Identification of a zebrafish model of muscular dystrophy

David Bassett* and Peter D. Currie†

**Institute of Human Genetics, Centre for Life,*
University of Newcastle upon Tyne,
Central Parkway, Newcastle upon Tyne, NE1 3BZ, United Kingdom
and
†Victor Chang Cardiac Research Institute,
384 Victoria St., Darlinghurst 2010,
Sydney, Australia

Summary

1. Large-scale mutagenic screens of the zebrafish genome have identified a number of different classes of mutations that disrupt skeletal muscle formation. Of particular interest and relevance to human health are a class of recessive lethal mutations in which muscle differentiation occurs normally but is followed by tissue-specific degeneration reminiscent of human muscular dystrophies.

2. We have shown that one member of this class of mutations, the *sapje* (*sap*), results from mutations within the zebrafish orthologue of the human *Duchenne muscular dystrophy* (*DMD*) gene. Mutations in this locus cause Duchenne or Becker muscular dystrophies in human patients and are thought to result in a dystrophic pathology by disrupting the link between the actin cytoskeleton and the extracellular matrix in skeletal muscle cells.

3. We have found that the progressive muscle degeneration phenotype of *sapje* mutant zebrafish embryos is caused by the failure of somitic muscle attachments at the embryonic myotendinous junction (MTJ).

4. Although a role for dystrophin at MTJ's has been postulated previously and MTJ structural abnormalities have been identified in the Dystrophin-deficient, *mdx* mouse model, *in vivo* evidence of pathology based on muscle attachment failure is thus far lacking. Therefore the *sapjre* mutation may provide a model for a novel pathological mechanism of Duchenne muscular dystrophy and other muscle diseases. In this review we discuss this finding in light of previously postulated models of Dystrophin function.

Introduction

Muscle development in the zebrafish

A number of attributes of the zebrafish embryo and larvae lend themselves to the study of skeletal muscle development. Zebrafish embryos develop externally, are optically transparent and are therefore accessible to *in vivo* embryological manipulations. As zebrafish employ precocious motor locomotor strategies, generating muscle load even before the completion of the first 24 h of development, mutations that disrupt muscle development are easily identifiable in large-scale mutagenic screens. Both embryological and genetic studies have taken advantage of these qualities to examine early stages of muscle development in the zebrafish with a particular focus on the mechanisms utilised to determine the different fibre types present within the embryonic myotome[1]. However, until recently the later stages of muscle development have been relatively little studied.

Axial muscle in fish initially forms from segmented paraxial mesoderm, the somites, which in turn give rise to the myotomes. In zebrafish, the different classes of muscle fibres, slow and fast twitch, are topographically separable in the embryonic myotome. The most medial cells of the forming myotome are specified by midline derived signals to form exclusively slow-twitch fibres. These cells subsequently migrate from their medial origin to traverse the entire extent of the myotome to form a subcutaneous layer of slow twitch muscle. The remainder of the myotome differentiates as fast twitch fibres behind this migration[2]. Regardless of fate or position within the myotome, muscle fibres initially differentiate to span an entire somite in the anterior-posterior axis (Fig. 1A). The somite adopts its distinctive chevron shape early on, by 24 hours post fertilisation (hpf), with the dorsal and ventral halves being separated by a sheet of extracellular matrix called the horizontal myoseptum and each pair of adjacent somites being separated by the vertical myoseptum which is similarly constructed (see Fig. 1A). The myosepta serve as the attachment sites for somitic muscle fibres. These muscle attachment sites have now come under the spotlight with the finding that their mechanical failure is the pathological mechanism in a zebrafish mutation that provides the first zebrafish model of an inherited disease of skeletal muscle.

A novel mechanism of pathology in a model of muscular dystrophy

The zebrafish dystrophic class mutants and human muscular dystrophy

Large-scale genetic screens in zebrafish have identified a large number of mutants that affect muscle formation, with one class showing a very specific degeneration of skeletal muscle[3]. Preliminary investigations revealed that mutations at several independent loci share the broad phenotype of developing visible lesions in the trunk muscle during the second day of development which gradually accumulate until the animals

die before reaching adulthood, a phenotype superficially similar to muscular dystrophy in humans. Consequently, this class of mutations have been identified by the authors as the "Dystrophic class mutants"[4].

In humans, muscular dystrophy is most often caused by mutations within genes encoding components of the Dystrophin associated protein complex (DAPC) which is a multi-protein assembly that provides a transmembrane link between the cytoskeleton and the extracellular matrix[5,6]. The complex consists of several sub-complexes, with the main structural link being provided by a series of three proteins that attach intracellular F-actin via the sarcolemma to laminin outside the cell. The laminin receptor within the DAPC is dystroglycan, which is formed by the cleavage of a precursor protein into extracellular α and transmembrane β subunits. Dystrophin is a large rod-like protein related to the spectrin family, which binds to β-dystroglycan at its C-terminus and to actin filaments via its N-terminus. A second sub-complex of the DAPC is comprised of a group of related transmembrane proteins called sarcoglycans, a third is based on syntrophin proteins and nitric oxide synthase, and several further proteins are known to associate with the complex. The DAPC is found at the membrane of skeletal muscle fibres and several other cell types in the body, while a similar complex in which dystrophin is replaced by the related protein utrophin is distributed widely throughout the body.

Dystrophin is the product of the DMD or Duchenne Muscular Dystrophy gene, which is responsible for a spectrum of X-linked conditions including Duchenne and the milder Becker muscular dystrophies, cardiomyopathies and mental retardation[7,8]. Although the exact pathological consequence of dystrophin loss has yet to be elucidated, the structural model of dystrophin function suggests that Duchenne muscular dystrophy results from sarcolemmal tearing during muscle contraction. This consequently leads to a cycle of death and replacement of muscle fibres which results in an accumulation of scar tissue in the muscle and a gradual loss of function and eventually to death[9,10]. Furthermore, many other muscular dystrophies and congenital myopathies are caused by mutations affecting other components of the DAPC complex, such as a congenital dystrophy linked to laminin-α2, and type-2 limb girdle muscular dystrophies (LGMD2) some of which are linked to sarcoglycans, calpain 3, caveolin 3 and dysferlin[11-15].

The sapje dystrophic class mutant results from mutations within the zebrafish (Zf) orthologue of dystrophin

An analysis of the expression of DAPC components within our zebrafish "dystrophic class" mutants has revealed loss of specific DAPC proteins within individual mutants. Within muscles of the zebrafish mutation *sap*, using antibodies raised against the mammalian dystrophin, which we have shown cross react with zf-dystrophin, we have found that zf-dystrophin is lost from the end of muscle fibres, confirming the class of muscle degeneration mutants may be valid genetic models of human muscular dystrophy phenotypes[4] (Fig. 1B and C). Histological examination and confocal imaging of skeletal muscle expressing green fluorescent protein (GFP) showed that the lesions occur where the ends of *sap* mutant muscle fibres become separated from their attachment sites (Fig. 1C and D). Many fibres are seen to detach at one end and contract to a fraction of their original length, showing compression or even collapse of the sarcomeric banding (Fig1E and F). Furthermore, electron microscopy has revealed nuclear condensations within detached fibres indicating that these cells are undergoing cell death, a process not evident within intact neighbouring cells. A subset of these detached fibres take up the vital dye Evans Blue, which only enters cells with compromised plasma membranes, indicating that some membrane tearing does occur (Fig. 1 G and H)[4]. Thus, despite sharing the phenotype of muscle degeneration at the whole organism level at the cellular level, the pathology of *sap* mutant zebrafish is different to the pathological process that is currently thought to be involved in human muscular dystrophies, where membrane damage occurs along the length of the fibre In zebrafish, the DAPC is localised embryonically to the ends of muscle fibres before it becomes detectable at the sarcolemma, suggesting that loss of the complex might compromise muscle attachments and possibly allowing detachment of the kind seen in *sap*. This is a surprising finding because this has not been reported in any human muscular dystrophy, and even in mouse mutants that show ultra-structural abnormalities of the cytoskeleton at the MTJ, there have been no reports of actual failure occurring *in vivo*[16-20].

By mapping analysis and mutation detection we have shown that *sap* is mutated at the zebrafish orthologue of the human Duchenne muscular dystrophy locus which encode zf-dystrophin. This finding therefore reveals a novel functional requirement for the DAPC in the stability of muscle attachments. We have identified a nonsense mutation within the N-terminal actin-binding domain of dystrophin that removes the large muscle-specific isoform and causes a progressive fatal muscle degeneration. Homozygous mutant *sap* embryos possess a far more severe phenotype than the mouse dystrophin mutant *mdx*, showing the same progression to early lethality as the human disease, perhaps because both human and zebrafish lack the regenerative capacity and compensatory levels of Utrophin that are thought to protect *mdx* mice[21-23]. Utrophin is not found at embryonic muscle attachments in zebrafish, and is absent from the non-specialised sarcolemma along the length of muscle fibres during embryonic development, but is present in the skin and pronephros[4]. This lack of either Dystrophin or Utrophin in embryonic muscle perhaps makes *sap* mutant embryos most similar to mouse models thought to lack any functional DAPC link, notably the laminin-α2 (*dy*), dystroglycan and *mdx*/utrophin double mutants[21-25]. In these mice, the MTJ lacks folding almost completely and may be weaker than in *mdx* animals, but it is unclear whether it ever fails completely. Only very slight folding of the sarcolemma is present in wild-type zebrafish muscle[26], indicating that *sap* resembles these mouse models in its anatomical details, and that the complex involutions of

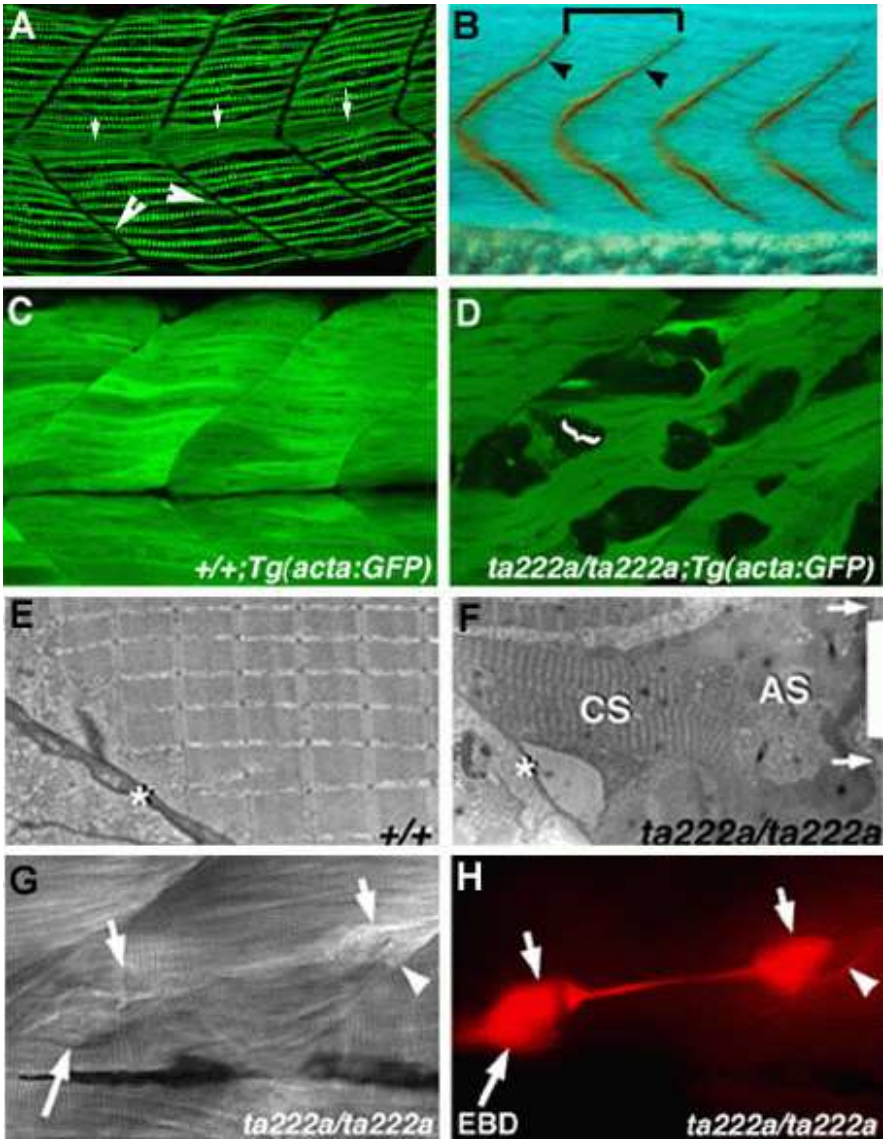

Figure 1. Phenotype of muscle detachment in* sapje *homozygote embryos.
A. Muscle fibres (Green) initially span the entirety of the myotome to attach to the vertical myosepta (Large arrows). The myotome is also bisected by the vertical myosepta, which separates the dorsal and ventral halves of the myotomes (Small arrows). Lateral view of a 24 hpf embryo stained with an antibody against slow MyHC. B. Dystrophin expression (yellow) is found exclusively at the end of muscle fibres at the vertical myosepta[4]*. C, D. Confocal microscopy of GFP expressed in C, Wildtype and D, sap homozygote muscle. In D is an example of muscle fibres that detach from the vertical myosepta in* sap *homozygotes. Fibres within* sap *homozygotes (D) exhibit a club-like or faceted appearance at their newly detached membranes, not evident in wild type embryos (C). E, F. Electron microscopy shows that wild type embryonic myofibrils align to form a regular sarcomeric array that attaches obliquely to the myosepta (asterisks (E). In* sap *homozygotes, fibres showing detached ends (arrows in F) and shortening of both the entire fibre and the sarcomeres, are visible. In these cells, the separation and regularity of sarcomeric banding is greatly reduced or collapsed compared to that in intact neighbouring cells, and absent in some places. G, H.* In vivo *observation of muscle attachment failure and molecular analysis of detached free ends. A single fibre (G, H short arrows) viewed in vivo in the process of detaching myosepta, under differential interference contrast (lateral view, 5 days post-fertilisation) and labelled with Evans blue dye. (H). A gap is visible between the separating posterior end of the fibre (right short arrow) and the myoseptum (arrowhead). A narrowed retraction zone has formed where the contractile apparatus has withdrawn from the centre of the fibre (between the short arrows). The anterior end of the fibre (left short arrow) is partly obscured by a second dye-positive detached cell (long arrow).*

the mammalian MTJ may have evolved to withstand the rigours of life on land. A similarly severe loss of the mechanical function might occur in the merosin (laminin-α2) deficient congenital muscular dystrophies[27], a group of very severe early muscle disorders, raising the possibility that such diseases might involve MTJ defects in human muscle.

