateral and injured muscles had areas of 0.96 ± 0.09 mm^2 and 1.37 ± 0.05 mm^2, contained a total number of 642 ± 70 and 712 ± 26 intact fibres, and individual fibre areas of 1367 ± 63 and 1445 ± 14 μm^2, respectively. For the muscles 3 days after exposure to the LCP, the fibre damage was 15 ± 2% (Fig. 3).

Table 1. Contractile parameters of EDL muscles administered the LCP and contralateral control muscles in WT and transgenic mice

	WT	*Tg(CAT)*	*Tg(SOD1)*	*Tg(CAT,SOD1)*	*Tg(SOD2)*	*Tg(SOD1,SOD2)*
Sample size	7	7	7	7	7	7
Body mass (g)	31.4 ± 0.8	30.9 ± 0.8	28.8 ± 0.6	31.6 ± 0.6	28.2 ± 0.7*	31.2 ± 0.7
Muscles of contralateral hindlimb not exposed to the LCP						
Muscle mass (mg)	11.3 ± 0.5	10.9 ± 0.3	10.5 ± 0.3	10.9 ± 0.2	10.5 ± 0.2	10.6 ± 0.3
L_f† (mm)	5.59 ± 0.09	5.54 ± 0.13	5.37 ± 0.07	5.44 ± 0.13	5.49 ± 0.07	5.33 ± 0.04
CSA† (mm^2)	1.95 ± 0.10	1.84 ± 0.05	1.85 ± 0.07	1.87 ± 0.02	1.81 ± 0.04	1.89 ± 0.07
P_o† (mN)	454 ± 27	417 ± 11	411 ± 22	454 ± 21	472 ± 31	456 ± 34
Specific P_o† (kN m^{-2})	233 ± 2	228 ± 9	222 ± 11	243 ± 10	261 ± 17	241 ± 13
Muscles exposed to the LCP						
Muscle mass at 3 days (mg)	12.9 ± 0.2	12.8 ± 0.4	12.3 ± 0.7	12.7 ± 0.7	12.2 ± 0.5	12.4 ± 0.7
Muscle mass at 3 days (% of contralateral muscle mass)	116 ± 7	117 ± 4	117 ± 4	117 ± 5	117 ± 7	117 ± 6
P_o before the LCP (mN)	463 ± 25	412 ± 20	438 ± 16	435 ± 27	442 ± 21	448 ± 13
P_o at 10 min (mN)	213 ± 26	242 ± 32	200 ± 12	224 ± 31	194 ± 12	242 ± 38
P_o at 3 days (mN)	262 ± 37	259 ± 39	203 ± 28	255 ± 44	179 ± 24	261 ± 36

Values are means ± S.E.M. EDL, extensor digitorum longus; LCP, lengthening contraction protocol; L_f, optimal fibre length; CSA, muscle cross-sectional area; and P_o, maximum isometric tetanic force. The CSA and specific P_o could not be determined at 3 days because of the oedema as a result of the inflammatory response. † Sample sizes for the parameter were 3 for WT mice, 5 for *Tg(SOD2)* mice and 6 for the remaining groups rather than 7 because for some of the contralateral control muscles either the measurment was not made or the peroneal nerve or muscle was damaged while preparing for the measurement. * Different ($P < 0.05$) from values for WT and *Tg(CAT,SOD1)* mice. No other differences ($P \leq 0.05$) were observed among the values for the different groups of mice.

