Influence of temperature-induced reactive oxygen species production on excitation-contraction coupling in mammalian skeletal muscle

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Summary

1. Here we review evidence obtained recently by us indicating that the poor longevity of isolated mammalian skeletal muscle preparations at temperatures in the normal physiological range is related to the increased production of reactive oxygen species (ROS) in the resting muscle.

2. Temperature-induced ROS production increases markedly above 32°C in isolated, resting skeletal muscle and is associated with the gradual and irreversible functional deterioration of the muscle.

3. The majority of the temperature-induced muscle ROS originate in the mitochondria and act on various sites involved in excitation-contraction coupling.

Introduction

Reactive oxygen species (ROS) is a term used to describe a wide range of molecules and free radicals (chemical species with one unpaired electron) derived from molecular oxygen. ROS are formed by several different mechanisms and are an unavoidable by-product of cellular respiration. Some electrons passing "down" the respiratory chain in mitochondria leak away from the main path (mainly through reverse electron transfer at the flavin mononucleotide group of complex I[1-3]) and go directly to reduce oxygen molecules to the superoxide anion ($O_2\bullet^-$). Primarily all ROS in skeletal muscle derive from $O_2\bullet^-$. Thus, dismutation of $O_2\bullet^-$ either spontaneously or through a reaction with superoxide dismutase (SOD) produces hydrogen peroxide (H_2O_2), which in turn can be fully reduced to water (H_2O) or partially reduced to form the most reactive of all oxidants, the hydroxyl radical (OH•).[4] ROS, in particular $O_2\bullet^-$, play an important role in many biological systems ranging from cell development[5] and metabolism to skeletal muscle contraction.[6-10] Darnley *et al*.[11] showed that application of $O_2\bullet^-$ to diaphragm muscle strips resulted in a reduced maximum Ca^{2+} activated force response and inhibition of SR Ca^{2+} release. This was also shown by Callahan *et al*.[12] who found that not only did $O_2\bullet^-$ affect maximum force but that it had no apparent effect on the sensitivity of the contractile apparatus to Ca^{2+}. Similar results in cardiac muscle were obtained by MacFarlane & Miller,[13] who found that $O_2\bullet^-$ decreased force production but did not affect Ca^{2+} sensitivity or SR function.

It has been shown that exposure of rat skeletal muscle to elevated temperature (>37°C) leads to a higher rate of ROS production in both the intracellular and extracellular regions of the muscle than at room temperature.[14-16] Figure 1 shows the amounts of $O_2\bullet^-$ produced by the resting isolated rat skeletal muscle (*extensor digitorum longus*, EDL) at different temperatures, as detected by the reduction of cytochrome C. Cytochrome C is membrane impermeant and therefore can be reduced only by ROS that are present in the same compartment. The method is based on the reaction of $O_2\bullet^-$ with oxidized cytochrome C (Fe^{3+}) to produce reduced cytochrome C (Fe^{2+}), which has a specific peak absorbance at 550 nm. As shown by Edwards *et al*.,[15] $O_2\bullet^-$ appears to be the only species of ROS or reactive nitrogen species (RNS) that can actually reduce cytochrome C. Therefore the use of the cytochrome C assay for measuring $O_2\bullet^-$ is rather conservative because if anything, it underestimates rather than overestimates the true rate of $O_2\bullet^-$ production in the presence of various ROS and RNS. EDL muscle from rats and mice produce relatively low and stable amounts of ROS between 22 and 32°C. However, when the temperature of the resting muscle is increased from 32°C to 37°C, there is a five fold increase in ROS production in EDL muscles from rats and mice[15] and here we review the evidence suggesting that the loss of muscle function when isolated skeletal muscle of mammals is incubated at physiological temperatures is related to the temperature-induced production of $O_2\bullet^-$.[15]

ROS scavengers and skeletal muscle function at physiological temperatures

Tempol is a stable, membrane permeable nitroxide that acts as a SOD mimetic and its action can therefore be compared to that of an enzyme.[17] Tempol is first oxidised by the protonated form of superoxide (•OOH) to yield H_2O_2 and an oxo-ammonium cation, which is then further reduced by another $O_2\bullet^-$ molecule to yield O_2 and regenerate the Tempol molecule, with the net removal of two $O_2\bullet^-$ molecules. Tempol is therefore, ideally suited as a tool to determine whether the increased $O_2\bullet^-$ production at 37°C plays a role in the decrease in tetanic force production at 37°C.