Within some mammalian muscles, dystrophin is also enriched at specialised myomuscular junctions (MMJs) that also transmit force between the ends of muscle fibres[28]. These occur as either intrafascicular fibre terminations, connecting single fibres into networks both end-to-end and end-to-side[29], or as fibrous sheets called tendinous intersections that separate segmented blocks of non-overlapping fibres. These, in particular, bear a striking structural resemblance to the dystrophin-dependent attachments between somites in zebrafish[29,30]. If MMJ failure was a significant factor in mammalian muscle disease, their differential utilisation might contribute to the observed variations in pathology between individual muscles affected in muscular dystrophies, and between humans and the different dystrophic animal models.

As well as accurately representing the progressive nature of DMD, *sap* closely resembles known Duchenne-causing nonsense mutations in the N-terminal, making *sap* a new model of the disease and raising the possibility that muscle attachment failure contributes to the pathology of either Duchenne or other muscular dystrophies. The known localisation of the DAPC to this site is consistent with this, but it seems possible that such pathology might so far have been overlooked, as muscle biopsies are often deliberately taken from sites at a distance from the tendon in order to simplify histological examinations. It remains to be seen to what extent this novel requirement for the DAPC in the stability of muscle attachments might contribute to human muscle diseases, but it might be prudent to re-examine these structures, especially myomuscular junctions.

Future research directions for zebrafish models of muscular dystrophy

The discoveries outlined here provided us with the tantalising possibility of applying the sophisticated embryological and genetic methodologies afforded in zebrafish to the study of the human dystrophic conditions. In particular, applying second site enhancer and suppressor screens to identify genes that may act to modulate the dystrophic condition is a particularly promising research direction. Furthermore, the molecular defects present within the remainder of the class of "dystrophic" mutants has yet to be elucidated, and it remains likely that these may represent potentially novel genes that may also be mutated in human muscular dystrophies.

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Author for correspondence:
Assoc. Prof. P.D. Currie
Victor Chang Cardiac Research Institute,
384 Victoria St.,
Darlinghurst
NSW 2010, Australia

Phone: +61 2 9295 8536
Fax: +61 2 9295 8537
Email: p.currie@victorchang.unsw.edu.au

Popping sarcomere hypothesis explains stretch induced muscle damage

David L. Morgan & Uwe Proske

Departments of Electrical Engineering and Physiology,
Monash University,
Clayton, Vic. 3800, Australia

Summary

1. Exercise that involves stretching a muscle while active cause microscopic areas of damage, delayed onset muscle soreness, and adaptation to withstand subsequent similar exercise.

2. Longer muscle lengths are associated with greater damage, and recent animal experiments show that it is the length relative to optimum that determines the damage.

3. In humans walking down stairs, taking two at a time increases the length of the muscle during the lengthening and increases the delayed onset muscle soreness.

4. The observed pattern of damage is consistent with explanations based on sarcomere length instabilities

5. The pattern of adaptation is consistent with the number of sarcomeres in series in a muscle being modulated by exercise, especially the range of muscle lengths over which eccentric exercise regularly occurs.

Muscle stretch

When muscle is stretched during active tension generation, the event is commonly, though not intuitively, described as an eccentric contraction. Other terms include pliometric contraction, or active stretch. The muscle is being forcibly lengthened while trying to shorten. Energetically the muscle is absorbing work, not performing it. In common terms, the muscle is being used as a brake, not a motor.

Eccentric contraction is an important function of muscle, occurring during activities ranging from lowering a load to walking down hill. It is present in many forms of exercise, such as running, particularly when down hill is included, horse-riding and skiing, where the action could be better described as shock absorbing rather than braking. Eccentric contractions seldom occur in cycling, rowing or swimming.

Stretch induced muscle damage.

It is a long standing observation that "unaccustomed" eccentric exercise can lead to stiff and tender muscles next day, known as Delayed Onset Muscle Soreness, or DOMS[1]. It has become apparent in recent years that "unaccustomed" can mean at unaccustomed length, as well as involving an unaccustomed number of repetitions or unaccustomed forces[2,3]. This is a key observation that will be reinforced later. DOMS is accompanied by microscopic muscle damage, with multiple areas of damage scattered throughout the muscle, but each confined to a single fibre[4]. The maximum force is also reduced, the dependence of force on stimulation rate is changed[5], and the optimum length is immediately shifted to longer lengths[6]. In some experiments, usually in frog fibres, the tension at long length has been shown to increase, making the changes in activation unable to fully account for the shift in optimum length[6].

Perhaps most importantly, eccentric exercise produces a rapid adaptation, so that a second similar bout of exercise produces substantially less soreness and injury[7].

Popping sarcomere hypothesis

The popping sarcomere hypothesis[8] states that stretch induced muscle damage results from very non-uniform lengthening of sarcomeres when active muscle is stretched beyond optimum length. If sarcomeres are beyond optimum length, then the longest sarcomeres will be the weakest, and so will be stretched more rapidly than the others, and so become weaker, until rising passive tension compensates for falling active tension. For at least some muscles, this corresponds to lengths beyond filament overlap. (The situation in other muscles is unclear, depending on the origins of the resting tension.) As the weakest sarcomeres are not at the same point along each myofibril, this non-uniform lengthening leads to shearing of myofibrils, exposing membranes, especially t-tubules, to large deformations. This is thought to lead to loss of calcium ion homeostasis, and hence damage, either through tearing of membranes or opening of stretch activated channels[9]. It is postulated[8] that the adaptation to eccentric exercise consists of increasing the number of sarcomeres in series, so that a given muscle length corresponds to a shorter sarcomere length. In particular, the adapted muscle confines eccentric activity to muscle lengths less than optimum.

Optimum length

For a simple muscle where all fibres have the same number of sarcomeres in series, the optimum length for tension generation during maximal activation will be given by the length of tendons, plus the product of the number of sarcomeres in series and the optimum length of a sarcomere. For a muscle containing fibres with a range of the number of sarcomeres in series, the above relation will approximately hold if the number of sarcomeres in series is replaced with the average number of sarcomeres in series. As the optimum length of a sarcomere is fixed, and tendons are slow to remodel, shifts in the optimum length with exercise that occur within a few days can be confidently assigned to changes in the number of sarcomeres in series.

In the popping sarcomere hypothesis, the optimum

muscle length takes on three very important roles. In the first, optimum length becomes a prime determinant of the susceptibility; damage is predicted to occur only when sarcomeres are used beyond optimum length. In the second, the immediate shift in optimum length after eccentric exercise is a measure of damage, which is largely specific to eccentric exercise damage, and independent of fatigue or damage that involves fibres ceasing to contract, both of which contribute to force drop. Here it is proposed that the immediate shift indicates the number of overstretched sarcomeres, an early stage in the process leading to DOMS. Experimentally, the shift in optimum is closely correlated with the fall in tension, but typically shows less scatter[2]. In its third role, the position of the optimum is a measure of adaptation. A muscle is expected to be protected from injury if the optimum length is near to or beyond the maximum length at which the muscle undergoes eccentric contraction.

The importance of long length in determining damage has been clearly demonstrated in frog sartorius muscles, rat vastus intermedius, and cat gastrocnemius. Furthermore it has been shown that damage to an individual motor unit within a muscle undergoing eccentric exercise depends on that motor unit's optimum length[10].

Walking down stairs two at a time

A recent unpublished experiment from our laboratories also showed a dependence of damage on length for human knee extensor muscles. When we walk down stairs, the knee extensors of the supporting leg undergo an eccentric contraction as they support the body as it is lowered to place the extended leg on to the next step. This does not normally cause muscle soreness, as it is an everyday exercise. Descending the same distance two steps at a time causes the muscles to undergo eccentric contractions at an unaccustomed longer length, while still generating the same forces and absorbing the same amount of energy.

Twenty one students walked down ten flights of 24 stairs with instructions to land on their heels rather than their toes. For alternate flights, they stepped down one step with leg A leading, and then brought leg B alongside, so that leg B absorbed all the energy of descending one flight in 24 eccentric contractions at short length, while the knee extensors of leg A were essentially inactive. For the other flights, they stepped down two steps with leg B leading, and then brought leg A alongside, so that A was the supporting leg that underwent eccentric contraction at long length. The same amount of energy was absorbed as for the other flights, but spread over only 12 eccentric contractions extending to longer muscle length. The relations between left and right, dominant and non-dominant, and A and B were randomized.

Subjects rated soreness in their left and right quadriceps muscles twice daily for the next week on a scale of 0 to 10, representing no pain and extreme pain respectively. Most experienced only mild pain. Figure 1 shows that the mean pain rating for leg A, the support leg during the double step, reached 2 between 24 and 48 hours, but leg B only reached 0.4. A General Linear Model of the 616 pain ratings was carried out for the following factors. The time of measurement was tested as a discrete variable, due to the rise and fall of pain with time, and reached $p<0.0001$. The time course is shown in Figure 1. The comparison between the A and B legs, that is whether the leg had been supporting the body during single or double stepping had $p<0.0001$, with the leg that was stretched to longer lengths showing more pain. The length of the subjects' legs, measured as the height of the iliac crest above the floor was significant as a linear factor with $p<0.003$, with taller people experiencing less pain, presumably because the fixed step requires smaller angles of flexion for taller people. Subjects also graded their regular participation in eccentric and concentric exercise on a five point scale. From these, an exercise factor was calculated as the difference between the index of participation in eccentrically biased exercise and the index for concentrically biased exercise. This showed $p=0.0001$, with high scores showing less damage. Gender had $p<0.0001$ when leg length was removed as a factor, but this became $p<0.02$ when leg length was included, suggestion that gender primarily acted through height. Weight was not a significant continuous factor. Measurements of optimum length were not made.

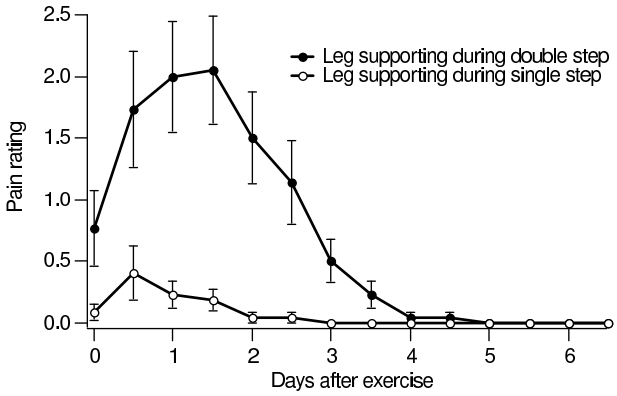

Figure 1. *Mean with SEM of pain ratings of knee extensor muscles after walking down stairs supported by one leg for five flights taken one step an a time, and the other leg for five flights taken two steps at a time. The larger steps stretched the muscles to longer lengths and produced more soreness.*

These results are all in accord with the hypothesis that damage only occurs when muscle is actively stretched beyond its optimum length, and that muscles adjust their optimum length by adjusting the number of sarcomeres so that active stretch normally occurs only at lengths less than optimum.