Discussion

The values for preinjury P_o, fibre damage, and initial and secondary force deficits of the WT and transgenic mice were consistent with data reported previously for *in situ* EDL muscles of WT mice before and after a 75 LCP (Zerba *et al.* 1990; Koh & Brooks, 2001; Koh *et al.* 2003; Koh & Escobedo, 2003). The ineffectiveness of the overexpression of antioxidants to reduce the initial force deficit was consistent with an initial mechanical injury to sarcomeres and reduced calcium release without any damage attributable to ROS (Zerba *et al.* 1990; Brooks *et al.* 1995; Balnave & Allen, 1995; Macpherson *et al.* 1996; van der Meulen *et al.* 1997). Three days after the LCP, the lack of any differences between the force deficits of muscles of transgenic and WT mice supported the null hypothesis that the presence of greater levels of intracellular antioxidants had no effect on the magnitude of the secondary ROS-induced injury. For the transgenic mice, three different genes of intracellular antioxidants were overexpressed in various combinations, and the levels of each of these antioxidants were increased twofold compared with those of WT mice. Consequently, the ineffectiveness of the overexpression was unlikely to be a result of deficient antioxidant levels. Rather, the results provide compelling evidence that the secondary ROS-induced injury did not occur at the intracellular locations where the antioxidants resided (Zerba *et al.* 1990; van der Meulen *et al.* 1997). The most valid interpretation of the data is that the undamaged portions of each injured muscle fibre were sealed off from the necrotic segments, segments that are contiguous with the ECM, prior to the release of ROS by inflammatory cells. The completion of sealing off prior to the influx of ROS resulted in the isolation of ROS-induced damage to the necrotic segments and ECM. Consequently, the intact segments of the fibre were not involved, and the overexpression of the genes for intracellular antioxidants had no bearing on either the magnitude or the time course of the damage.

The underlying mechanisms of sealing off have been investigated previously in studies of micropuncture (Carpenter & Karpati, 1989; Eddleman *et al.* 2000; McNeil *et al.* 2000) or transection (Echeverria *et al.* 1987). Following micropuncture (Carpenter & Karpati, 1989) or transection (Echeverria *et al.* 1987) of muscle, an influx of extracellular calcium into the cytosol leads to the hypercontraction of sarcomeres proximal and distal to the focal damage of injured fibres. The process of vesicle accumulation and fusion that follows hypercontraction has been studied most extensively in sea urchin eggs (McNeil *et al.* 2000) and axons (Eddleman *et al.* 2000). For the resealing of membrane disruptions 1–1000 μm^2, the influx of calcium activates the fusion of yolk granules in sea urchin egg to rapidly, within seconds, form a 'patch' that with exocytosis subsequently fuses with the surface membrane (McNeil *et al.* 2000). In contrast, neuronal resealing of disruptions requires endocytosis of plasma membrane to form vesicles and the gradual (15–60 min) plug-like accumulation of the vesicles at the disrupted site (Eddleman *et al.* 2000). Whether sealing off after contraction-induced injury of muscle fibres requires exocytosis of a 'patch' of fused vesicles (McNeil *et al.* 2000) or endocytosis of the plasma membrane and gradual

accumulation of vesicles (Eddleman *et al.* 2000) is not clear. What is apparent from the reports regarding sea urchin eggs (McNeil *et al.* 2000) and axons (Eddleman *et al.* 2000) is that the sealing off process is rapid; therefore, following a LCP, sealing off could potentially precede the inflammatory cell infiltration.

The lack of any protection through the overexpression of intracellular antioxidants was in contrast to findings regarding an extracellular antioxidant, polyethylene glycol-superoxide dismutase (PEG-SOD; Zerba *et al.* 1990). Treatment with the extracellular PEG-SOD reduced the secondary injury by 50% (Zerba *et al.* 1990). Polyethylene glycol-superoxide dismutase is a large, 100 kDa, molecule that does not readily diffuse across lipid membranes (Beckman *et al.* 1988). Therefore, this finding established that ROS damage occurs in the ECM or in portions of the fibres rendered continuous with the ECM because of disruption of the plasma membrane. The study did not clarify whether the sealing off process was rapid enough to limit the extent to which a fibre becomes continuous with the ECM before the arrival of the inflammatory cells. When the previous results are considered along with the present data, the sealing off process is likely to have confined the action of the PEG-SOD to the injured fibre segments and the contiguous ECM, where the inflammatory cells and the damaging ROS originated.