At 37°C the tetanic force produced by isolated mouse and rat EDL muscles is markedly less than that produced at 22°C (Figure 1). At 37°C and in the presence of 1 mM Tempol, tetanic force production was significantly greater

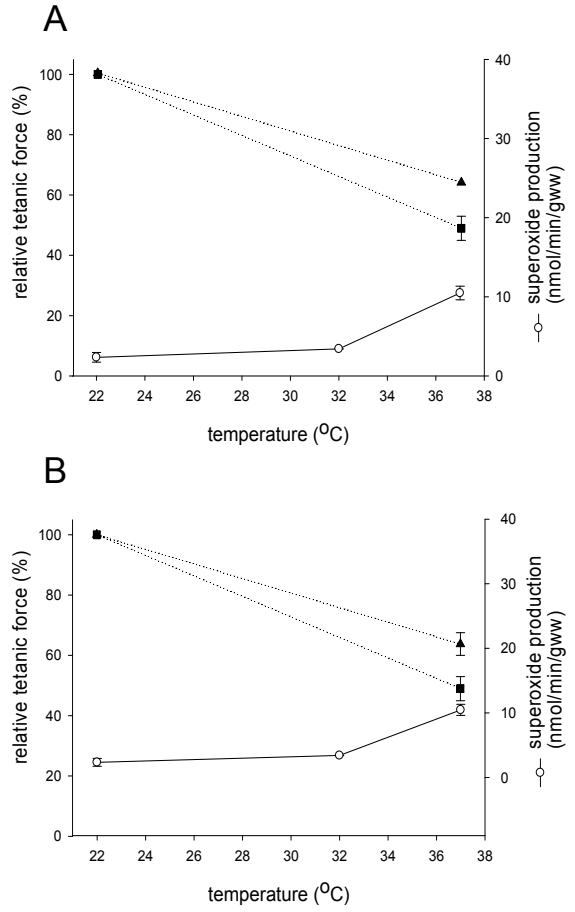

Figure 1. Tetanic force at 22 and 37°C (filled symbols) in EDL muscles of rat (A) and mouse (B) and O_2•⁻ production (open circles) at 22, 32 and 37°C in resting EDL muscles of rat (A) and mouse (B). *The decline in tetanic force was partially prevented in muscles incubated at 37°C in the presence of 1mM Tempol (filled triangles), a superoxide dismutase mimetic. Tetanic force production remained stable at 22°C in both isolated intact rat and mouse EDL muscles (data from Edwards* et al.*, 2007[15]). Note that the lines connecting the data points for tetanic force at 22°C and 37°C are arbitrarily drawn and do not reflect the force production between 22°C and 37°C.*

than when no Tempol was present in solutions (Figure 1A and B). This protective effect of Tempol on excitation-contraction (E-C) coupling at 37°C was also demonstrated at the single fibre level.[18,19] Whereas action potential induced force responses of rat EDL mechanically skinned single fibres dropped by 80% after 8 min at 37°C without Tempol in the system, when 1mM Tempol was present, the force response at 8 min dropped by only 40%. Thus, the results obtained with Tempol strongly suggest that a large part of the decrease in the force production in intact rat and mouse muscle fibres at 37°C is related to the increased production of O_2•⁻ which can act at different sites in the process of E-C coupling.