Sarcomere addition as a protective mechanism

The idea that adaptation to eccentric exercise consists of adding sarcomeres in series in fibres has a number of supporting observations. It ties in closely with the observations that damage depends critically on the range of lengths of the stretch compared to optimum. It also explains why biopsy studies have been unable to show differences with adaptation, as the sarcomeres are unchanged. It is consistent with previous observations that the number of sarcomeres in series is capable of relatively rapid change[11], though the mechanisms are yet to be fully elucidated. It also gives a reason for the reversal of training. Extra sarcomeres in series increase the energy consumption for isometric force development. This provides an incentive to shed un-needed sarcomeres in the interests of efficiency of tension generation. Extensive exercise involving only concentric contractions would be expected to increase this, supporting the anecdotal observation that "couch potatoes" are less prone to eccentric exercise damage to knee extensors than regular cyclists. In this view, the number of sarcomeres is continuously modulated up or down to maximise efficiency while avoiding damage during "normal" activity. Investigating the mechanisms of the adaptation is beyond our expertise.

Evidence for sarcomere number modulation

Direct evidence for sarcomere number modulation has come from rat vastus intermedius muscles, the postural knee extensors[12]. Rats were trained by running on an climbing or descending treadmill for about 20 mins per day for five days, in a protocol that had been previously shown to cause damage to these muscle in the descending group but not the climbing group[13]. The muscles were fixed and then digested in acid. Intact fibres were identified in serial dilutions of the digested muscle, and the number of sarcomeres estimated from fibre length and sarcomere length, measured by laser diffraction. From these the number of sarcomeres in fibres was calculated. All groups had quite broad distributions of sarcomere numbers, but the means were significantly different between training groups. The descending trained animals had the largest sarcomere count, the climbing trained rats had the smallest counts, and sedentary rats had intermediate counts, though closer to the climbing group. This could be interpreted as the response to concentric exercise being either smaller or slower than the response to eccentric exercise.

In another series of experiments[14], vastus intermedius muscles of treadmill trained rats were tested mechanically while still in situ, that is attached to the bones, but with all other muscles about the knee joint removed. This avoids introducing uncertainties into muscle length. In descending trained rats the knee angle for optimum torque generation corresponded to longer muscle lengths than in climbing trained rats, and the muscles of descending trained rats suffered less damage from an acute bout of eccentric contractions over the same range of knee angles.

In humans the hamstring muscles are of particular interest, because they are used relatively rarely for eccentric contractions, and because they are subject to muscle tears, which are thought to occur during eccentric contractions in activities such as sprinting where they act as brakes on the forward swing of the leg, particularly the lower leg. They are also a convenient muscle to use experimentally, as passive tension about the knee is small over most of the anatomical range. The optimum knee angle for torque generation can be reliably measured by isokinetic dynamometry, where an angle torque curve is measured during maximum voluntary contraction with constant velocity shortening. Doing a number of cycles and averaging improves the reliability.

Using this measure before and after a series of eccentric exercises produced a significant shift of about 7° in optimum knee angle for torque generation[15]. The exercise was a series of "hamstring lowers", where the subject knelt on a pad with ankles restrained and hips straight, and leaned slowly forward from the knees as far as possible before collapsing. This led to muscle soreness in the hamstring group. Some subjects repeated the exercise ten days later, and suffered much less soreness. Again eccentric exercise, DOMS, increased optimum length and adaptation all occurred together.

A less well investigated consequence of the hypothesis is that the unloaded shortening velocity should increase with eccentric training, as the unloaded shortening velocity of a fibre is the sum of the velocities of the sarcomeres. Some indications of this have been reported[16], though the long time course makes it difficult to rule out fibre type change as an alternative explanation.

It has been noted in tetanically stimulated animal muscles with multiple fibre types that the damaged fibres are largely fast twitch fibres[17]. In sub-maximal voluntary activity the opposite is true[13], presumably because the fast twitch fibres are not likely to be activated. Hence the fast fibres can be seen as being susceptible in tetanic contractions because they are not recruited, and hence not trained, during normal submaximal activity. If training consists of increasing the number of sarcomeres, then slow motor units should have longer optima than fast units, or more precisely, the most susceptible units should have the shortest optima and be fast type. This was tested for cat gastrocnemius motor units[10]. It was found that all motor units with optimum length less than the whole muscle optimum had a time to peak twitch of less that 50msec, that is all slow motor units had long optima. Interestingly, a small number of fast twitch units had long optimum lengths, suggesting that they have adapted to, and hence were subject to, regular eccentric exercise. However as the experimental observation was that all damaged fibres were fast, not all fast fibres were damaged, the existence of units that are both fast and well adapted is not inconsistent.

Different optimum lengths may be due to different tendon lengths as well as different numbers of sarcomeres. Where a relatively rapid (hours to days) shift of optimum is shown, it is more likely that sarcomeres are involved than tendon. This is less clear with experiments such as the above comparison of motor units, where the adaptation has taken place over a lifetime, so that tendon adaptations

cannot be ruled out. However, the basic hypothesis that muscle adapts optimum length to avoid eccentric contractions beyond optimum is not affected by this complication.

Are other mechanisms involved?

It is possible that adaptation of optimum length does occur, but that other adaptation mechanisms are also important. This was tested by training more rats as described above, either climbing or descending. All animals were then anaesthetised, and the vastus intermedius subjected to a series of 20 eccentric contractions. All stretches had an amplitude of 27° of knee angle, but beginning at different knee angles. In some animals stretch began at the measured optimum angle, while in others it began from 90° of knee angle[3]. One way of analysing these data is shown in Figure 2. Damage has been quantified by the immediate shift in optimum angle, but the tension decrease shows similar results. When the damage is plotted against the absolute knee angle from which the eccentric contractions began, the climbing and descending trained muscles clearly fall on two distinct lines, confirming both the existence of a training effect and the dependence of damage on muscle length. When the same damage data are plotted against the angle relative to optimum from which the acute eccentric contractions began, the two groups fall on the same line. Damage still depends on sarcomere length, but not independently on training. Statistical analysis of the data confirmed these conclusions. If training group (discrete) and absolute knee angle (continuous) were used as factors in a General Linear Model, group was highly significant. If absolute knee angle was replaced by relative knee angle, group was no longer significant. This indicated that effect of training attributable to mechanisms other than the shift in optimum length was not statistically significant.

These ideas and observations have been useful in directing current research into eccentric exercise, such as examination of the role of transverse tubules[9] and in muscle injury prevention[18].

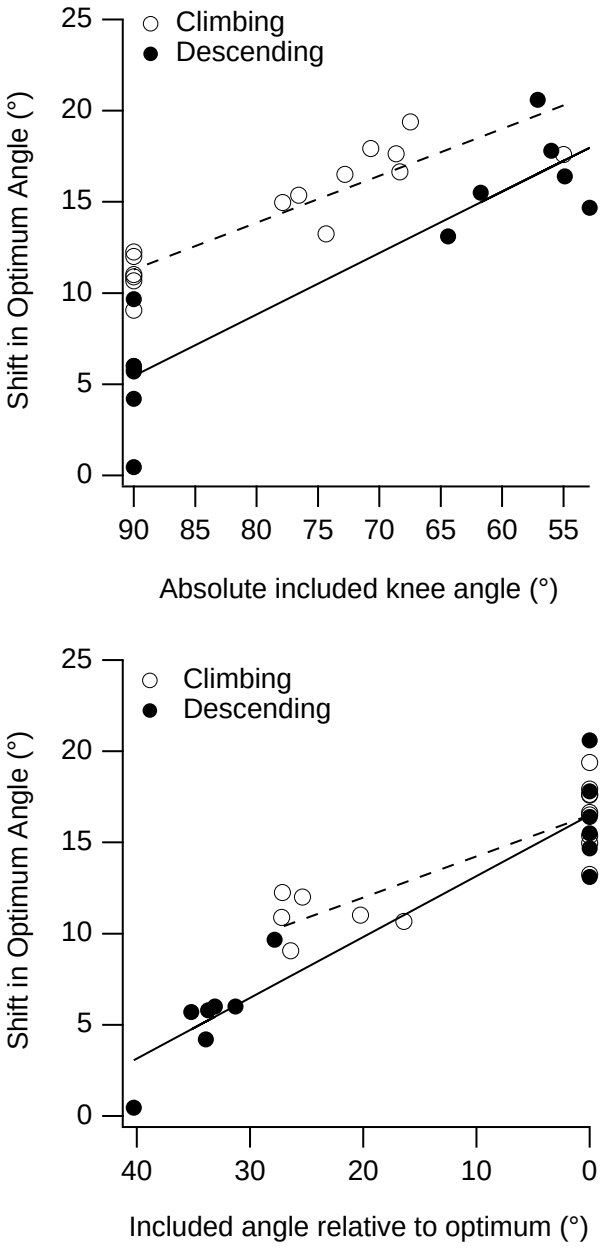

Figure 2. *The shift in optimum angle after acute eccentric contractions from various initial lengths, in climbing and descending trained rats. In the upper panel, data are plotted against the absolute knee angle from which stretches began. The muscles stretched from 90° are on the left, and those stretched from optimum length were generally longer and more damaged. Most importantly, the climbing and descending animals are clearly separated. In the lower panel the same data are plotted against angle relative to optimum. In this case the muscles stretched from optimum all appear at zero, and those stretched from 90° are generally stretched from less than optimum and damaged less. In this case, the climbing and descending trained animals are not separated.*

Summary

There is now an extensive body of evidence that damage from eccentric exercise is strongly dependent on the sarcomere lengths over which the stretching occurs. Adaptation in a number of preparations has been shown to be accompanied by a shift in optimum length, and the shift has been shown to account for the major portion of the adaptation. All of this provides evidence that damage occurs when sarcomeres are beyond optimum, the central prediction of the popping sarcomere hypothesis. This is true whether the stretch progresses to damage by tearing of structures or by opening of stretch activated channels.

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Author for correspondence:
Prof. David L. Morgan
Department of Electrical Engineering
Monash University,
Clayton, Vic. 3800, Australia

Tel: +61 3 9905 3483
Fax: +61 3 9905 1820
Email: david.morgan@ieee.org

Identifying athletes at risk of hamstring strains and how to protect them

U. Proske, D.L. Morgan*, C.L. Brockett & P. Percival*

Department of Physiology
and
**Department of Electrical and Computer Systems Engineering,*
Monash University,
Clayton VIC 3800,
Australia

Summary

1. One common soft-tissue injury in sports involving sprinting and kicking a ball is the hamstring strain. Strain injuries often occur while the contracting muscle is lengthened, an eccentric contraction. We have proposed that the microscopic damage to muscle fibres which routinely occurs after a period of unaccustomed eccentric exercise, can lead to a more severe strain injury.

2. An indicator of susceptibility for the damage from eccentric exercise is the optimum angle for torque. When this is at a short muscle length, the muscle is more prone to eccentric damage. It is known that subjects most at risk of a hamstring strain have a previous history of hamstring strains. By means of isokinetic dynamometry, we have measured the optimum angle for torque for 9 athletes with a history of unilateral hamstring strains. We also measured optimum angles for 18 athletes with no previous history of strain injuries. It was found that mean optimum angle in the previously injured muscles was at a significantly shorter length than for the uninjured muscles of the other leg and for muscles of both legs in the uninjured group. This result suggested that previously injured muscles were more prone to eccentric damage and therefore, according to our hypothesis, more prone to strain injuries than uninjured muscles.

3. After a period of unaccustomed eccentric exercise, if the exercise is repeated a week later, there is much less evidence of damage because the muscle has undergone an adaptation process which protects it against further damage. We propose that for athletes considered at risk of a hamstring strain, as indicated by the optimum angle for torque, a regular program of mild eccentric exercise should be carried out. This approach seems to work since evidence from one group of athletes, who have implemented such a program, shows a significant reduction in the incidence of hamstring strains.

Introduction

Hamstring strains are a common soft-tissue injury in sports such as Australian football and track and field events, including sprinting and hurdling. The Australian Football League (AFL) has the hamstring strain at the top of its injury list with 16% of all injuries attributed to it. Worse still, the re-injury rate for players who have at some time incurred a hamstring strain currently lies at 34%.[1] These figures indicate that current preventative strategies for this kind of injury remain inadequate.

Epidemiological evidence suggests that hamstring strains are associated with eccentric contractions, where the contracting muscle is lengthened.[2,3] Hamstrings undergo eccentric contractions during sprinting, kicking the ball and picking up the ball. Indeed, it is regularly observed that during these activities, players incur hamstring strains. This fact has led us to propose a new approach to hamstring strains, indeed, to all muscle strains, based on recent research.

We have been studying the mechanical changes in a muscle subjected to a series of eccentric contractions. Eccentric exercise is the only form of exercise which is routinely accompanied by muscle damage. For a review of the topic see Proske and Morgan.[4] Here we have gone one step further and proposed that under certain conditions, the microscopic damage at the level of muscle fibres from eccentric contractions may, at times, progress to a more major strain injury.