The data of the present study provided evidence that was indirect and supportive, rather than conclusive for a rapid sealing off process. Following transection (Echeverria *et al.* 1987) and micropuncture (Carpenter & Karpati, 1989), electron micrographs provided conclusive evidence for the timing of sealing off. This evidence was possible because the location of the focal injury was controlled

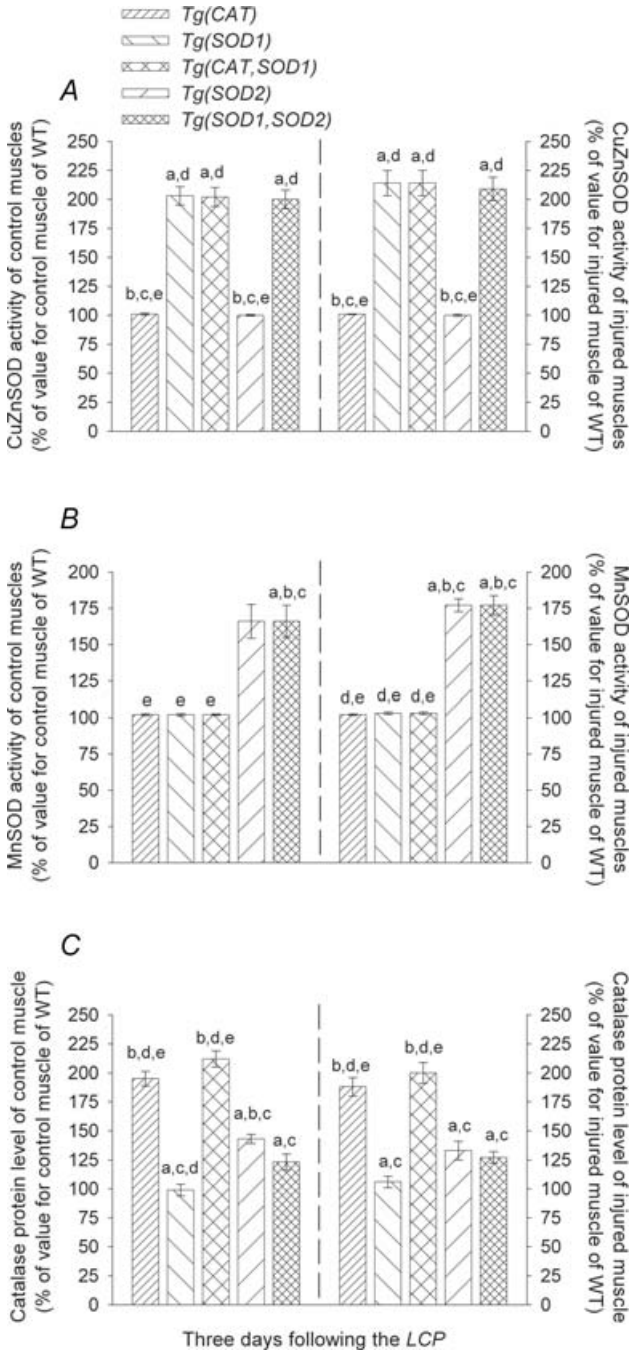

Figure 1. CuZnSOD activity levels (*A*), MnSOD activity levels (*B*) and catalase protein levels (*C*) of contralateral control muscles and injured muscles for transgenic mice 3 days after the LCP
Values, means ± S.E.M. ($n = 4$ per group), are expressed as percentages of the values for the WT mice. [a,b,c,d,e] Value different ($P \leq 0.05$) from data for [a]*Tg(CAT)*, [b]*Tg(SOD1)*, [c]*Tg(CAT,SOD1)*, [d]*Tg(SOD2)* or [e]*Tg(SOD1,SOD2)* mice.