Sites of O_2•⁻ action in muscle

The ROS–induced decline in tetanic force after exposing muscles to 37°C in the absence of Tempol could be due to effects on various sites involved in E-C coupling, such as the contractile apparatus, the SR, or the plasma membrane.

Contractile apparatus. If the ability of the contractile apparatus to generate maximum Ca^{2+}-activated force and/or the sensitivity to Ca^{2+} were reduced, then the twitch and the tetanic force responses would also become smaller. In rat EDL muscles the ability of the contractile apparatus *per se* to produce maximum Ca^{2+} activated force was not different after exposure to 37°C in the presence / absence of Tempol (Figure 2A).[19] However, skinned fibres from the 37°C treated mouse EDL muscles produced ~55% less specific force than those from the 22°C control muscles (Figure 2B).[15] Importantly, this decline in specific maximum Ca^{2+}-activated force was not seen in the presence of 1 mM Tempol, as the maximum Ca^{2+}-activated force was not significantly different from that in fibres kept at 22°C.[15] There were also no changes in the Ca^{2+} sensitivity of the contractile apparatus from both rat and mouse EDL muscles after the muscles were incubated in the temperature range 37-46°C.[15,20] Thus, a large proportion of the depression in tetanic force in mouse EDL muscles exposed to 37°C compared with controls involving muscles maintained at 22°C can be explained by the reduced ability of the contractile apparatus to produce maximal force due to O_2•⁻ production. However, this was not the case for the rat EDL muscle where the ability of the contractile apparatus to produce force was not impaired following exposure of the muscle to 37°C (note though that increasing the temperature above 40°C also caused force depression in rat EDL muscle[15,20]). Therefore, while the loss of force at 37°C in isolated skeletal muscle of the mouse can be largely explained by effects of ROS production on the contractile apparatus, this is not the case for the rat skeletal muscle where ROS production at 37°C must primarily affect steps in E-C coupling before Ca^{2+} activation of the contractile apparatus.

The SR. It has long been recognised that proteins in the SR are sensitive to ROS.[21] ROS and other oxidants have been shown to increase the probability of ryanodine-sensitive Ca^{2+} release channel (RyR) opening and thereby promoting Ca^{2+} release from the SR.[22,23] ROS have also been shown to modulate SR Ca^{2+}-ATPase pump function, with very high ROS concentrations decreasing the reuptake of Ca^{2+} into the SR.[24,25]

The ability of the SR to accumulate Ca^{2+} was significantly reduced by about 20-40% after the EDL muscle of the rat was exposed to 37-40°C due to a marked increase in SR Ca^{2+}-leak, which persisted for at least 3 hours after treatment (see Figure 2C and van der Poel & Stephenson, 2007[26]). The increased Ca^{2+}-leak was not through the SR Ca^{2+}-release channel or the SR-Ca^{2+}-pump, although it is possible that the leak pathway was *via* oligomerised Ca^{2+}-pump molecules.[27] No significant change in the maximum SR-Ca^{2+}-ATPase activity was