Muscle damage from eccentric exercise

Eccentric exercise, in someone unaccustomed to it, produces stiffness and soreness next day. This is because the exercise has led to muscle damage which, in turn, leads to sensitisation of nociceptors.[5] Why eccentric exercise produces muscle damage can be explained in terms of a theory based on sarcomere dynamics.[6] This proposes that the descending limb of the length-tension curve for skeletal muscle is a region of instability. When a sarcomere, which is weaker than it neighbours, lengthens on the descending limb, it becomes progressively weaker. In addition, when the yield point of the force-velocity relation is reached, lengthening is rapid and uncontrolled, without the development of additional force. The rapid lengthening will only stop when tension in passive structures associated with the sarcomere has risen sufficiently to balance the tension being generated in adjacent still-functioning sarcomeres. Then the next weakest sarcomere begins to lengthen uncontrollably. This process continues for the duration of the applied lengthening during the eccentric contraction.

Once a sarcomere has been stretched to the point of no overlap, when the muscle relaxes, the sarcomere risks becoming disrupted, that is, the myosin and actin filaments no longer interdigitate properly.[7] A non-functioning, disrupted sarcomere represents a point of weakness in the muscle. During repeated eccentric contractions the area of

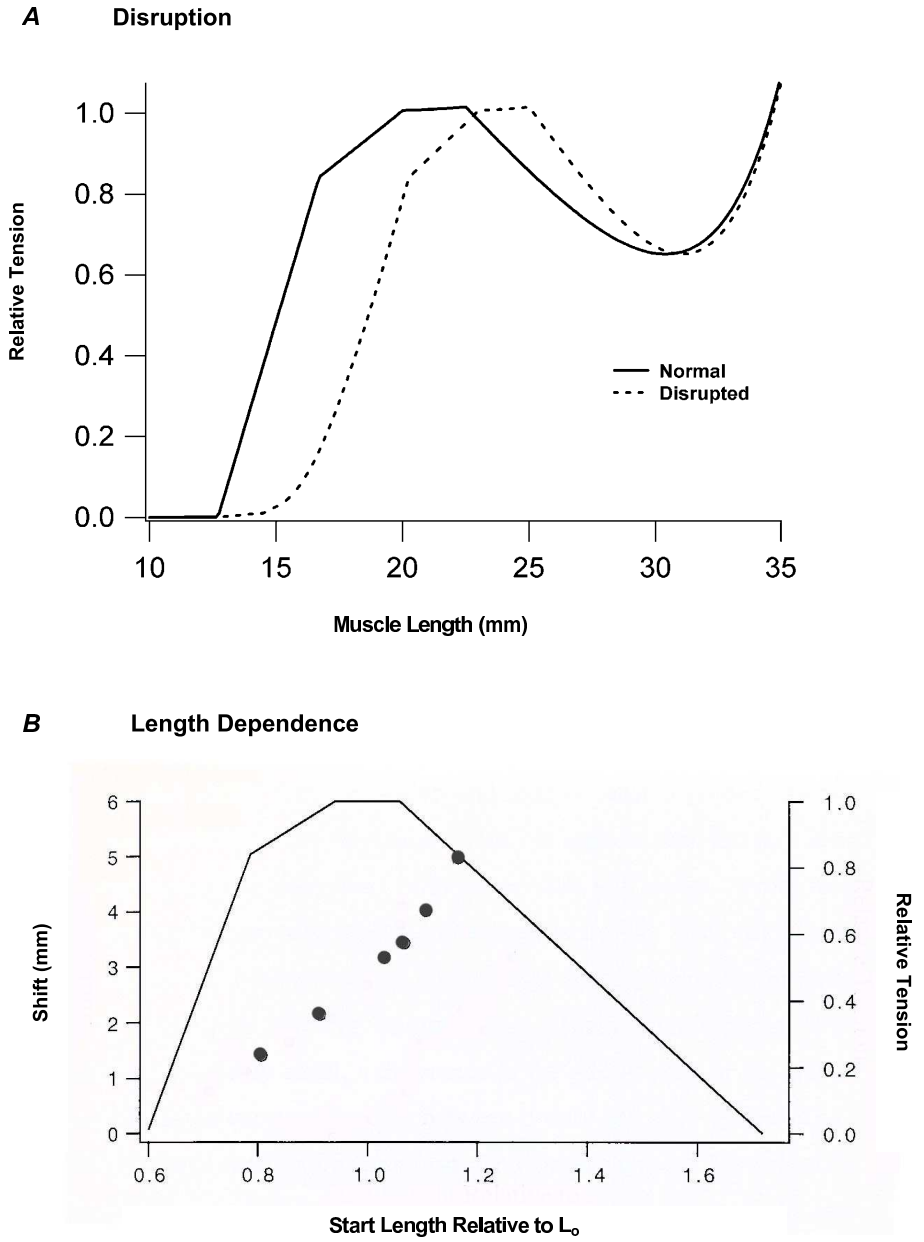

Figure 1. A: Changes in sarcomere length-tension relation following a series of eccentric contractions. *A computer-simulated curve of total sarcomere tension has been represented, based on the active length-tension relation[11] to which the estimated, exponentially rising passive tension has been added. Tension has been normalized relative to the maximum active tension. Length is of a fibre postulated to comprise 10,000 sarcomeres with a sarcomere length of 2.5 μm at optimum length. The control curve (solid line) is on the left. After a series of eccentric contractions, 10% of sarcomeres have their tension output set to zero to simulate disruption. That shifts the length-tension relation in the direction of longer lengths by 3 mm, as shown by the dashed curve on the right. Redrawn from Proske and Morgan.[4]*

B: Dependence of the shift in length-tension relation on the starting length. *The filled circles represent the data from each of 6 toad sartorius muscles subjected to 20 eccentric contractions. These were active stretches of 3 mm (10% L_o) at 3 muscle lengths s^{-1}. They were applied at progressively longer lengths, the first at a starting length of 0.8 L_o, the last at 1.2 L_o. At longer starting lengths, the resultant shifts in optimum were larger. To provide an indication of where on the sarcomere length-tension relation these shifts lay, a superimposed active sarcomere length tension curve has been shown (from Gordon* et al.*[11]) Figure redrawn from Talbot.[12]*

disruption is likely to grow and a point is reached where membranes are torn and the muscle fibre begins to contract uncontrollably, leading to a rise in whole-muscle passive tension.[8] Ultimately some of these fibres are likely to die.[4]

Signs of damage

Evidence for the presence of disrupted sarcomeres in series with still functioning sarcomeres is provided by a

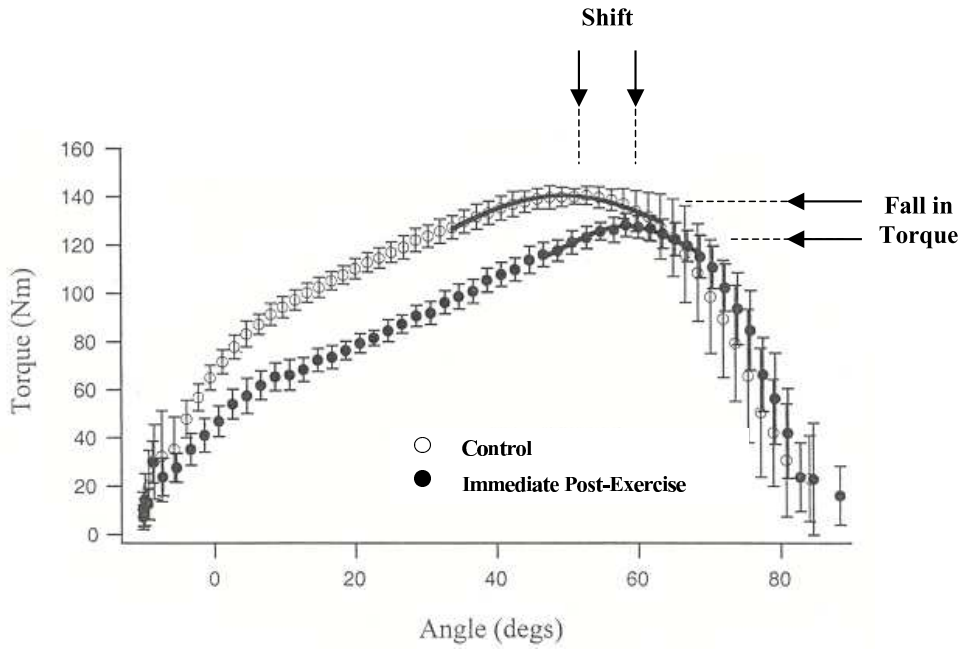

Figure. 2. Torque-angle curves for human hamstring muscles, before and after a series of eccentric contractions. *Torque and angle values were obtained from a series of maximal knee extensions carried out on an isokinetic dynamometer. Gaussian curves were fitted by computer to the top 10% of the digitised and averaged values (continuous lines). These gave values for peak torque and optimum angle. Open circles (± S.E.M.), data acquired immediately before the exercise (Control), filled circles (± S.E.M.), immediately after the exercise (Immediate Post-Exercise). The exercise consisted of a series of controlled forward falls, using hamstrings to brake the fall. Figure redrawn from Brockett* et al.*[13] Downwards directed arrows indicate the shift in optimum angle (7.2°). Horizontal arrows show the drop in torque (12.6 N).*

shift in the muscle's length-tension relation in the direction of longer muscle lengths.[9,10] This can be modelled by means of a sarcomere length-tension curve based on Gordon, Huxley and Julian.[11] A disruption of 10% of 10,000 sarcomeres, each with a length of 2.5 μm at optimum, produces, in a muscle 25 mm long, a shift of 3 mm, in the direction of longer lengths (Fig. 1A).

Since the instability of sarcomeres is present only on the descending limb of the length tension curve, the single, most important determinant of the amount of damage and disruption from eccentric contractions is the length range over which the muscle is stretched. For amphibian muscle, the size of the shift in optimum length is directly dependent on the starting length for the stretch. The shift is small (1 – 2 mm) when the starting length is below the optimum. It increases steeply up to 5 mm when it exceeds the optimum (Fig. 1B).[12]

There has been much debate over the reliability of the various damage indicators after eccentric exercise. In our view the drop in force is not as reliable an indicator as a shift in optimum length. This is because during repeated eccentric contractions, as occurs in most sports, the force drop may be confounded by fatigue effects. The shift in optimum is present immediately after the exercise, not delayed like soreness, and the size of the shift is a direct indication of the amount of damage that has occurred.[4]

Our approach to the problem of hamstring strains is based on the proposal that damage at the level of single muscle fibres can, at times, lead to a major tear, the muscle strain.[13] If we are right, it means that evidence for a predisposition for eccentric damage is also an indication of vulnerability for strain injury.

We have tested this proposal by, first of all, measuring torque-angle curves for hamstring muscles in untrained subjects. Curves were constructed using an isokinetic dynamometer. Subjects were asked to carry out a series of isokinetic contractions and the torque and angle signals during each contraction were digitised, sorted according to length and averaged. A computer fitted a curve to values above 90% of torque to determine the optimum angle. Subjects were then asked to carry out a series of eccentric contractions with their hamstring muscles. For this they were asked to kneel on a padded board with their feet strapped to the board at the ankle. Subjects were instructed to lower their trunk down onto the board, using their hamstrings to brake the fall. Subjects carried out a series of such 'hamstring lowers' and then a second torque-angle curve was constructed immediately afterwards.

An example of a pair of torque angle curves constructed before and after a period of eccentric exercise is shown in Figure 2. For 10 subjects tested it was found that there was a fall in optimum torque, by an average of 25% (± 4%), and a shift of the optimum angle of 7° (± 3°).[13] This result confirmed that it was indeed possible to obtain

evidence for muscle damage in hamstrings after a series of eccentric contractions.

Previously injured athletes are more prone to injury

As mentioned earlier, more than one third of all AFL players with a previous history of hamstring strains, subsequently re-injure. If we are right in our predictions, a greater-than-normal vulnerability for eccentric damage should mean an increased likelihood for a strain injury. This leads to the prediction that previous hamstring-injured subjects should show a greater-than-normal susceptibility for eccentric damage. It was decided to test this hypothesis.

Hamstring angle-torque curves, as described previously, were measured for 9 elite athletes, 5 AFL players and 4 track and field athletes all of whom had previously incurred one or more hamstring strains in one leg, 4 weeks or more previously. At the time of testing they had all returned to full training and no one experienced any soreness during testing. Measurements made on the previously injured hamstrings were compared with hamstrings of the other, uninjured leg. In addition measurements were made on both legs of 18 AFL players, none of whom had a previous history of hamstring strains.[14]

The values for optimum angles revealed dramatic differences. The mean optimum angle for the previously injured hamstrings was 12.1° (± 2.7°) shorter than for the uninjured muscle (Fig. 3). A shorter-than-normal optimum length means that more of the muscle's working range is on the descending limb of the length-tension relation, the region of instability and damage. Interestingly, the uninjured muscles of the other leg not only had longer optima but these were not significantly different from values for both legs of the uninjured subjects. So the uninjured muscles of subjects with a history of unilateral hamstring strains show no signs of a susceptibility for damage. If, as experience shows, differences between hamstrings on the two sides are usually small, this finding suggests that at-risk subjects within a population of uninjured players may not always be identified by their optimum angles. It also suggests that for the initial injury other factors are likely to play a role.