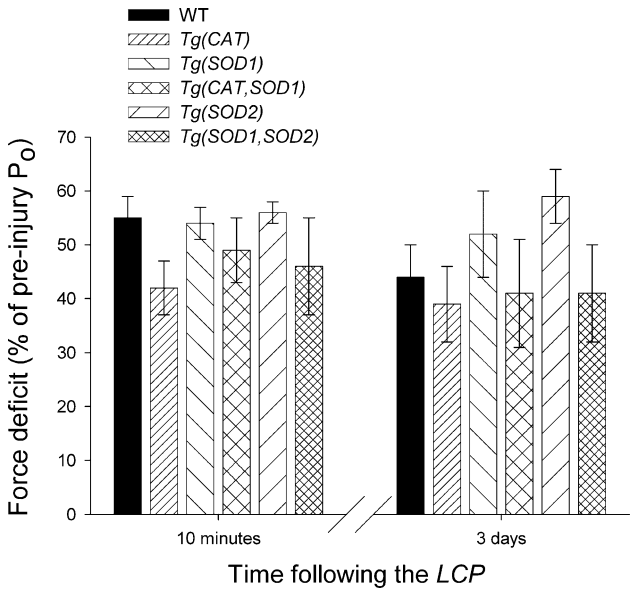

Figure 2. Force deficits of EDL muscles 10 min and three days after the 75 LCP for WT and transgenic mice
Values are plotted as means ± S.E.M. (n = 7 per group) No differences ($P \leq 0.05$) were observed among the values for the different groups of mice at each time point.

by the investigator and the investigator could observe the sealing off process at the site of injury. In contrast, for the present study and prior investigations of contraction-induced injury, the damage from contraction-induced injury was dispersed throughout the length and breadth of the muscle (McCully & Faulkner, 1985). Consequently, the monitoring of the precise timing and location of sealing off was not possible. Despite these limitations, the existence of a sealing off process was supported in prior studies (Komulainen *et al.* 1998; Friden & Lieber, 1998; Hamer *et al.* 2002). The present study was unique and valuable in that the data supported a proposal regarding the timing and necessity of sealing off relative to the inflammatory response.

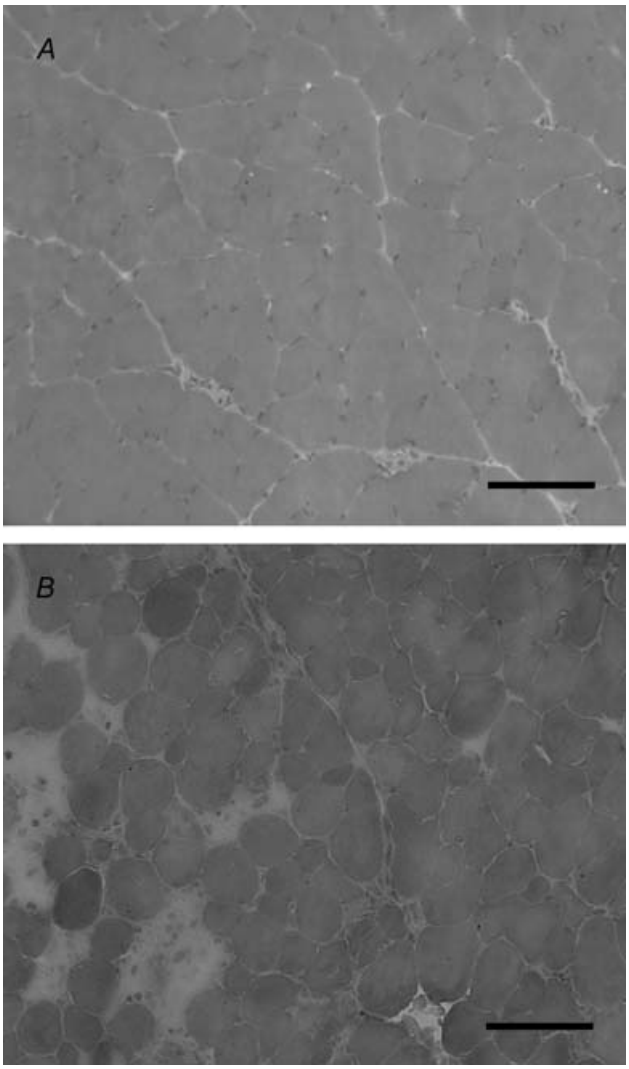

Figure 3. Representative transverse sections of the EDL muscles of WT mice
The sections are from a contralateral control muscle (*A*) and a muscle 3 days after the 75 LCP (*B*). Sections were stained with Haematoxylin and Eosin. Scale bars represent 100 μm.