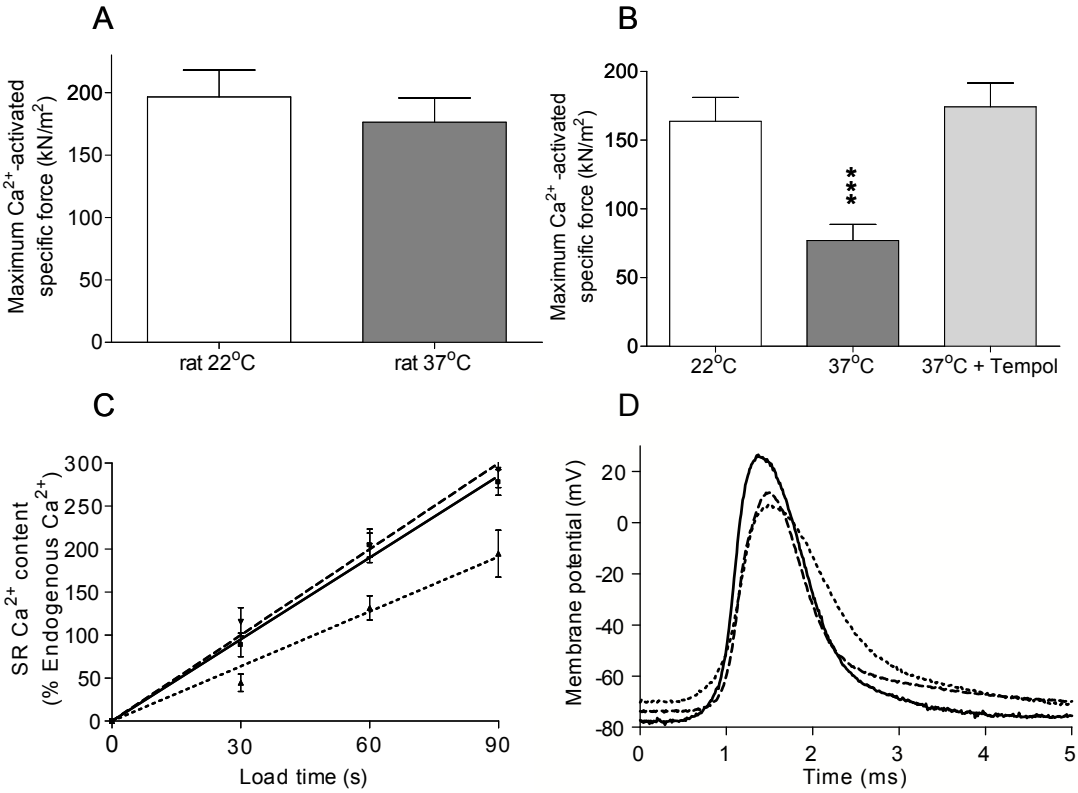

Figure 2. Effects of temperature on the maximum Ca^{2+}-activated force (A,B), rate of SR Ca^{2+} accumulation (C) and action potential properties (D) of EDL muscles from rat (A, C, D) and mouse (B). *Maximum Ca^{2+}-activated force and SR Ca^{2+} accumulation measurements were made at 22°C from mechanically skinned muscle fibres obtained from EDL muscle kept either at 22°C (open bars), or after exposure to 37°C for 30 min (dark grey bars). The light grey bar in (B) shows maximum Ca^{2+}-activated force production in mechanically skinned muscle fibres from mouse muscles exposed to 37°C for 30 min in the presence of 1mM Tempol. After exposure to 37°C the ability of the SR to accumulate Ca^{2+} was significantly reduced (dotted line in C) compared with controls (continuous line in C). Exposure to 37°C in the presence of 1 mM Tempol did not reduce the ability of the SR to accumulate Ca^{2+} (broken line in C). Changes in the AP parameters are seen in representative traces from muscles that were exposed to 37°C for 40 min (dotted line) compared to muscles kept at 22°C (solid line) and partial recovery from muscles that were exposed to 37°C for 40 min in the presence of 1 mM Tempol (broken line) (D). (Panels A and B are reproduced with permission from Edwards* et al. *(2007)[15]; data in panel C are original and were obtained using procedures described in van der Poel & Stephenson (2007),[26] and AP traces in D are from the studies presented in Edwards* et al. *(2007)[15] and van der Poel* et al. *(2007).[19]*

observed after the temperature treatment.[26] The observed changes in SR properties were fully prevented by the $O_2^{\bullet -}$ scavenger Tiron, indicating that the production of $O_2^{\bullet -}$ at 37-40°C is responsible for the increase in SR Ca^{2+}-leak. These lasting changes in SR properties with respect to Ca^{2+} handling mediated by $O_2^{\bullet -}$ production at 37°C are not sufficient to explain the more marked depression observed in the tetanic force in isolated intact rat muscle preparations kept at 37°C, because 30% SR depletion of endogenous Ca^{2+} would produce only a minor drop in the twitch and tetanic response.[28] Thus, the decrease in force production in rat muscle cannot be explained by changes in the ability of the contractile apparatus to produce force or the SR Ca^{2+} handling properties. Therefore, ROS production in the rat muscle at 37°C must primarily affect steps in EC coupling before Ca^{2+} release from the SR.