It has previously been proposed that a measure of susceptibility for hamstring strains is the quadriceps:hamstrings torque ratio.[15] Other observations suggest that this ratio is not a reliable predictor of strain injuries.[16] We have calculated quadriceps:hamstrings torque ratios for the muscle of the previously injured leg, the muscle of the other, uninjured leg and for the muscles of both legs in the athletes with no history of hamstring strains. Plotting ratios for muscles of one leg against the other leg showed no significant difference for the previously injured leg (Fig. 3B).

The "repeated bout effect" from eccentric training

We have all had the experience that a period of unaccustomed exercise, biased towards eccentric exercise, like walking downhill, leaves us stiff and sore next day. However the same exercise a week later is followed by much less stiffness and soreness. This is the "repeated bout effect".[17] Following damage from the first period of exercise, the muscle adapts to prevent further damage. It has been proposed that the adaptation process involves the incorporation of additional sarcomeres, in series, in muscle fibres.[6] It is known that such an addition of sarcomeres can occur in less than a week.[18] The presence of additional sarcomeres in myofibrils means that the average sarcomere length for a given fibre length becomes less, leading to a shift in the direction of longer muscle lengths of the optimum length for force. That, in turn, makes it less likely for the muscle, within its normal working range, to be stretched onto its descending limb, the region of potential damage.

We have measured the training effect in hamstrings. In non-athletic subjects optimum angle shifted by 7° immediately after a first period of eccentric exercise (see above). By 8 days after the exercise, there remained a persistent 6° (±4°) shift. Following a second period of exercise at day 8, there was a further 1° shift accompanied by a smaller-than-previous drop in force and less soreness.[13] Our interpretation is that the shift in optimum length after the first period of eccentric exercise reverses only partially since repair of damaged muscle fibres is accompanied by incorporation of additional sarcomeres. This, in turn, means that the second period of exercise is not stretching muscle fibres quite as far as previously, so avoiding the descending limb of the length-tension curve. As a consequence there is less damage and disruption. If we are right, and a susceptibility for eccentric damage signals a vulnerability for strain injuries, the training effect is likely to be a means of providing protection against further injury.

Training with eccentric exercise reduces the incidence of hamstring strains

In order to test some of our ideas, over the last 3 years we have been collaborating with one of the AFL clubs. In cooperation with the club's fitness coordinator a new training program has been implemented for all players. Our approach is based on the proposition that the precursor event to a hamstring strain is microscopic damage in muscle fibres from eccentric exercise. It follows that if it is possible to reduce the muscle's susceptibility for eccentric damage, this will lead to a reduced incidence of strain injuries.

Pre-season training before we began our study was mainly aimed at achieving greater aerobic fitness. The new program emphasised kicking and other exercises that stretched the active hamstrings. In addition, players carried out some specific, targeted eccentric exercises. These included "straight-legged deadlifts" and carrying out "knee-curls" on a GHG (gluteus-hamstrings-gastrocnemius) machine. For players who had incurred a hamstring strain, initially a rather mild program of exercise was given and this was gradually increased as the subject recovered from the injury.

In the 2001 season, which was before we began working with the club, they had reported a total of 16

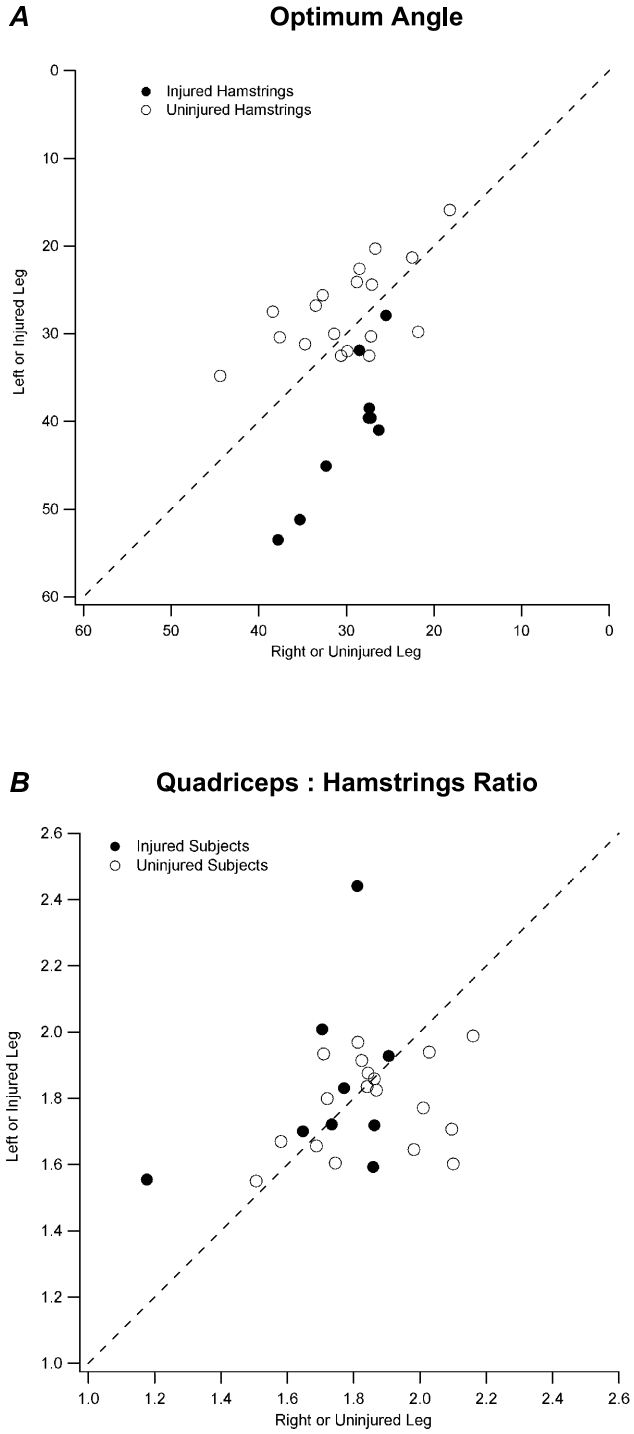

Figure 3. A: *A plot of the optimum angle for torque in hamstrings for the left, or the injured leg against optimum angle for the right or the uninjured leg. Data from 9 athletes with a previous history of a unilateral hamstrings strain (filled circles) and from 18 athletes with no previous history of strain injuries (open circles). The dashed line indicates where values would lie if they were equal. Optimum angle has been expressed in degrees of knee flexion, where 0° is when the knee is fully extended and 110° when it is fully flexed. Optimum angles for the previously injured muscles were at significantly shorter muscle lengths, that is, a more flexed knee, than for muscles with no history of injury (Figure redrawn, in part, from Brockett* et al.[14]*).*

B: *Ratio of peak torque in quadriceps to peak torque in hamstrings. Values for athletes with a previous history of unilateral hamstring strains shown as filled circles. Values from athletes with no history of injury shown as open circles. Differences in ratios between injured and uninjured muscles were not significant.*

hamstring strains amongst players. After introduction of the new training program, 5 hamstring strains were reported during the 2002 season. For the 2003 season, the club reported only 2 hamstring strains. This data remains preliminary, but is encouraging. Currently testing is continuing and other AFL clubs are beginning to participate. So we are hoping that in the future widespread implementation of a program of targetted eccentric exercise will lead to a dramatic fall in the incidence of hamstring strains.

Conclusions

The outcome of this first cooperative study has obviously delighted the AFL club. But it has also provided additional supporting evidence for our proposal of a link between the microscopic damage routinely incurred after eccentric exercise and development of a more major muscle tear. If protection against eccentric damage can be achieved with a regular program of eccentric exercise, this will be the means of preventing the occurrence of all such strain injuries. The evidence suggests that in athletes with a history of unilateral hamstring strains, the uninjured muscle is indistinguishable in its properties from muscles of athletes with no history of such injuries. It may, therefore, make it difficult, at times, to detect at risk athletes in an uninjured population. It means that all participants in sports known to be associated with the occurrence of hamstring strains should be subjected to regular eccentric exercise programs, carried out in combination with measurements of optimum angle for torque, to make sure that the exercise has led to the desired adaptive changes.

Now it remains for other AFL clubs, indeed, other sporting bodies confronted with the problem of hamstring strains, to participate in similar training programs, to help eliminate this kind of injury.

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Author for correspondence:
Prof. U. Proske
Department of Physiology
Monash University,
Clayton VIC 3800, Australia

Tel: +61 3 9905 2526 Fax: +61 3 9905 2531
Email: uwe.proske@med.monash.edu.au

Stretch-activated channels in stretch-induced muscle damage - role in muscular dystrophy

Ella W. Yeung & David G. Allen*

Department of Rehabilitation Sciences,
The Hong Kong Polytechnic University,
Hung Hom, Kowloon, Hong Kong
and
**Institute for Biomedical Research and*
Department of Physiology,
University of Sydney F13,
NSW 2006, Australia

Summary

1. Stretch-induced muscle injury results in the damage which causes reduced force and increased membrane permeability. This muscle damage is partly caused by ionic entry through stretch-activated channels and blocking these channels with Gd^{3+} or streptomycin reduces the force deficit associated with damage.

2. Dystrophin-deficient muscles are more susceptible to stretch-induced muscle injury and the recovery from injury can be incomplete. We have found that Na^+ entry associated with stretch-induced injury is enhanced in dystrophin-deficient muscles and that blockers of stretch-activated channels are capable of preventing the ionic entry and reducing the muscle damage.

3. A model is presented which proposes links between stretch-induced injury, opening of stretch-activated channels, increased levels of intracellular ions and various forms of muscle damage. While changes in Na^+ accompany stretch-induced muscle injury, we believe that changes in Ca^{2+} probably have a more central role in the damage process.

Introduction

Muscles which are stretched during contraction are susceptible to damage particularly when the exercise is prolonged and unaccustomed. It is widely agreed that stretch-induced injury includes both structural disorganization and changes to ionic regulation of the muscle fibres. The structural and functional changes include focal sarcomeric disorganisation, increase membrane permeability, reduction in force, delayed muscle soreness and reduction in joint range.[1-3] Severely damaged fibres may degenerate and this is then normally followed by regeneration as evidenced by force recovery.[4] In normal individuals, this cycle of damage and repair lasts 4-6 days and is associated with symptoms of transient muscle soreness, stiffness and weakness. However in patients with debilitating muscle diseases, the muscle damage more frequently leads to degeneration and the regeneration is insufficient to compensate for damage.

Duchenne muscular dystrophy (DMD) is an X-linked genetic disease caused by the absence of the protein dystrophin. This devastating disease affects approximately 1 in 3500 male births. The disease is characterized by progressive muscle wasting and weakness. Affected boys are usually confined to a wheelchair before the age of 12 and die in their late teens or early twenties through respiratory muscle failure. Several studies have shown that *mdx* muscle fibres (an animal model for human DMD) are more vulnerable to stretch-induced injury and the increases in membrane permeability were greater.[5-7] Dystrophin, the protein absent in DMD and the *mdx* mouse, connects the cytoskeletal network to the sarcolemma, and is thought it to provide mechanical reinforcement to the sarcolemma and minimize damage induced by contractile activity in normal muscles.[8] However the reasons why absence of dystrophin cause increased in susceptibility to stretch-induced injury and the sequence of events leading to muscle necrosis remain unclear.

In recent years, attempts have been made to replace or transform the defective dystrophin gene using genetic approaches such as viral and plasmid vector therapy and corrective gene conversion therapy. While such approaches have been effective in the *mdx* mice,[9-10] in humans these therapeutic strategies have not so far been of therapeutic value because of inefficiencies in the delivery and expression of the very large dystrophin gene and because of immune responses to parts of the expressed dystrophin.[11]

In this review we focus primarily on the mechanisms of damage associated with stretch-induced damage in both wild-type and *mdx* muscle fibres. Specifically, we discuss the evidence that the activity of the stretch-activated (or mechanosensitive) channels after stretch-induced contraction injury is enhanced. Given the higher opening probability of stretch-activated channels in *mdx* myotubes,[12] we postulate that this abnormally high activity provides a leak pathway for Ca^{2+} to enter the cell causing cellular damage. Blocking these channels may provide a therapeutic approach for reducing muscle damage in DMD patients.