The results of the present study, considered with data from past reports, are suggestive of the following sequence of events after a severe LCP (Fig. 4). An influx of calcium as a result of membrane ruptures (Lovering & De Deyne, 2004) activates proteases (Jackson *et al.* 1984) and induces hypercontraction of myofibrils within minutes of the severe focal injury (Echeverria *et al.* 1987; Carpenter & Karpati, 1989). The hypercontracted myofibrils begin to be degraded while vesicles accumulate at the borders between the viable and necrotic segments (Carpenter & Karpati, 1989). Neutrophils and macrophages infiltrate

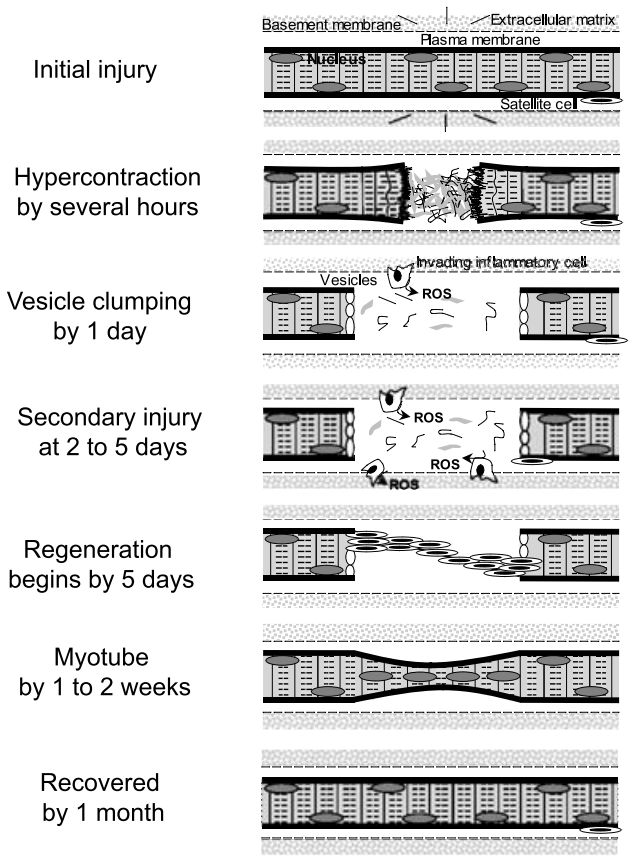

Figure 4. Schematic diagram of the sequence of events for a typical muscle fibre following a severe LCP
Within several hours following focal injury, the plasma membrane is damaged, an influx of calcium activates proteases intrinsic to the muscle fibre, and myofibrils hypercontract, resulting in a zone of necrosis. The freely permeable basement membrane remains intact. By 1 day, the hypercontracted myofibrils degenerate while vesicles accumulate to seal off the viable portions from the necrotic segments of the fibre. Neutrophils infiltrate at this time. Between 2 and 5 days, macrophages infiltrate, releasing more cytotoxic substances such as ROS that break down damaged tissue further, as well as previously uninjured tissue, resulting in a secondary injury. Satellite cells migrate to the site of injury. At 5–30 days, satellite cells proliferate and fuse across the necrotic segment so that recovery takes place. With permission of the McGraw-Hill Companies, the figure is reproduced with modifications based on a previously published figure (Bischoff, 1994).

the injury site and release ROS (Pizza *et al.* 2002). The result is the accumulation of ROS exclusively in the ECM and necrotic portion of the fibre. By 1 week after a protocol of lengthening contractions, myoblasts and myotubes are present (McCully & Faulkner, 1985) and the demarcating membranes fuse with the muscle precursor cells so that regeneration across the necrotic segment occurs (Carpenter & Karpati, 1989). Normal mechanical function of muscle fibres is gradually restored over a period of several weeks (Brooks & Faulkner, 1990; Faulkner *et al.* 1995; McArdle *et al.* 2004*a*). A greater understanding of the role of the sealing off process in limiting the extent of damage following lengthening contractions may be useful for improving the recovery from contraction-induced injury. The focus should be on antioxidants that function in the ECM or on therapeutic agents that target the sealing off process rather than events restricted to the cytosol.

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