Plasma membrane. Upstream sites from the SR involved in E-C coupling are located in the plasma membrane represented by the sarcolemma and the t-tubular membrane where the dihydropyridine receptors (DHPRs)/ voltage sensors are located.[29] The plasma membrane plays a crucial role in maintaining muscle fibre excitability which refers to the ability of the fibre to trigger and propagate action potentials (APs). In turn, fibre excitability is sensitive to the functional state of the Na^+, K^+ and Cl^- channels and resting membrane potential (RMP).[30] How muscle fibre excitability is affected by temperature-induced ROS production is discussed in the following section.

Skeletal muscle excitability and ROS production

At 37°C the RMP of rat EDL muscle was

significantly depolarised by about 10 mV (from approximately -80 to -70 mV) and the amplitude of the AP was reduced (Figure 2D and Edwards *et al.*, 2007[15]). Addition of 1 mM Tempol at 37°C prevented the depolarisation of the RMP at 37°C, and the amplitude of the AP was not different from that of control muscles that were kept at 22°C. The maximum rate of depolarisation of the action potential was markedly slower in fibres incubated at 37°C when compared to fibres kept at 22°C and the rate of repolarisation of fibres at 37°C was significantly slower than of fibres at 22°C. The presence of 1 mM Tempol significantly prevented the decrease in the maximum rate of repolarisation but had little effect on the maximal rate of depolarisation.[15,19]

A direct effect of ROS on sarcolemmal and t-tubule membrane properties has been postulated.[31] Oxidation of thiol groups has been shown to irreversibly inactivate the DHPR[32] which would ultimately result in a decrease in DHPR mediated SR Ca^{2+} release. More recently it has been demonstrated that pre-treatment with N-acetylcysteine (NAC; antioxidant and reduced thiol donor) improves Na^+/K^+-pump activity, lessens changes in circulating K^+ levels, and delays fatigue during prolonged cycling exercise.[33,34]

This provides strong evidence that ROS production in rat skeletal muscle at 37°C reduces the excitability of the muscle fibres by causing membrane depolarisation, which, in turn, causes slow inactivation of the Na^+ channels,[35] reduction in the amplitude of the action potential, and impaired AP propagation along the t-system.[36]

Source of the temperature-induced ROS

ROS production in whole muscle arises not only from muscle fibres but also from other cellular structures such as nerve terminals, blood cells, and capillaries.[4] Specifically within skeletal muscle sites of $O_2^{\bullet -}$ production include the mitochondria, 5-lipoxygenase, cyclooxygenase, xanthine oxidase, and NAD(P)H oxidase.[37] It has been hypothesised that the major site of intracellular $O_2^{\bullet -}$ at elevated temperatures was the sarcolemmal NAD(P)H oxidase,[37] however this has been challenged recently where evidence suggests that isolated muscle mitochondria produce considerable amounts of $O_2^{\bullet -}$.[38,39] To determine the source of $O_2^{\bullet -}$ production, we used specific substrates and inhibitors known to induce and inhibit $O_2^{\bullet -}$ production in mitochondria.