Stretch-induced muscle injury in wild-type fibres

Changes following stretch-induced muscle injury

It has long been recognized that cellular and ultrastructural damage occur following stretch-induced muscle injury in humans and animals. This includes myofibrillar disruption especially at the Z-lines and loss of

the cytoskeletal proteins, such as titin and desmin.[13-15] In addition to the morphological abnormalities, a decrease in the force production and the shift of optimum length (L_o) have been observed following stretch-induced muscle injury.[16-18]

The 'popping sarcomere hypothesis' proposed by Morgan[19] suggested that when muscles are stretched during contraction on the descending limb of the tension-length relation the sarcomeres show non-uniform increases in length. In general the weakest sarcomere will stretch first making it weaker still and then it will stretch rapidly to some maximum set by structural proteins in the sarcomere i.e. it will 'pop'. If lengthening continues after the weakest sarcomere has 'popped', then the next weakest sarcomere will elongate. It is likely that repeated lengthening contractions lead to increased numbers of disrupted sarcomeres in which the myofilaments fail to re-interdigitate. Furthermore, the overstretched sarcomeres increase the series compliance so that there is a shift in the active length-tension relation to longer muscle lengths. This shift following stretch-induced damage was first described by Katz[20] and subsequently confirmed in whole muscles and humans.[21-24] Structural evidence of overstretched sarcomeres was obtained by electron microscopy of fibres fixed during contraction, and supported the non-uniform sarcomere hypothesis. The occurrence of over-stretched sarcomeres after a single bout of lengthening contraction has been quantified and has been shown to account for more than half of the stretch[25].

The primary injury in stretch-induced muscle damage appears to be mechanical in nature with localised regions of sarcomere inhomogeneities. There is also evidence that changes in excitation-contraction (E-C) coupling may play a role in triggering the biomechanical and biochemical changes following stretch-induced injury. Calcium has long been thought to play a central role in muscle damage.[26] Earlier study in our laboratory on single wild-type muscle fibres[27] showed that both tetanic $[Ca^{2+}]_i$ and force were reduced following lengthening contractions. Furthermore, a persistent elevation in resting $[Ca^{2+}]_I$ was also observed though the mechanism of this increase was unclear. Another indication of increases in resting $[Ca^{2+}]_I$ arises from measurements of passive tension which has been shown to rise after a series of eccentric contractions.[23]

One possible cause for the increase in resting $[Ca^{2+}]_I$ would be if T-tubules were forcibly disconnected from the surface membrane during stretch-induced injury causing increased membrane permeability. To study this possibility we performed experiments on single muscle fibres and used an extracellular fluorescent dye (sulforhodamine B) which enters the T-tubules. Following a series of eccentric (or stretched) contractions the T-tubules were found to be distorted and extracellular vacuoles attached to T-tubules were observed. Previously it had been shown that such vacuoles were a consequence of increased activity of the Na^+ pump as it removes excess Na^+ from the intracellular space[28] and we confirmed that the vacuoles observed after stretch-induced damage were also prevented by inhibiting the Na^+ pump with ouabain.[17]

Does $[Na^+]_i$ increase following stretch-induced muscle damage?

If the mechanism proposed above for the development of vacuoles is correct, then a rise in $[Na^+]_i$ should followed stretch-induced muscle damage. To test this idea we measured $[Na^+]_i$ with a fluorescent indicator (SBFI). Following the stretch protocol, $[Na^+]_i$ rose from the resting level of 7.3 ± 0.2 mM reaching a new level of 16.3 ± 1.6 mM. It is interesting to note that the rise of $[Na^+]_i$ occurred slowly taking 5-10 minutes to reach to a steady state.

To determine if the rise was caused by increased Na^+ influx, the stretch protocol was performed in a low-Na^+ solution. This solution eliminated the rise in $[Na^+]_i$ suggesting that Na^+ influx from the extracellular space was responsible. To determine whether inhibition of the Na^+ pump, for instance by damage to the pump or isolation of the pump within sealed off T-tubules, contributed to the rise of $[Na^+]_i$ we performed experiments in ouabain. Blocking the Na^+ pump with ouabain caused a slow rise of $[Na^+]_i$ but the rise of $[Na^+]_i$ produced by stretched contractions was further increased suggesting that the this rise was not caused by inhibition of the Na^+ pump.[18]

Does Na^+ enter through tears in the membrane?

The SBFI experiments establish that $[Na^+]_i$ rises following stretch-induced muscle injury but they do not identify whether the increase in $[Na^+]_i$ was via membrane tears. Earlier experiments have established that resting $[Ca^{2+}]_i$ also rises following stretch-induced damage and this rise may be the stimulus that trigger calcium-activated proteases which initiate muscle fibre degeneration.[29] If Ca^{2+} entry were at sites of membrane damage one would expect to observe localised elevations of $[Ca^{2+}]_i$ in the region of overstretched sarcomeres. However published studies showed that the distribution of $[Ca^{2+}]_i$ was uniform.[30-31] One possible explanation for these findings is that the sarcoplasmic reticulum rapidly sequesters Ca^{2+} which enters the fibre. For this reason, imaging $[Na^+]_i$ might be a better strategy since there is no large and active sink for $[Na^+]_i$ in skeletal muscles and detection of localised elevations might therefore be easier.

We imaged $[Na^+]_i$ with a confocal microscope having loaded the fibres with sodium green. $[Na^+]_i$ increased after the stretch protocol consistent with the SBFI results but, similar to the earlier $[Ca^{2+}]_i$ experiments, no localised elevations of $[Na^+]_i$ were detected.[18] These results probably exclude large membrane tears but obviously small, multiple, transient tears might have escaped detection. Another possibility is that the Na^+ entry occurs through a class of Na^+ permeable channels which are open for many minutes after stretch-induced muscle damage.

Stretch-activated ion channels in skeletal muscle fibres

One possible contributor to the increased membrane permeability following stretch-induced muscle damage is the involvement of stretch-activated channels. Involvement

of the stretch-activated channels leading to membrane depolarization in rat muscle fibres after lengthening contractions has been reported.[32] These results suggested that membrane depolarization was due to an increase in Na^+ permeability and the accompanying increase in Na^+ influx. Stretch-activated channels of various ionic selectivities have been found in many cell types including striated muscles.[33] These channels were first described in cultured chick embryonic skeletal muscle cells.[34] They respond to mechanical stress by increasing the open probability[12,35] and act as membrane-embedded mechano-electrical switches, opening a large water-filled pore in response to lipid bilayer deformations. This process is important in a wide array of cellular activities such as volume regulation, electrolyte homeostasis and sensory transduction, and is critical to the response of living organisms to mechanical stimulation.[35-36] Non-selective stretch-activated cation channels pass Ca^{2+} as well as Na^+ and K^+, whereas others classes of mechanosensitive channels are selectively permeable to K^+ or Cl^-.[37-38]

Gadolinium (Gd^{3+}) and streptomycin have been reported to block stretch-activated channels.[36,39-42] They have been used to inhibit cation-permeable stretch-activated channels in cardiac and skeletal muscle cells. Although Gd^{3+} is the most widely used blocker of stretch-activated channels,[42-43] it is relatively non-specific blocking L-type Ca^{2+} channels,[44] store-dependent Ca^{2+} channels[45] and Cl^- channels[46] though generally with lower potency. Identification of the physiological role of stretch-activated channels has been hampered by the absence of specific channel blockers or activators. The spider venom peptide GsmTx-4 described by Sachs and colleagues[47] is the most potent and specific inhibitor of stretch-activated ion channels described so far.

Given the failure to detect localised Na^+ entry in our imaging experiments, a blocker of stretch-activated channels (Gd^{3+} or streptomycin) was applied for 10 min following stretch-induced damage. Not only did the blockers prevent the increase of $[Na^+]_i$ after the stretch protocol but it also prevented part of the force deficit.[18] We hypothesise that stretched contractions open stretch-activated channels and allow influx of Na^+ and Ca^{2+} ions. The consequent rise in $[Na^+]_i$ activates the Na^+-K^+ pump and the efflux of Na^+ and H_2O through the T-tubules is thought to cause the vacuoles. The increased $[Ca^{2+}]_i$ may activate proteases and phospholipases which cause membrane damage and the increase in membrane permeability (see Fig. 2).[48-49]

Stretch-induced muscle damage in *mdx* muscle fibres

Function of dystrophin in the prevention of stretch-induced muscle damage

Duchenne muscular dystrophy (DMD) is caused by mutations in the dystrophin gene which prevent dystrophin expression. Lack of dystrophin causes disruption of the dystrophin-glycoprotein complex (DGC) and results in sarcolemmal instability. Studies performed on dystrophic muscles have shown that lengthening contractions induce greater damage than in wild-type muscle fibres.[5,7]

The cytoskeletal protein dystrophin is thought to provide mechanical reinforcement to the sarcolemmal membrane and minimize damage induced by contractile activity in normal muscles. Since dystrophin normally links the contractile proteins to the DGC membrane complex it may prevent the relative movement between the myofibrils and the surface membrane. In the absence of dystrophin there may be relative movement between the myofibrils and the T-tubules which attach to the surface membrane and then pass perpendicularly through the myofibrils. Relative movement would therefore be expected to damage both the T-tubules and the surface membrane. Another possibility is that the dystrophic cells are often abnormal in shape (branching, tapering)[5] so that stretch might be more prone to cause cell damage because of the non-uniformity in shape.

Apart from mechanical reinforcement, it has been suggested that dystrophin is involved in the clustering of ion channels within the sarcolemma. Using the manganese quench technique, the membrane permeability for cations has been shown to be two times higher in resting *mdx* muscle fibres than the wild-type fibres.[50] These authors showed that this leak channel was blocked by 50 µM Gd^{3+} raising the possibility that a stretch-activated channel might be involved. This idea is also supported by patch-clamp recordings from *mdx* myotubes in which single channel activity was recorded and stretch-sensitivity tested by applying suction. The results showed a 3-fold higher opening probability of the stretch-activated channels[12] in *mdx* compared to wild type fibres. As these channels have been shown to be present in the sarcolemma, and are non-specific cation channels allowing influx of Ca^{2+} and Na^+, this pathway could contribute to the elevated resting $[Ca^{2+}]_i$ and $[Na^+]_i$. Furthermore, stretched contractions can result in activation of these channels, and the resulting Ca^{2+} influx can trigger Ca^{2+}-dependent proteolysis and lead eventually to muscle necrosis.

Stretch-activated channel blockers in the prevention of stretch-induced muscle damage

The above results suggest that Ca^{2+} leak pathways which may be stretch-activated are more prevalent in *mdx* fibres. We therefore tested the changes in $[Na^+]_i$ in *mdx* fibres following stretch-induced muscle damage. Single *mdx* muscle fibres were isolated and loaded with sodium green for examination under confocal microscopy.[51] We show that $[Na^+]_i$ in *mdx* muscles (15.4 ± 1.1 mM) was higher than control (9.7 ± 1.1 mM). Similar to other *mdx* muscle studies,[5-7] there was a greater reduction in force following lengthening contractions than in wild-type fibres. We also observe a significantly greater rise in $[Na^+]_i$ following lengthening contractions than in wild-type fibres (see Fig. 1A). Just as in wild-type fibres, the increase in $[Na^+]_i$ was uniformly distributed across the cell and there was no detectable localized elevations. Given that Gd^{3+} and streptomycin reduced the elevated $[Na^+]_i$ in wild-type fibres, we applied these stretch-activated channel blockers

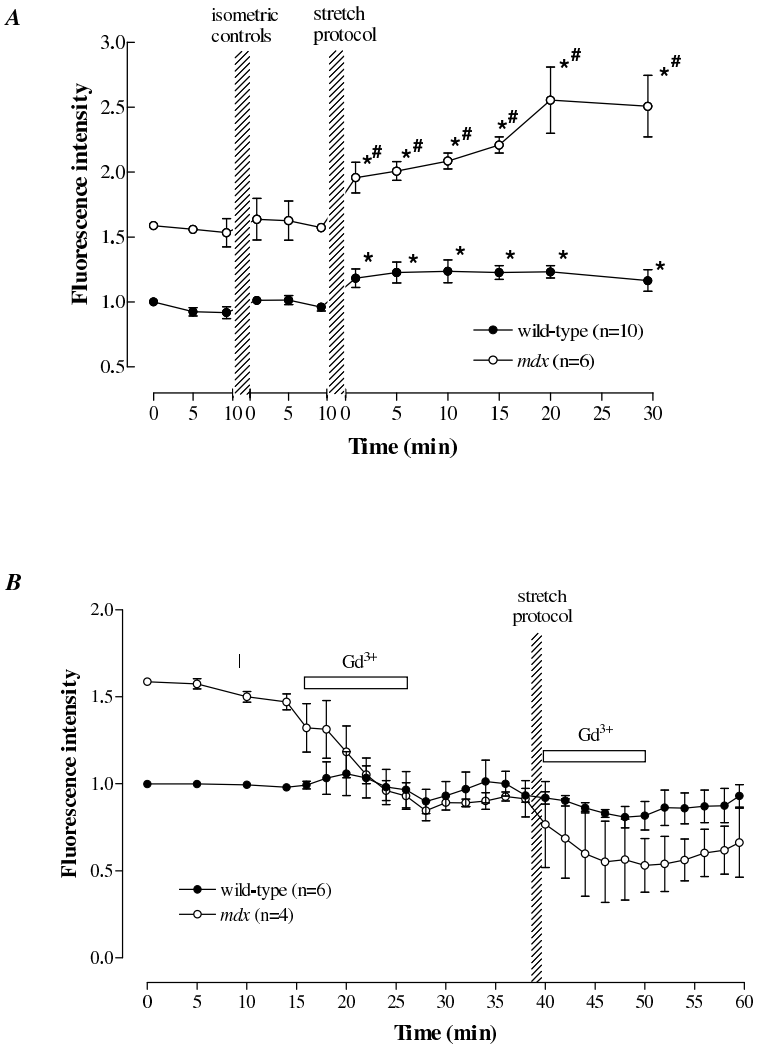

Figure 1. Na^+ fluorescence following stretch-induced injury in wild-type and* mdx *muscle fibres. *The fibres were loaded with sodium green and the fluorescence intensity was normalised to the initial value at the start of the experiment. Note that the initial fluorescence signal was adjusted for* mdx *muscle fibres based on the* in vivo *calibration procedures.*
Panel A, *there was a rise in Na^+ fluorescence after the stretch-induced injury protocol in both wild-type and* mdx *fibres but the rise in* mdx *fibres was significantly higher. * significantly larger than initial wild-type control: # significantly larger than initial* mdx *control (*$P < 0.05$*).*
Panel B, *under control conditions, Gd^{3+} has no effect on wild-type fibres but lowered the Na^+ fluorescence in the* mdx *fibres to the level of the wild-type fibres. The fibres underwent a stretch-induced protocol and Gd^{3+} was applied immediately for 10 min (as indicated by the bars). Gd^{3+} eliminated the rise of sodium green fluorescence following the stretch in both wild-type and* mdx *fibres. Values are mean ± S.E.M.*

to the *mdx* fibres immediately after the stretch protocol (Fig. 1B). Similar to the wild-type muscle data, not only did both agents eliminated the rise of $[Na^+]_i$ but, in addition, the force deficit was reduced. After the stretch protocol on *mdx* muscle fibres, the force was reduced to 23 ± 3 % of the control value at the original length but when Gd^{3+} was applied during or immediately after the lengthening contractions, the force was improved to 46 ± 4 %. When the muscle fibres were stretched to the new optimum length, the force was improved to 96 ± 5 % of the control.

Based on these results, we postulate that the increase in Na^+ permeability is caused by increased opening of the stretch-activated channels, and blocking these channels is capable of preventing the rise of $[Na^+]_i$ and, more interesting, the force deficit. Furthermore, it is possible that the increased resting $[Ca^{2+}]_i$ observed[27] was caused by increased activity of these stretch-activated channels. This increased $[Ca^{2+}]_i$ may be responsible for the initiation of

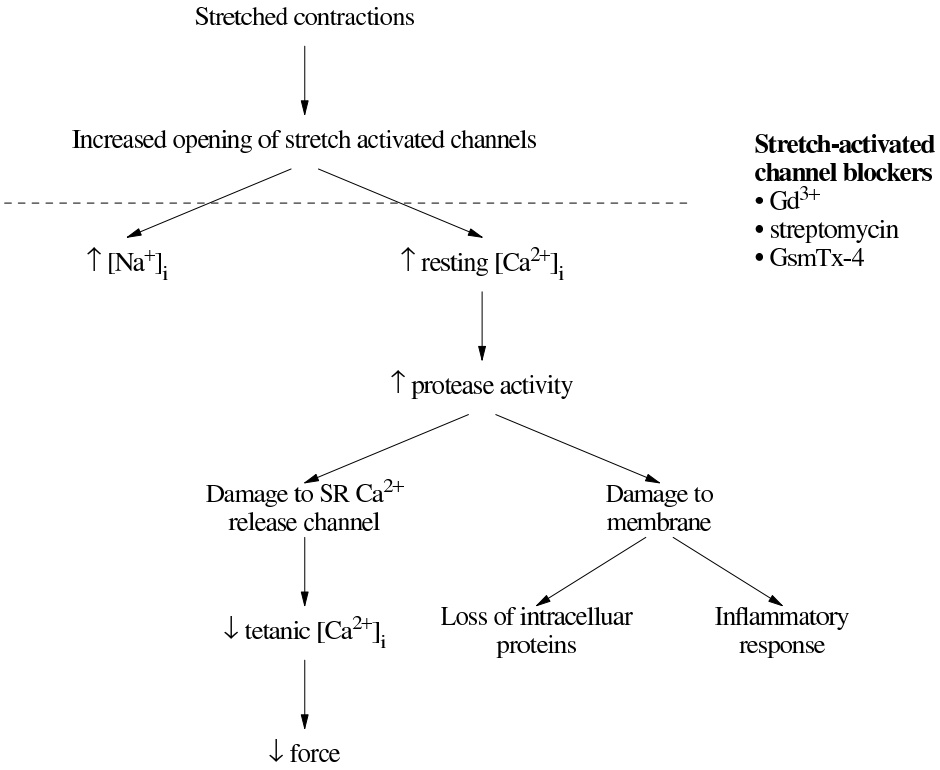

Figure 2. Working hypothesis linking stretch-activated channels, ionic changes and muscle damage. *The causes of force reduction following stretch-induced muscle injury could be: (i) sarcomere inhomogeneity (not shown here) and (ii) damage arising through increased opening of the stretch-activated channels. It is likely that the opening of stretch-activated channels is a consequence of sarcomere inhomogeneity. In wild-type or* mdx *muscle fibres, elevated levels of* $[Na^+]_i$ *has been shown caused by the increased activity of the stretch-activated channels. One possibility is that elevations in resting* $[Ca^{2+}]_i$ *which occur following stretch-induced injury are a consequence of* Ca^{2+} *influx through the stretch-activated channels. It is possible that the associated inflammatory response, muscle weakness and pain observed following stretch-induced injury is caused by increased* Ca^{2+}*. If the stretch-activated channel blockers (*Gd^{3+}*, streptomycin, GsmTx-4) are capable of preventing the cascade of events following stretch-induced injury, they may prove valuable in reducing muscle damage.*

protease activity causing damage to the SR Ca^{2+} release channel and to the membrane. Elevations in $[Ca^{2+}]_i$ have been shown to induce inactivation of E-C coupling associated with a decrease in tetanic $[Ca^{2+}]_i$ and reduction in force.[52-53] The disruption to the membrane allows leakage of the intracellular contents out of the muscle cells and as evidenced by elevated serum levels of muscle enzymes and accumulation of inflammatory cells.[1,54] An overview of this hypothesis is summarised in Fig. 2.

The force deficit provides an indication of the extent of muscle damage and the results seem to suggest that the stretch-activated channel blockers can protect against muscle damage at least over the 30 min post injury. Whether these blockers are capable of exerting protection for longer term damage is uncertain. Nevertheless these findings have potential implication in the treatment of DMD patients.

Clinical applications

DMD is a chronic, degenerative disease and there is no effective treatment at present despite attempts using different genetic approaches. The exact role of dystrophin in the regulation of stretch-activated channels is not certain but we have evidence that these channels are abnormal in muscular dystrophy and cause part of the stretch-induced muscle damage. The various blockers of the stretch-activated channels and their capabilities of preventing Ca^{2+} and Na^+ meant that they hold as potential therapeutic agents to overcome irreversible muscle damage in DMD patients. However much work remains to be done before this therapy can be applied to patients.

Conclusion

Dystrophin deficiency is the precipitating feature in the muscle pathology of patients with Duchenne muscular dystrophy. The role of dystrophin in normal muscles remains unclear; possible roles include contributing to structural stability by connecting the cytoskeleton to the dystrophin-asssociated proteins in the membrane and contributing to channel function by anchoring and/or regulating ion channels. One channel whose function is altered in the absence of dystrophin is the stretch-activated

channel which allows increased influx of Na^+ and Ca^{2+} following stretch-induced injury. The alteration of Ca^{2+} homeostasis in muscular dystrophy may be responsible for muscle degeneration. Consequently blockers of stretch-activated channels may have therapeutic potential by reducing stretch-induced muscle damage in DMD patients.

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Author for correspondence:
Professor D. G. Allen
Department of Physiology
University of Sydney F13,
NSW 2006, Australia

Tel: +61 2 9351 4602 Fax: +61 2 9351 2058
Email: davida@physiol.usyd.edu.au

The role of contraction-induced injury in the mechanisms of muscle damage in muscular dystrophy

Gordon S. Lynch

Department of Physiology,
The University of Melbourne,
Victoria 3010,
Australia

Summary

1. Duchenne muscular dystrophy (DMD) is a severe disease of skeletal muscle, characterised by an X-linked recessive inheritance and a lack of dystrophin in muscle fibres. It is associated with progressive and severe wasting and weakness of nearly all muscles, and premature death by cardiorespiratory failure.

2. Studies investigating the susceptibility of dystrophic skeletal muscles to contraction-mediated damage, especially after lengthening actions where activated muscles are stretched forcibly, have concluded that dystrophin may confer protection to muscle fibres by providing a mechanical link between the contractile apparatus and the plasma membrane. In the absence of dystrophin, there is disruption to normal force transmission and greater stress placed upon myofibrillar and membrane proteins, leading to muscle damage.

3. Contraction protocols (involving activation and stretch of isolated muscles or muscle fibres) have been developed to assess the relative susceptibility of dystrophic (and otherwise healthy) muscles to contraction-induced injury. These protocols have been used successfully to determine the relative efficacy of different (gene, cell, or pharmacological) interventions designed to ameliorate or cure the dystrophic pathology. More research is needed to develop specific 'contraction assays' that will assist in the evaluation of the clinical significance of different therapeutic strategies for muscular dystrophy.

Duchenne muscular dystrophy and the *mdx* mouse

Duchenne muscular dystrophy (DMD) is a severe X chromosome-linked myopathy caused by a variety of mutations and deletions in the dystrophin gene.[1,2] In the absence of dystrophin expression, the skeletal muscles of boys with DMD undergo continuous cycles of degeneration and insufficient regeneration that leads to progressive muscle wasting and weakness. Patients are confined to wheelchairs by their early teens and die of respiratory or heart failure by their early twenties.[3] The *mdx* mouse, a commonly used animal model for DMD, carries a mutation in the dystrophin gene and lacks the native protein similar to the human condition, but exhibits a more benign pathological phenotype. The diaphragm muscles of *mdx* mice show progressive structural and functional deterioration consistent with DMD, whereas limb muscles exhibit a relatively mild pathology for much of the life span.[4-7] Despite an early period of severe degeneration in the limb muscles of *mdx* mice at 3-4 weeks of age, the muscles regenerate extremely well. In fact, despite ongoing cycles of (less severe) degeneration and regeneration throughout adulthood, the muscles of *mdx* mice are actually hypertrophied compared to wild type mice. However, despite their larger size they are comparatively weaker, since their maximum force output per muscle cross-sectional area is usually lower.[8]

Dystrophin and the costamere

Dystrophin links actin in the cytoskeleton through the transmembrane dystrophin-associated glycoprotein complex (or dystrophin-glycoprotein complex, DGC) to laminin in the extracellular matrix (ECM).[9] The DGC and other cytoskeletal proteins form rib-like lattices on the cytoplasmic face of the sarcolemma, called costameres. Costameres help stabilise the cytoskeleton to the ECM; they act as mechanical couplers to distribute contractile forces from the sarcomere through to the sarcolemma and basal lamina; and they help facilitate uniform sarcomere length between fibres, at rest and during contraction.[10,11] Dystrophin has also been found at the myotendinous junction and has therefore been postulated to play a role in the transmission of force to tendons.[12,13]

The precise functional role of dystrophin and the DGC has not been described definitively, but it has been postulated that its primary role is to anchor the sarcolemma to costameres and thus stabilize the sarcolemma against physical forces transduced through costameres during muscle contraction, most especially when muscles are activated and stretched forcibly. Such muscle lengthening actions usually occur when muscles act as brakes during slowing movements (e.g. when running downhill), and they are commonly referred to as 'eccentric' or 'pliometric' contractions.[14,15]

In addition to its membrane stabilising role, the DGC is postulated to play a role in the regulation of intracellular calcium, molecular signalling, and in signal transduction, such as neuronal nitric oxide synthase (nNOS)-mediated regulation of blood flow to contracting muscles.[16] For the purpose of this review I will limit my discussion to dystrophin's role in protecting muscle fibres against contraction-induced injury.