When succinate, which is known to increase $O_2^{\bullet -}$ production,[39] was present in the solution in which mechanically skinned fibres were incubated, a large increase in the rate of cytochrome C reduction was observed compared with controls (Figure 3A). Addition of rotenone and succinate together is known to prevent reverse electron transport into complex I of the electron transport chain which reduces $O_2^{\bullet -}$ production of isolated mitochondria. When succinate and rotenone were added to mechanically skinned fibres at 37°C there was no cytochrome C reduction indicating no $O_2^{\bullet -}$ produced by the muscle fibres (Figure 3A).[19]

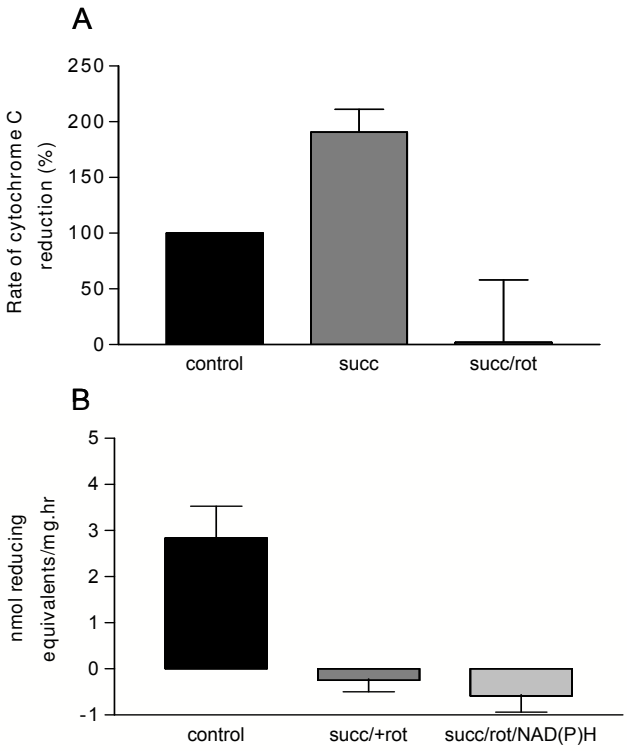

Figure 3. Influence of mitochondrial substrates and inhibitors as well as NADH on the rate of $O_2^{\bullet -}$ production of mechanically skinned single fibres from rat EDL muscles at 37°C. *The presence of 5 mM succinate significantly increased the amount of $O_2^{\bullet -}$ detected by cytochome C, which was completely abolished with the addition of 50 μM rotenone (A). In the presence of succinate, rotenone and 0.1 mM NADH, the rate of $O_2^{\bullet -}$ produced by single fibres was not significantly different from that without NADH indicating the presence of a very low NOX activity. Reproduced with permission from van der Poel* et al., *2007.*[19]

A transverse tubule NAD(P)H-dependent plasma membrane oxidase (NOX) activity has been recently discovered,[40] but it is unlikely to contribute in any significant way to the temperature induced $O_2^{\bullet -}$ production, as suggested by results from experiments where mitochondria $O_2^{\bullet -}$ production was blocked with succinate and rotenone, and a NOX substrate, NADH was present to induce NOX activity. Under these conditions no measurable amounts of $O_2^{\bullet -}$ were produced in either mechanically skinned fibres, where the t-system remained intact or whole EDL muscles, indicating that compared with mitochondria, $O_2^{\bullet -}$ production *via* the plasma membrane NAD(P)H oxidase activity is not a major source of temperature induced $O_2^{\bullet -}$ production in skeletal muscle (Figure 3B).[19]

Physiological temperature and isolated mammalian skeletal muscle preparations

Most *in vitro* studies on isolated mammalian skeletal muscles are conducted at sub-physiological temperatures,