Evidence for a functional role of dystrophin

Contraction-induced injury is associated with a mechanical disruption of sarcomeres that are stretched

excessively. Whether dystrophin helps maintain sarcomere stability is not known, but there are several lines of evidence supporting a functional role of dystrophin in skeletal muscle fibres, including: increased susceptibility to osmotic stress[17,18]; increased permeability of the sarcolemma in *mdx* mice indicated by increased serum concentrations of muscle enzymes (e.g. creatine kinase); and elevated intracellular Ca^{2+} concentration.[19] An uptake of Evans blue dye (EBD) by fibres in quiescent muscles of *mdx*, but not control mice, provides further support for an increased permeability of the sarcolemma of fibres lacking dystrophin.[20] Furthermore, when *mdx* and wild type mice are subjected to downhill running exercise, there is extensive EBD uptake in muscle fibres of *mdx* but not wild type mice, indicating increased sarcolemmal fragility and permeability in the absence of dystrophin.[21]

Intact Muscles

A number of different contraction protocols[6,22-26] have demonstrated that skeletal muscles of *mdx* mice have a greater susceptibility to injury, particularly when maximally activated muscles are stretched. Whether whole muscles are studied *in vitro*, *in situ*, or *in vivo*, the overwhelming evidence indicates that intact skeletal muscles of adult *mdx* mice show a greater susceptibility to contraction-induced injury than muscles of control mice. Interestingly, the muscles of very young (9-12 day old) *mdx* mouse pups are relatively resistant to injury from acute mechanical injury, suggesting that the early onset of the dystrophic process might be independent of a mechanical perturbation to the sarcolemma.[13] The few reports that muscles of adult *mdx* and control mice do not differ in their susceptibility to contraction-induced injury involved protocols with hundreds of these lengthening actions.[27,28] These arduous protocols may have produced such severe damage to muscles in both *mdx* and control mice that they did not discriminate the differences between the two.

It should be noted that the majority of these studies have not reported the sarcomere length range or the region of the length tension curve over which the damaging contractions occurred. This is important since recent studies have indicated that this is a major determinant of the extent of damage in normal muscles.[15] Whether the optimum length of a muscle corresponds to the same joint angle in normal and dystrophic muscles has not been described. In examining the relative susceptibility of normal and dystrophic muscles to contraction-mediated damage, experiments conducted over the same joint angle, the same part of the length-tension curve (relative to optimum), or the same range of sarcomere lengths, are worthy of consideration and would provide interesting information about the differences and similarities between normal and dystrophic muscles.

Studies have recently focused on developing contraction-induced injury 'assays', with some employing as few as two lengthening contractions, to differentiate between the injury susceptibility of muscles from dystrophic and wild type mice, especially after gene therapies such as injection of viruses carrying full-length dystrophin or microdystrophins.[29,30] DelloRusso and colleagues[31] developed an assay based on the high susceptibility to injury of limb muscles in *mdx* mice for use in evaluating such therapeutic interventions. The assay involved two stretches of maximally activated tibialis anterior (TA) muscles *in situ*. The stretches of 40% strain relative to muscle fibre length were initiated once peak isometric force was attained. Damage (injury) was assessed one minute later by the deficit in isometric force. They found that the force deficits were four- to seven-fold higher for muscles of *mdx* compared with control mice. Such an *in situ* lengthening contraction protocol was used to assess whether intramuscular injection of gutted adenoviral vectors expressing full-length dystrophin into TA muscles of *mdx* mice could confer protection from contraction-mediated injury. The force deficit after each of the two stretches was used to determine the muscle resistance to injury. Despite a relative inefficiency of the intramuscular injection delivery leading to only 25% of the muscle cross-sectional area being transduced, this level of dystrophin expression conferred an ~40% correction of the functional difference between muscles of *mdx* and wild type mice.[32]

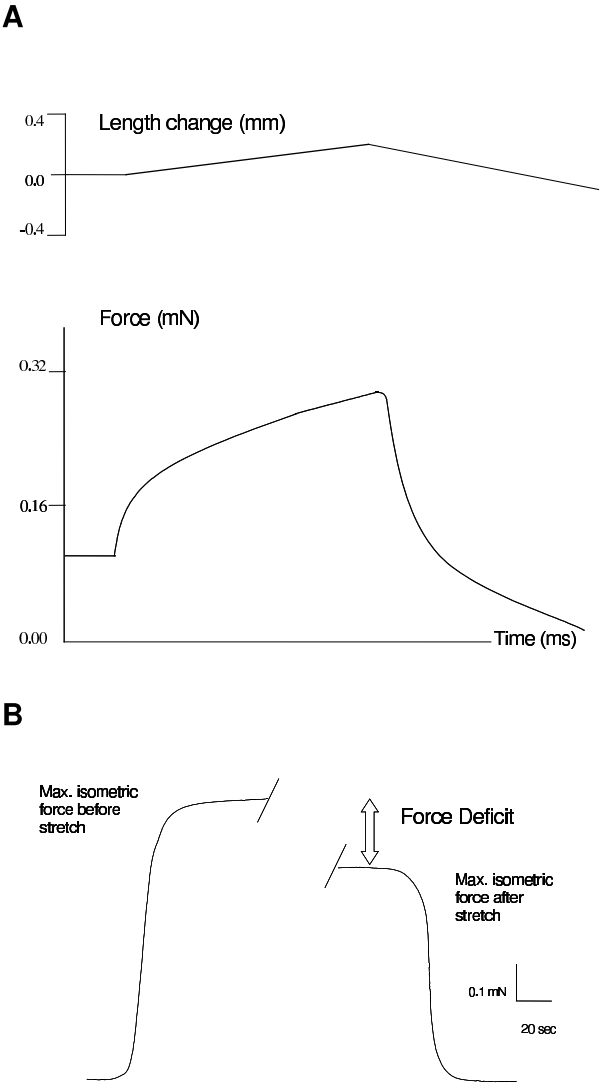
More recently, Consolino and Brooks[33]examined the susceptibility to sarcomere injury induced by single stretches of maximally activated muscles of *mdx* mice. Single stretch protocols are less likely to result in fatigue or depletion of energy stores, factors that can complicate the mechanistic interpretation of muscle injury after protocols involving many repeated contractions. In this elegant study, the authors hypothesised that on the basis that muscles of *mdx* mice would be more susceptible to injury, stretches of lesser strains would be expected to cause more damage (i.e. cause a greater force deficit) to muscles of *mdx* compared with wild type mice.[33] In fast extensor digitorum longus (EDL) muscles of wild type mice, single stretches of 30% strain were necessary to cause a significant deficit in isometric force, whereas in *mdx* mice, single stretches of only 20% strain caused significant loss of force producing capacity. After stretches of 30, 40, and 50% strain, force deficits were two- to three-fold greater for EDL muscles of *mdx* than for wild type mice.[33] Interestingly, analysis of dye uptake into muscles following the single stretch protocols revealed no membrane damage. The authors concluded that on the basis of greater force deficits, in the absence of fatigue, depletion of energy stores, or significant membrane damage, the differences in the force deficits from single stretches were due to differences in the extent of disruption to the ultrastructure of force-generating or force-transmitting structures within or between sarcomeres, and that in addition to a compromised membrane, the lack of dystrophin in EDL muscles of *mdx* mice results in a mechanically compromised cytoskeleton.[33] These findings support a role for the DGC in the maintenance of the structural stability of sarcomeres and hence "activities involving either single or repeated contractions that are innocuous for muscles in control animals may be injurious to dystrophic muscles".[33] However, it should be noted that the precise mechanism for the protective role of the DGC

remains elusive. Other contributing mechanisms to the loss of force transmission after damage, including alterations in excitation-contraction coupling, cannot be ruled out.[34]

Single Fibres

Similar studies have investigated the susceptibility of dystrophic muscle to contraction-induced injury at the cellular (single fibre level) using membrane permeabilized and intact single muscle fibre preparations. Yeung and colleagues[35] reported that single (flexor brevis) muscle fibres from *mdx* mice were more susceptible to stretch-induced damage and showed an associated rise in intracellular sodium concentration that was greater than in wild type mice. Each muscle fibre was subjected to 10 isometric tetani followed by 10 eccentric tetani of 40% strain relative to muscle length. Following the stretch-induced injury protocol, isometric force decreased to ~34% of the control in fibres from wild type mice and to ~23% in fibres from *mdx* mice.[35]

Chemical permeabilization of muscle fibres disrupts the integrity of the sarcolemma severely.[36] In a study comparing the susceptibility of muscle fibres from *mdx* and wild type mice to contraction-induced injury, Lynch and colleagues[37] proposed that since the integrity of membranes of muscle fibres from *mdx* and control mice would be compromised equally, any protection conferred by dystrophin and the DGC to intact fibres from muscles of wild type mice would be eliminated, and thus the susceptibility to contraction-induced injury (as determined from the force deficit) would not be different (Fig. 1). Fibres from EDL muscles of wild type and *mdx* mice were maximally activated by Ca^{2+} and then subjected to a single stretch of either 10, 20, or 30% strain relative to muscle fibre length. The observation of no difference in the force deficits of fibres from muscles of *mdx* and wild type mice provided indirect evidence that the protection conferred on skeletal muscle fibres by dystrophin and the DGC is a stabilisation in the alignment of sarcomeres through the lateral transmission of force from the myofilaments to the laminin 2 and, eventually, collagen IV in the ECM. Taken together, the findings on permeabilized fibres and membrane-intact fibres indicate that dystrophic symptoms do not arise from factors within the myofibrillar structure of fibres but, rather, through a disruption of sarcolemmal integrity that normally confers significant protection from contraction-induced injury. The greater force deficits for single permeabilized fibres compared with intact muscles (following single stretches of identical magnitude) indicates the significance of the overall protection from injury afforded the myofibrils by the linkages among the myofibres, the sarcolemma, and the ECM.[9-11,21,38] The findings also supported the premise that the dystrophin and DGC are major factors in the stabilisation of the membrane,[21] the lateral transmission of force,[10] and the alignment of sarcomeres, particularly during stretches of activated muscles.[33,37] One other possibility, not immediately apparent when using permeabilized fiber preparations, is that the susceptibility of dystrophic muscles to contraction-mediated damage could also disrupt normal excitation-contraction coupling, and thus subsequently affect (post-stretch) force generation.



A. *Typical force trace of a maximally activated single permeabilized fibre before and after a single stretch of 20% strain. Upper trace shows the magnitude (20% strain relative to muscle fibre length) and duration (400 ms) of the ramp stretch, performed at 0.5 fibre lengths/s. Lower trace shows the corresponding force response during stretch. Note that the fibre has attained maximum isometric force before the stretch has been imposed.* ***B.*** *Force deficit is calculated as the difference in maximum isometric force (P_o) after stretch compared with before stretch, expressed as a percentage of the pre-stretch maximum isometric force.*

New directions for clinical strategies: Protecting dystrophic muscles from contraction-induced injury

For clinical application, any therapy for muscular dystrophy, whether it be gene-based, cell-based, or

pharmacological in nature, must not increase the likelihood of contraction-mediated damage. This is especially relevant for therapies that do not replace the functional protein and serve to ameliorate the dystrophic pathology and either increase or decrease muscle fibre size. A long-held contention was that larger, fast muscle fibres were most susceptible to contraction-induced injury and that this explained why smaller calibre fibres were relatively spared from the dystrophic pathology.[39,40] This notion has been challenged more recently by studies in mice that have blocked the myostatin gene product (a negative regulator of muscle size) either through transgenic approaches or through the use of antibodies, and produced *mdx* mice with larger and stronger muscles and with an attenuated dystrophic pathology.[41,42] Although assessments of muscle function were not performed on the more severely affected diaphragm, the lesser dystrophic pathology highlighted the possibility that larger muscle fibres might be less susceptible to contraction-mediated damage.[43] This is an important question that needs to be addressed carefully through future experiments employing the contraction-induced injury assays described earlier. One approach could be to increase muscle fibre size through administration of anabolic agents, such as a β_2-agonist. In a preliminary study, Lynch and colleagues[44] examined whether long-term (18 weeks') clenbuterol treatment in mice affected muscle fibre susceptibility to contraction-induced injury. After a single stretch of 20% strain relative to fibre length, no difference was evident in the force deficit of permeabilized fibres from EDL muscles of treated and untreated mice. These preliminary findings suggest that although β_2-agonists increase skeletal muscle mass and fibre size, they do not increase muscle fibre susceptibility to contraction-induced injury.[44]

Given the continual development of new therapeutic strategies for treating neuromuscular disorders, assessments of muscle (fibre) susceptibility to contraction-induced injury will become increasingly important as a tool for evaluating treatment efficacy and their overall clinical significance.

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Author for correspondence:
Assoc. Prof. Gordon S. Lynch
Department of Physiology
The University of Melbourne
Victoria 3010, Australia

Tel: +61 3 8344 0065
Fax: +61 3 8344 5818
Email: gsl@unimelb.edu.au