between 22 and 30°C, because at temperatures above about 32°C there is a gradual, irreversible deterioration in muscle function. For many decades, this decline in muscle function at physiological temperatures was thought to be due to the relatively slow diffusion rate of oxygen into the muscle as a consequence to the removal of vascular perfusion, resulting in an anoxic region in the centre of the isolated muscle preparation. Yet recent work by Barclay[41] has shown that diffusive oxygen supply would be adequate to support resting metabolism and energy requirements at low duty cycles in isolated intact rat and mouse EDL muscle at 37°C. Moreover, Lännergren & Westerblad[42] showed that the irreversible decline of muscle function also occurs in single mouse muscle fibres exposed to 37°C, where oxygen supply to the muscle cell is not limited. Furthermore, a marked difference in ability to respond to electrical stimulation at 22-25°C and 37°C also occurs in mechanically skinned single fibres in which stable electrically induced responses can be elicited at 22-25°C for up to 30 min but not at 37°C, where electrically induced force responses drop to 20% of the initial force response after only 8 min.[19] These observations indicate that the deterioration in isolated mammalian skeletal muscle function at physiological temperatures is not simply due to the lack of oxygen supply or to a number of other potential factors such as mechanical trauma during surgery or inadequate stimulation to recruit all motor units in the muscle.

Clearly, an irreversible, temperature-dependent process must be responsible for the gradual irreversible loss of muscle function at 37°C in isolated mammalian muscle preparations and this review has highlighted the link between an increased rate of production of reactive oxygen species (ROS) at 37°C and deterioration of muscle performance at 37°C.[15,19,20]

An explanation for functional differences at 37°C between mammalian skeletal muscles *in vivo* and isolated preparations

There are major differences between the prevalent conditions for the skeletal muscle in the body of the animal and the experimental conditions normally used with the isolated skeletal muscle *in vitro*. One of the major differences refers to the partial O_2 pressure in solutions used in experiments on isolated muscle preparations *in vitro* (710-750 mmHg) compared with that in the blood capillaries of the resting rat muscle (<30 mmHg).[43] The much greater partial O_2 pressure in solutions than in the muscle capillaries may result in increased $O_2\bullet^-$ production in at least some parts of the muscle compared with the situation *in vivo* since hyperoxia has been associated with increased ROS production.[44]

Also, compared with the *in vitro* isolated skeletal muscle, *in vivo* skeletal muscle should also be better protected from $O_2\bullet^-$-induced stress by the presence of extracellular superoxide dismutase (EC-SOD), a SOD isoform present in plasma and in the extracellular fluid which binds reversibly, with relatively high affinity, to multiple sites on collagen and the basement membrane of muscle fibres through a heparin-binding carboxy terminal on its tail.[45] When the muscle is removed from the body of the animal and placed in a physiological saline, the EC-SOD is gradually lost and this renders the isolated muscle more sensitive to $O_2\bullet^-$. The importance of EC-SOD in the protection of the skeletal muscle from injury caused by generation of $O_2\bullet^-$ was recently demonstrated *in vivo* using EC-SOD knockout mice.[46]

Thus, perfusion of the muscle with blood through capillaries, compared with bathing the muscle in a grossly hyperoxic physiological solution, would result in less ROS produced in the muscle and more efficient removal of $O_2\bullet^-$ through continuous blood flow aided by the presence of EC-SOD. This would prevent accumulation of $O_2\bullet^-$ in the muscle above levels causing the deterioration in the force response *in vivo* at 37°C. It is therefore the accumulation of ROS, rather than lack of oxygen due to diffusional constraints, that is likely responsible for the observed decrease in the force response in the isolated mammalian skeletal muscle at 37°C.

Concluding Remarks

It is clear from the growing number of different studies that there is a strong link between the increased temperature-induced ROS production in mammalian muscle and the gradual, irreversible loss of force observed in isolated skeletal muscles *in vitro* at physiological temperatures. The increased temperature-induced ROS production at physiological temperatures can lead to damage to the various sites involved in EC coupling in isolated skeletal muscle *in vitro*, thus essentially restricting experimentation *in vitro* to sub-physiological temperatures. The development of methodologies that reduce ROS accumulation in muscle at physiological temperatures is essential for using skeletal muscle experimentation *in vitro* for optimising strategies in clinical settings to treat conditions in which skeletal muscle is negatively affected, such as cancer cachexia, respiratory disease, heart failure, muscle dystrophies and ageing.

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